Evaluation of antioxidant properties of some commercially available culinary and medicinal mushrooms from Taiwan

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Abstract

A selection of commercially available mushrooms was obtained from Taiwan and screened for phenolic contents and antioxidant activity in aqueous extracts using various chemical measurements, namely scavenging of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (TEAC), Ferric reducing antioxidant power (FRAP), scavenging of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and Folin-Ciocalteu reaction. According to the antioxidant activity perceived, *Cordyceps militaris*, *Pleurotus citrinopileatus*, *Trametes versicolor*, *Hericium erinaceus*, *Ganoderma lucidum* and *Auricularia auricula-judae* were selected for *in vitro* digestion and cellular antioxidant assay. After the *in vitro* digestion steps, the antioxidant activity in the extracts of *C. militaris* had significantly decreased, (in TEAC 22% and 27 % decrease, in hot- and cold-water extracts, respectively, in FRAP 42% and 21% decrease, in hot- and cold-water extracts, respectively and in DPPH 78% and 21% decrease in hot- and cold-water extracts, respectively). The hot-water extract of *A. auricula-judae* and cold-water extracts of *H. erinaceus* showed no significant increase in TEAC assay after enzymatic digestion. There was a significant increase in antioxidant activity in the other mushroom extracts after in vitro enzymatic digestion. *P. citrinopileatus* exhibited the most potent antioxidant activity in the TEAC (from 24 to 2 times higher and 10 to 1.5 times higher than other mushrooms in hot- and cold-water extracts, respectively) and DPPH assays (from 6.4 to 1.2 times higher and from 27 to 1.6 times higher than the other five mushrooms in hot- and cold-water extracts, respectively) after digestion steps. *T. versicolor* showed the most potent ferric reducing power after digestion steps (from 29 to 5 times higher and 14 to 1.1 times higher than the other five mushrooms in hot- and cold-water extracts, respectively). These results indicate that most of the potential antioxidant compounds within the mushroom extracts could be released after digestion steps, whereas the potential antioxidant compounds of *C. militaris* might be degraded after digestion steps.

The results suggest that determination of antioxidant activity in selected mushroom extracts may underestimate the real antioxidant activity that may be in close contact with the intestinal lumen. Chemical estimates of potential antioxidant compounds within the mushroom extracts may not accurately

indicate the complex nature of the antioxidant activity of mushroom extracts within cells. In this study, human hepatoma cell lines (Huh 7) were used to measure cellular antioxidant activity using 2′, 7′- dichlorodihydrofluorescein diacetate as a fluorescent probe. In artificially induced peroxyl radicals, among the selected mushroom extracts tested, *C. militaris* and *T. versicolor* had the highest cellular antioxidant activity, whereas *H. erinaceus* had the lowest. In addition, in chemical assays (TEAC and DPPH), the antioxidant activity of *T. versicolor* was less than that of *P. citrinopileatus* (64% and 67 % less in TEAC in hot-and cold- water extracts, respectively and 70% and 82% less in DPPH in hot- and cold-water extracts respectively). Even though the antioxidant activity of *C. militaris* was decreased after digestion steps, *C. militaris* exhibited far stronger cellular antioxidant activity than the other five mushrooms ($p < 0.001$). Based on the different antioxidant assay methods, the antioxidant activity of each antioxidant assay gave different antioxidant trends and antioxidant activity value depending on the type of extract method (hot- and cold-water extracts). Using cellular antioxidant assays may produce bioactivity results of the antioxidant activity of mushroom extracts within cells. These findings could suggest that the aqueous extracts from *C. militaris* and *T. versicolor* associated with health benefits and other traditional remedies, at least in part, might be their potent antioxidant activity.

List of Abbreviations

A3	Amyloid 3-protein
ABAP	2, 2'-azobis(2-methylpropionamide)dihydrochloride
ABTS	2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammoniun salt
AD	Alzheimer’s disease
AFR	Acute renal failure
ATP	Adenosine triphosphate
CAT	Catalase
DCFH-DA	2', 7'-dichlorofluorescin diacetate
DCF	Dichlorofluorescein
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid
DW	Dried weight
EC_{50}	The concentration to scavenge the 50% of initial free radicals.
ETC	Electron transport chain
FBS	Foetal Bovine Serum
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
GAE	Gallic acid equivalent
GI	Gastrointestinal tract
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
HBSS	Hank’s Balanced Salt Solution
HIV-1	Human immunodeficiency virus type 1
LC	Lucigenin
LM	Luminol
LD_{50}	The amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals.
LDL	Low-density lipoprotein
MnSOD	Manganese superoxide dismutase

mtDNA	Mitochondrial DNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NFTs	Neurofibrillary tangles
NOS	Nitric oxide synthase
PC	Phosphatidylcholine
PD	Parkinson's disease
PE	Phosphatidylethanolamine
Prx	Peroxiredoxins
QE	Quercetin equivalents
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SD	Standard deviation
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant capacity
TPTZ	2,4,6-tris(2-pyridyl)-*s*-triazine
TR	Thioredoxin reductase

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Chapter 1 Introduction

1.1 Oxygen and Free radicals

1.1.1 *The history of oxygen and the fact that oxygen is poisonous*

In the Earth's crust, oxygen is the prevalent element whose accumulation into the atmosphere has forever changed the surface chemistry of the Earth resulting in the development of photosynthesis over three billion years ago. *Cyanobacteria* were the most primitive oxygenic photosynthetic organisms, and with their presence, the dawn of oxidative stress, one of the greatest selective pressures imposed on primordial life, commenced. In all living organisms, oxygen is essential for energy production, except for those organisms that live under anaerobic conditions. Oxidative stress is a universal phenomenon in all three domains of life, the archaea, the bacteria and the eukaryote (Canfield, 2005; Wiedenheft et al., 2005; Venturi and Venturi, 2007). However, the presence of a greater concentration of oxygen than normal is toxic to plants, to animals, and to aerobic bacteria. In plants, an inhibition of chloroplast development and membrane damage caused by high concentrations of oxygen has been observed (Wroblewska et al., 1994). In *Escherichia coli*, growth is inhibited when the oxygen tension is increased *in vitro* (Brown et al., 1966).

1.1.2 *Definition of Free Radicals*

The outermost shell of a ground-state atom has complementary electrons which spin in opposite directions. An atom is considered to be 'ground' when its electrons fill the lowest energy orbital completely, and are arranged in pairs with antiparallel spin states before they begin to occupy higher energy orbitals. By definition, free radicals are any chemical species possessing one or more unpaired electrons in the outermost shell, and are capable of independent existence (Hanson, 2005; Fridovich, 2006) (Table 1).

Name	Formula	Comments
Hydrogen atom	$H^{\bullet}$	The simplest free radicals
Trichloromethyl	CCl_3	A carbon-centred radical react rapidly with O_2
Superoxide	$O_2^{\bullet-}$	Oxygen-centred radicals
Peroxyl, alkoxyl	$RO_2^{\bullet}$, $RO^{\bullet}$	Oxygen-centred radicals formed during the breakdown of organic peroxides.
Hydroxyl	$OH^{\bullet}$	Highly reactive oxygen
Thiyl / perthiyl	$RS^{\bullet}$ / $RSS^{\bullet}$	Radicals with unpaired electrons residing sulphur.
Nitrogen-centred radicals	$C_6H_5N{=}N^{\bullet}$	Formed during oxidation of phenylhydrazine.
Chlorine radical	$Cl^{\bullet}$	Produced by homolytic fission of chloride, e.g. by UV light

Table 1: Different types of free radicals (Halliwell and Gutteridge, 2007).

1.1.3 *The Octet rule*

According to Lewis (1916), each bonded atom shares two or ‘paired’ electrons to form a stable molecule. Lewis (1916) called this discovery the ‘rule of two’, and subsequently Langmuis suggested the ‘octet rule’ (Jensen, 1984; Gillespie and Silvi, 2002). When a valence shell is filled completely and with eight electrons, the electron configuration corresponds to that of a noble gas, and the atoms are thought to be as stable as those of noble gases (Hanson, 2005; Fridovich, 2006).

1.1.4 *Free radical reaction*

When unpaired electrons are present, the free radicals are usually highly reactive, and not selective about what they react with. In chemistry, the classic route of the oxidative reactions of free radicals is divided into three distinct processes: initiation, propagation and termination (Hanson, 2005).

Initiation: When free radicals react with surrounding compounds or molecules, the

reaction creates more free radicals.

Propagation: The newly formed free radicals react with other compounds or molecules to form more free radicals.

Termination: Theoretically, one free radical will encounter another free radical eventually. The reaction will be terminated when the unpaired electrons form a new covalent bond (Hanson, 2005; Fridovich, 2006).

1.2 Reactive Oxygen Species (ROS)

1.2.1 *Definition of ROS*

Reactive oxygen species (ROS) are chemical structures, either stable or transient, containing oxygen atoms which are highly reactive in character due to the presence of unpaired valence shell electrons, and mediate extensive biological interaction. The free radicals which form reactive oxygen species are superoxide radicals ($O_2^{\bullet -}$), hydroperoxyl radicals ($HO_2^{\bullet}$), hydroxyl radicals ($OH^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and alkoxyl radicals ($RO^{\bullet}$). However, not all ROS are free radicals. Several non-radicals are also designated as ROS. These non-radicals include hydrogen peroxide (H_2O_2), singlet oxygen (O_2^1), hypochlorous acid (HOCl) and ozone (O_3). Other types of reactive species are reactive nitrogen species (RNS). Just like ROS, not only do RNS also have free radical forms such as nitric oxide ($^{\bullet}NO$) and nitrogen dioxide radical ($^{\bullet}NO_2$), but also non radicals such as peroxynitrite (ONOO), peroxynitrous acid (ONOOH), peroxynitrate (O_2NOO^-), nitronium cation (NO_2^+) and dinitrogen trioxide (N_2O_3) (Aruoma, 1998; Halliwell and Gutteridge, 2007; Angelopoulou et al., 2009).

1.2.2 *Biological functions related to ROS*

ROS and RNS are ubiquitous and play an important role in all living organisms; ROS are by their nature by-products of normal metabolism, and both play a dual role as both deleterious and beneficial species. In normal circumstances, the endogenous production of ROS and RNS is essential for life (Droge, 2002). Small amounts of ROS and RNS are continuously produced in a normal biological system, and both play important roles in different biological functions such as signal transduction, neurotransmission, smooth muscle

relaxation, peristalsis, platelet aggregation, blood pressure modulation, the immune system control, learning and memory, production of energy, phagocytosis, the regulation of cellular growth, cellular signalling, the synthesis of important biological compounds and the metabolism of xenobiotics (Droge, 2002; Caporaso, 2003; Cooke et al., 2003).

In the case of macrophage phagocytosis, when a foreign cell is engulfed during the processes of phagocytosis, the phagocyte increases its oxygen consumption, a process which is called oxidative or respiratory burst. In respiratory burst, superoxide is rapidly released and is converted from oxygen by NADPH oxidase and then converted to hydrogen peroxides by superoxide dismutase (SOD). In a third step, hydrogen peroxide is combined with a chloride ion to form bleach, ClO^- by myeloperoxidase. On the other hand, ROS at

low/moderate concentration also induces mitogenic response (Hanson, 2005; Valko et al., 2007). The oxygen molecule is capable of accepting an additional electron to form more reactive oxygen superoxide. The ROS molecules contain oxygen ions that derive and present higher reactivity than molecular oxygen with redox activity such as $O_2{\bullet}^-$ and $OH^{\bullet}$ (Hanson, 2005).

Type of radicals	Sources of radicals	Physiological process
Nitric oxide (NO●)	Nitric oxide synthase	Smooth muscle relaxation (control of vascular tone) and various other cGMP-dependent functions
Superoxide and related ROS	NAD(P)H oxidase	Control of ventilation, control of erythropoietin production and other hypoxia-inducible functions. Smooth muscle relaxation, signal transduction from various membrane receptors/enhancement of immunological functions
Superoxide and related ROS	Any source	Oxidative stress responses and the maintenance of redox homeostasis

Table 1.1 Important physiological functions that involve free radicals (Droge, 2002).

1.3 The source of ROS

Free radicals are generated both naturally and from man-made sources (Halliwell, 1996). Antoine Lavoisier (1743-1794), the father of modern chemistry and a pioneer oxygen chemist who identified and named oxygen (1778) and hydrogen (1783), pointed out that "animals that respire are true combustible bodies that burn and consume themselves". They operate just like an internal combustion engine producing nitric oxide and nitric dioxide (NO_2) to pollute our atmosphere. However, the combustion in a biological system is much more complex than an internal combustion engine and harmful intermediates, the ROS, are produced (Sies, 1997; Finkel and Holbrook, 2000; Ou et al., 2003).

1.3.1 *Environmental*

It is well known that the production of ROS may be compared to exposure to certain noxious factors such as some xenobiotic, infectious agents, pollution, UV light, cigarette smoke and radiation. In the environment, there are several causes of ROS generation directly or indirectly by physical, chemical and biological reactions (Hanson, 2005; Valko et al., 2006). For instance, when a molecule is exposed to a specific wavelength such as ultraviolet light, bond homolysis occurs and two electrons are divided equally to generate free radicals (Jurkiewicz and Buettner, 1994). In the body, water can be split to generate the hydroxyl radical ($OH^{\bullet}$) by low-wavelength electromagnetic radiation such as gamma rays from the environment. Although ultraviolet light is unable to split water, it can cleave the oxygen covalent bond in H_2O_2 (Halliwell, 1996). The human body can also generate free radicals such as $O_2^{\bullet-}$. Pollutant gases and smoking can lead to the increase of ROS activity in the human body (Abou-Seif, 1996; Proteggente et al., 2006; Valko et al., 2006).

1.3.2 *Cellular*

There are several other sources of ROS in living organisms. The mitochondrion is one of the major cellular sources of ROS production in the cell. In the mitochondrial electron transport chain (ETC), approximately 1% to 3% of total mitochondrial oxygen is incompletely consumed and that which is left leaks out of the pathway and passes directly to oxygen, leading to the generation of $O_2^{\bullet-}$ (Batandier et al., 2002). These redox reactions are carried out by four electron transporting complexes (I to IV) and one H^+ - translocating ATP synthetic complex (V). In mitochondria oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors. During the electron transport chain, when ubiquinone (coenzyme Q) accepts electrons to form ubisemiquinone, a highly reactive compound leaks out from the electron transport chain and ends up as stray superoxide. Two of the four mitochondrial complexes (I and Ш), the NADH-uiquinone oxidoreductase and the ubiquinone-cytochrome *c* oxidoreductase, are important sources of ROS production (Raha and Robinson, 2000). In addition, based on methods using nematodes, Ishii *et al.* (1998) suggested that the second mitochondrial complex (II) also produces superoxides. Under normal

physiological conditions, the rate of ROS generation is limited by high concentrations of the enzyme manganese superoxide dismutase (MnSOD) which ensures that the ROS can be efficiently removed keeping the rate of ROS generation at the basal level and resulting in little damage to biological systems (Raha and Robinson, 2000). It has been identified that within the mitochondrial complex I, the semiquinones act as donors for transforming O_2 to $O_2^{\bullet-}$. Although the detailed mechanisms by which this mitochondrial complex converts electrons to vectorial protons are not apparent yet, laboratory evidence suggests that the increase of superoxide and hydrogen peroxide production in the mitochondrial complex might be due to blockage of the site where semiquinone donates an electron to an acceptor (Pitkanen et al., 1996; Pitkanen and Robinson, 1996; Raha and Robinson, 2000) (Figure 1).

Enzymes such as xanthine oxidase are also capable of the production of $O_2^{\bullet-}$ (Kellogg and Fridovich, 1975). The important event in free radical generation in a biological system is the redox process that includes two reactions: oxidation and reduction. Oxidation is a process that donates electrons to an atom or molecule (donor) and reduction is a process that accepts electrons in an atom or molecule (acceptor). ROS also can be generated by metal redox systems such as Fe^{2+}/Fe^{3+}. Although H_2O_2 is not the major ROS, $OH^{\bullet}$ can be generated via the Fenton reaction ($Fe^{2+} + H_2O_2 \leftrightarrow Fe^{3+} + OH^- + OH^{\bullet}$) (Kirkinezos and Moraes, 2001) in which Fe^{2+} is a donor and H_2O_2 is an acceptor; the by-product $OH^{\bullet}$ is a strong free radical. The major generation of $OH^{\bullet}$ is from the interaction of hydrogen peroxide with transition ions, for instance Fe^{2+} and Cu^{2+} (Mantle and Preedy, 1999).

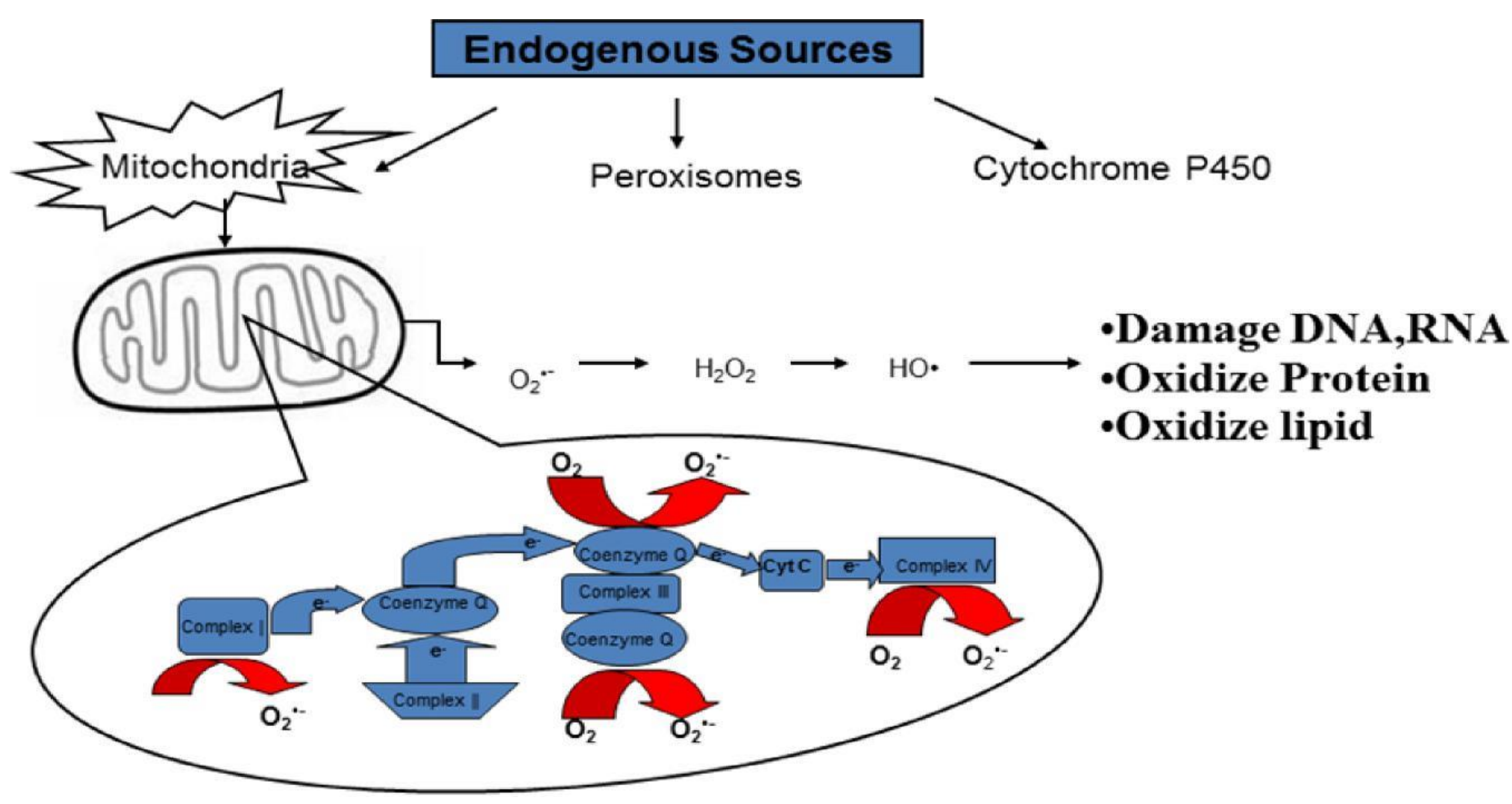


Figure 1: Intercellular ROS sources (Batandier et al., 2002).

1.4 Free radical damage

1.4.1 *Oxygen Stress*

In biological systems, maintaining the equilibrium of peroxidant/antioxidant operates like a balance. When ROS are over-produced in one site, enzymatic and non-enzymatic antioxidants maintain the balance in other site. When the balance is disturbed, the cumulative damage to proteins, lipids and DNA caused by excess ROS produced in the body leads to the occurrence of what is called ‘oxidative stress’, and this is believed to cause ageing and many human diseases (Sies, 1997; Droge, 2002; Ou et al., 2003). Numerous important metabolic and cellular disturbances are being linked to ROS when ROS overwhelm the normally efficient protective mechanism of cells and tissues. There is no doubt that ROS can cause lipid, DNA and protein damage (Hanson, 2005), and these will be discussed in greater detail next.

1.4.2 *Lipid damage*

Unsaturated fatty acids are the most oxidative labile components in a biological system due to unstable electrons near double bonds which are sensitive to lipid peroxidation where lipid peroxidation increases exponentially with the number of double bonds. (Barja, 2004; Valko et al., 2007). The CH_2 group between carbon-carbon double covalent bonds is the major site in which ROS reaction occurs. The ROS can react with one of the CH_2 giving water and a new radical species, lipid peroxyl radicals ($LOO^\bullet$). If lipid peroxyl radicals are formed, they can react with

other lipids to form a lipid hydroperoxide (LOOH) and another lipid radical ($L^\bullet$) which can continue the oxidation reaction. This chain reaction can be thousands of events long, as demonstrated in the following reaction (Pardini, 1995; Hanson, 2005):

$$LH + OH^\bullet \qquad H_2O + L^\bullet$$

$$L^\bullet + O_2 \qquad LOO^\bullet$$

$$LOO^\bullet + LH \longrightarrow LOOH + L^\bullet$$

1.4.3 *DNA damage*

Free radicals can result in DNA damage which can cause mutation in oncogene or tumour suppressor gene generation. All components of DNA are known to react with hydroxyl radicals which result in single- and double-strand DNA breaks, purine,

pyrimidine base and deoxyribose modification, and DNA cross linkage (Halliwell and Gutteridge, 2007). Carcinogenesis is associated with the arrest or induction of transcription, the induction of a signal transduction pathway, replication error and genomic instability, which are all related to oxidative damage (Davey et al., 2000). Evidence of the production of DNA lesions has been accumulated to prove the relationship between oxidative stress and cancer (Hanson, 2005). For example, by studying 8-hydroxyguanine (8-OH-G), in cancer patients, the 8-OH-G extracted into urine is about 50% higher than in the control group (Rozalski et al., 2002). Exposure to asbestos, cadmium, hexavalent chromium, arsenic and tobacco smoke increases oxidative stress level and is considered to be potentially carcinogenic (Davey et al., 2000; Hanson, 2005).

1.4.4 *Protein damage*

Compared with lipids, proteins are much larger molecules and have various structures and functions. When proteins are damaged, proteases hydrolysis the damaged proteins and recycle the atoms instead of repairing them. ROS damage proteins by two different mechanisms; the protein backbone is damaged first, followed by the side chains. For example, the sulphydryl or thiol groups (-SH) can be oxidized to form a disulphide bridge (-S=S-). However, when a -SH group cannot be formed into a disulphide bridge due to oxidation, the protein cannot fold up into its functional form. If some of the protein genes which turn off growth regulation are damaged, the faulty proteins may result in cells irregularly growing to form a cluster of cells and become cancerous (Hanson, 2005).

1.5 Pathological Problems and Free Radical Generation

In 1954, Gershman published the theory of oxygen toxicity of free radicals, and there are large evidences of proof that free radicals are not only adapted within the living biological system but also used in various physiological functions (Droge, 2002; Valko et al., 2007). Commoner *et al* (1954) discovered free radicals in biological material. Harman (1956) described free radicals as a Pandora's Box of evils that may account for gross cellular damage, mutagenesis, cancer and the degenerative process

of biological ageing (Droge, 2002). Recently, ROS have been suggested to be the causative agents involved in a number of pathological processes such as diabetes mellitus, atherosclerosis, neurodegenerative disease, ischemia/reperfusion injury and many other diseases. Therefore, finding a critical concept for maintaining the balance between antioxidation and oxidation in biological systems is believed to be the most popular area in modern medicine (Droge, 2002; AshokKumar, 2004).

1.5.1 *Cardiovascular disease*

It has been suggested that atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure are linked with oxidative stress (Kukreja and Hess, 1992; Lakshmi et al., 2009). ROS-induced stress modifies phospholipids and thiol groups of proteins by peroxidation and oxidation, leading to changes in membrane permeability, membrane lipid bilayer disruption and the functional modification of various cellular proteins (Valko et al., 2007). Also, by incubating rat heart sarcolemma with hydrogen peroxide and Fe^{2+}, Ca^{2+}/Mg^{2+} ecto-ATPase and sarcolemmal ATP independent Ca^{2+} binding activity are inhibited (Kaneko et al., 1989). A previous study had shown that exposure to xanthine plus xanthine oxidase or hydrogen peroxide caused rat heart mitochondrial creatine kinase activity to decrease (Hayashi et al., 1998). It may be concluded that increasing oxidative stress results in myocyte abnormalities, and this is suggested to be related to the effects of ROS on subcellular organelles (Angelopoulou et al., 2009). ROS induce intercellular Ca^{2+} overload which is directly or indirectly related to neointimal hyperplasia of atherosclerosis, vasoconstriction for hypertension, myocardial cell damage in ischemia reperfusion, as well as cardiac hypertrophy in heart failure, as has been revealed by many studies (Irani et al., 1997; Pérez et al., 1998; Podrez et al., 2000; Souza et al., 2000; Yuan and Li, 2003). As well as Ca^{2+} increase, Na^{+} concentration and long-chain fatty acid accumulation in cardiac membrane are also suggested to be relevant (Valko et al., 2007).

Lipid peroxidation leads to a decrease of membrane fluidity and results in damage to membrane proteins, inactivating enzymes and ion channels. The oxidized lipids can have a similar effect to H_2O_2, such as stimulating proliferation, blocking proliferation and inducing apoptosis and necrosis which occur in atherosclerosis, and maybe also be in the gastrointestinal (GI) tract (Halliwell and Gutteridge, 2007). Lipid

oxidization participates in orchestrating atherosclerosis (Farbstein et al., 2010). Enzymatic and free radical oxidations play important roles in cardiovascular disease. Because of oxidizable olefinic or double bonds, lipids are primary targets of oxidation (McIntyre and Hazen, 2010).

The oxidized low-density lipoprotein cholesterol has been suggested as the atherogenic factor that contributes to cardiovascular disease. In the low-density lipoprotein (LDL) hypothesis, in the presence of high concentrations of LDL, the artery wall will be infiltrated by LDL, and the increasing LDL can be oxidized by free radicals. When LDL is taken up by a macrophage scavenger receptor, the formation of inflammatory cytokines and foam cells, the promotion of cell proliferation and cholesterol ester accumulation occur. When those cells accumulate in the blood vessels forming a fatty streak, this causes further endothelial injury and leads to atherosclerotic disease (Hanson, 2005; Liu and Finley, 2005; Singh and Jialal, 2006) (Figure 1.2).

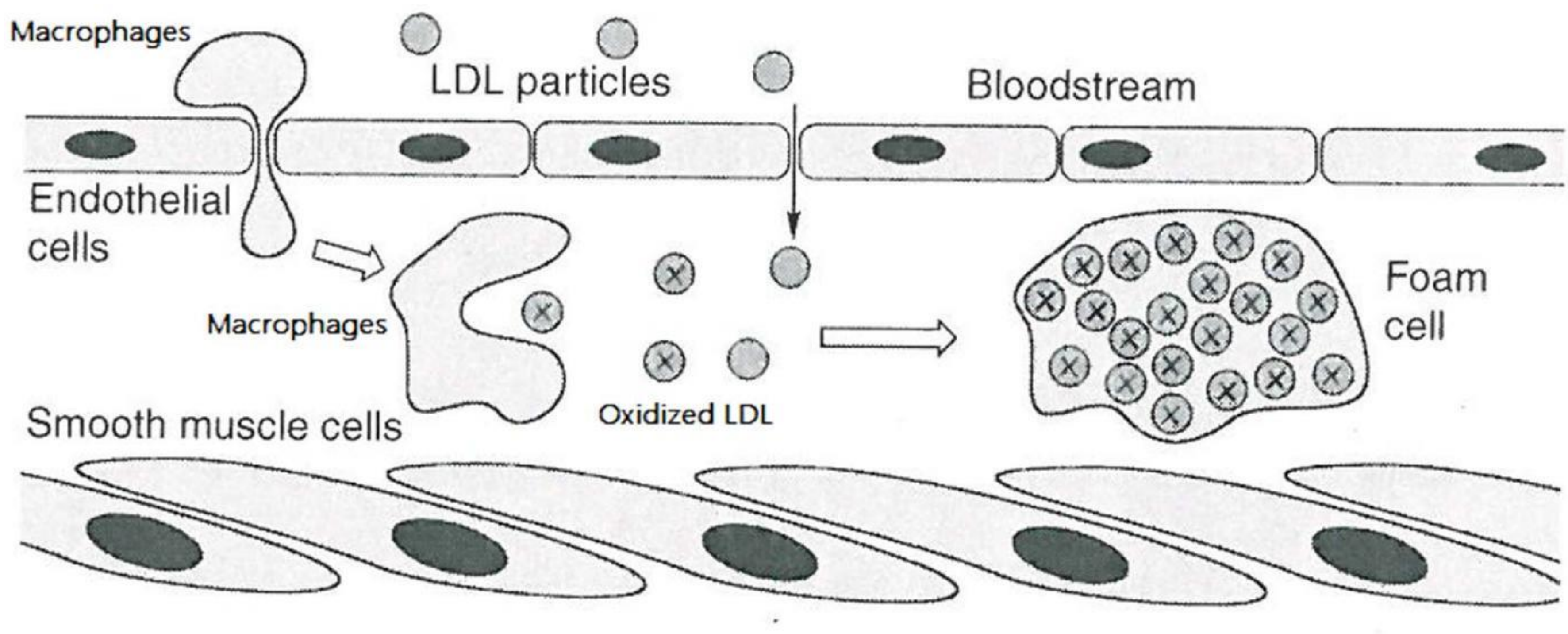


Figure 1.2: The oxidized LDL process of atherosclerosis ◯X : oxidized LDL (Hanson, 2005).

1.5.2 *Diabetes*

Diabetes is caused by faulty production of, or tissue response to insulin. Insulin signaling is a key part of the regulation of metabolism which may involve ROS. It has been suggested that increased activity of ROS and oxidative cellular damage are linked with diabetes (McCune and Johns, 2002; Praticņ, 2005). Oxidative phosphorylation, glucose autooxidation, NADPH oxidase, lipooxygenase, cytochrome P450 monooxygenases, and nitric oxide synthase (NOS) formation is stimulated by hyperglycemia caused by diabetic complications (Niedowicz and Daleke, 2005).

1.5.3 *Neurological disorders*

The brain is particularly highly susceptible to oxidative damage and this is considered to be related to several neurodegenerative diseases (Klein and Ackerman, 2003; Ono et al., 2006). The brain is a high oxygen utilising organ: in adult humans, the brain consumes about 20% of basal O_2 (Halliwell, 2006). With its high aerobic metabolic activity, polyunsaturated fatty acids and redox-active metals (such as Cu and Fe) make the brain particularly susceptible to oxidative damage.

1.5.4 *Alzheimer's disease*

Evidence directly supports the belief that oxidative stress increases in the brain as a consequence of Alzheimer's disease (AD) which includes an increase in the content of lipid peroxidation, a decrease in polyunsaturated fatty acids, protein and DNA oxidation, and the presence in activated microglia surrounding the most senile plaques of nitrotyrosine, formed from peroxynitrite ($ONOO^{\bullet -}$) (Halliwell and Gutteridge, 2007; Valko et al., 2007).

Human brain studies have indicated that oxidative stress plays an important role in neuronal degeneration in AD. In AD, soluble amyloid beta-protein (Aβ), Aβ fibrils, neurofibrillary tangles (NFTs), mitochondrial abnormalities, and ageing are major contributors to increased oxidative stress. Imbalance in oxidative homeostasis has been suggested to be important factors in neurogenerative disorders such as AD (Chauhan and Chauhan, 2006) (Figure 1.3 and 1.4).

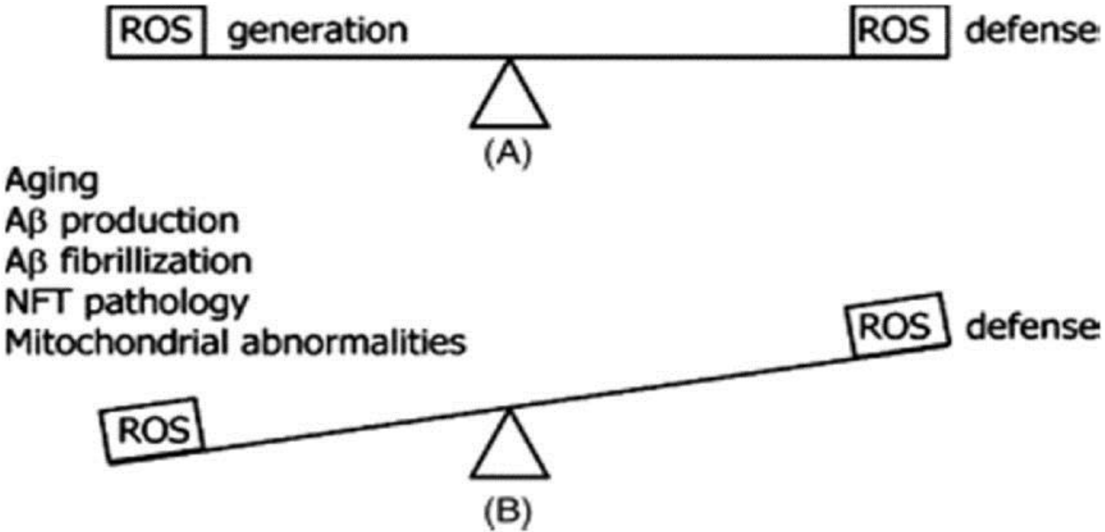


Figure 1.3: Possible mechanism of oxidative stress in AD: (A) under normal homeostasis conditions; (B) when ROS production overpowers the defence mechanism (Chauhan and Chauhan, 2006).

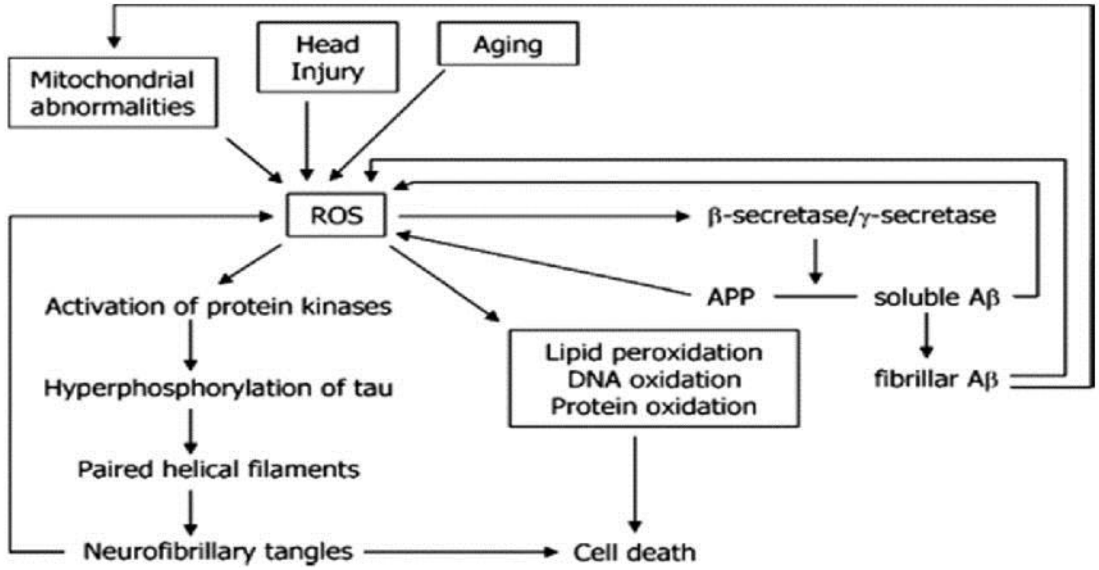


Figure 1.4: Factors possibly involved in oxidative stress induced cell death in AD (Chauhan and Chauhan, 2006).

1.5.5 *Parkinson's disease*

In Parkinson's disease (PD), studies of post-mortem brains have shown that PD patients are subjected to oxidative challenge. PD involves the progressive death of *substantia nigra* cells which use dopamine (the neurotransmitter-chemical messenger

between the brain and nerve cells) to communicate with the stratium. Decreasing nigral dopamine levels lead to a decrease in stratial dopamine, which is believed to cause PD (Jenner, 2003). Oxidative stresses can cause dopamine cell degeneration in PD (Tretter et al., 2004). Mitochondrial dysfunction in complex I is also suggested by current evidence to play a potential and central role of excitotoxicity and oxidative stress in PD. The complex I inhibition is due to the production of nitric oxide ($NO^{\bullet}$) which interacts with the protein within complex I (Evans, 1993; Koutsilieri et al., 2002).

1.5.6 *Ageing*

Two main theories have been proposed as processes forming ageing: damage-accumulation and genetic theories. The damage-accumulation theory suggests that the accumulation of free radical damage to DNA, lipids and proteins produced by free radical random deleterious effects is probably the most complex approach to explain the process of ageing. However, DNA damage is proposed as the most important process for ageing (Barja, 2004). Mitochondrial DNA (mtDNA) is the primary site of oxygen damage by superoxides. The damaged mtDNA cannot be readily fixed and the aftermath of the accumulation of damaged mtDNA causes cell death and organism ageing (Valko et al., 2007). Studies of maximum longevity and fatty acid unsaturation have shown a negative correlation (Pamplona et al., 2002).

1.6 Defense system

To help to counteract the oxidative challenge, the human body has a highly complicated antioxidant defence system composed of enzymes and vitamins to counteract the damaging power of ROS. In aerobic environments, there are three principal levels that maintain a steady-state of metabolites and functional integrity: prevention, interception and repair (Ou et al., 2003).

1.6.1 *Definition of Antioxidants*

Antioxidants have been defined by Halliwell as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and thus significantly delay or inhibit the oxidation of these substrates (Halliwell and

Gutteridge, 2007). Antioxidant defence systems in the human body can be categorised into endogenous antioxidant defences, and exogenous antioxidant defences (Hanson,2005).

1.6.2 *Endogenous antioxidants*

The endogenous antioxidant defences include enzymatic and non-enzymatic antioxidants. Among the enzymatic defences are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), peroxiredoxins (Prx) and thioredoxin reductase (TR) (Kahl et al., 2004).

The $O_2\bullet-$ is converted to O_2 and H_2O_2 by SOD by the following reaction (Hanson, 2005):

$$2\,O_2\bullet- + 2H+ \xrightarrow{SOD} H_2O_2 + O_2$$

H_2O_2 is still an ROS and it is a powerful oxidant. Another enzyme, CAT, then converts H_2O_2 to water and O_2 by following reaction (Hanson, 2005):

$$2\,H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

GPx also can react with H_2O_2 by using glutathione (GSH) to form water and a dimer of glutathione (GS-SG). The GS-SG consists of two GSH linked by a disulphide bridge which can be converted back to GPx by GR in the following reaction (Hanson, 2005):

$$H_2O_2 + 2\,GSH \xrightarrow{GPx} 2\,H_2O + GS\text{-}SG$$

$$GS\text{-}SG + NADPH + H^+ \xrightarrow{GR} 2\,GSH + NAD^+$$

Prx reduces H_2O_2 and organic peroxides, and TR plays an important role in maintaining protein in reduced states. The non-enzymatic antioxidants include hormones, lipoic acid, uric acid, bilurbin, melanins, transferrin and lactoferrin (Tan et al., 2003; Halliwell and Gutteridge, 2007).

1.6.3 *Exogenous antioxidants*

Plants produce numbers of antioxidant compounds (Hanson, 2005). Exogenous dietary antioxidants such as vitamin C, carotenoids and polyphenols are found in plants, fruits and vegetables. Epidemiological evidence suggests that consumption of an antioxidant-rich diet such as vegetables, fruits and tea may confer a significant effect on the prevention of chronic human disease coronary heart disease (Agarwal and Rao, 2000; Asplund, 2002).

1.6.4 *Polyphenolic compounds*

Polyphenolic compounds are a diverse group of ubiquitous compounds which can be found in all vascular plants. The common feature of phenolic compounds is at least a hydroxy-substituted aromatic ring. Several thousand different phenolic compounds have been characterized based on hydroxylated aromatic ring(s). Phenolic compounds are secondary metabolites that evolve in response to environmental stress such as pests and root parasitic nematodes (Boudet, 2007). Phenolic compounds are considered to have multiple biological effects in edible and non-edible plants. When plants are under oxidative stress, an abundance of antioxidants are produced such as tocopherol, ascorbate, glutathione, betanin, proline and many others regarded as plant defence compounds (Knekt et al., 1996; Kähkönen et al., 1999; Hanson, 2005; Shao et al., 2008).

Any compound with one or more aromatic rings containing one or more hydroxyl groups is referred to as a phenolic compound. In 1800, Joseph Lister first used phenol as an antiseptic in surgical processes (Fisher and Arnold, 2004). In *in vitro* studies, most phenols act as chain-breaking peroxyl radical scavengers to inhibit lipid peroxidation. Phenols also inhibit other ROS such as $OH^{\bullet}$, NO_2, N_2O_3, ONOOH and HOCl. Phenolic compounds form the majority of dietary antioxidants such as tocopherol, isoflavones in soybean, taxifolin in peanuts, resveratrol in wine, curcumin in turmeric and catechins in green tea (Halliwell and Gutteridge, 2007) .

These antioxidant compounds are concentrated in the oxidation prone site of the plants. For example, the concentration of ascorbic acid found in chlorophyll can be up to 50 mM which is approximately 1000 times higher than the ascorbic acid found in human plasma (Davey et al., 2000). Several antioxidant compounds can neutralize ROS and pass along the extract electron until it resides in the stable molecule. For

example, when vitamin E reacts with ROS, the phenolic hydrogen is rapidly transferred to the ROS and forms a phenoxyl radical from vitamin E. Due to the aromatic amine structure, the phenoxyl radical is resonance stabilized and relatively unreactive to lipid or oxygen. Therefore, the chain reaction does not continue (Burton and Ingold, 1984) (Figure 1.5).

Figure 1.5: The role of Vitamin E in quenching ROS and resonance stabilization (Hanson, 2005).

It has been reported that polyphenolic families have different antioxidant abilities depending on their structure characteristics. Polyphenols are derived from two main synthetic pathways: the shikimate pathway and phenylpropanoid metabolism. At least ten different main classes of polyphenols have been listed according to their basic chemical structure. For example, simple phenol (C_6) includes such compounds as phenol, cresol, thymol, resorcinol, orcinol, phenolic acid, with a C_6-C_1 structure, such as gallic, vanillic, syringic, *p*-hydroxybenzoic, and xanthones (C_6-C_1-C_6), and the single most important group, the flavonoids ($C_6C_3C_6$) (Figure 1.6) (Robards and Antolovich, 1997; Tapiero et al., 2002; Bravo, 2009).

Chemical structure of flavonoids

		R_1	R_2	R_3	R_4	R_5
Falavones	Apigenin	H	H	H	OH	H
	Chrysin	H	H	H	H	H
	Luteolin	H	H	OH	OH	H
Flavonols	Datiscetin	OH	H	OH	OH	H
	Quercetin	OH	H	OH	OH	H
	Myricetin	OH	H	OH	OH	OH
	Morin	OH	OH	H	OH	H
	Kaempferol	OH	H	H	OH	H
Flavanones	Hesperetin	H	H	OH	OCH_3	H
	Naringenin	H	H	H	OH	H
Flavanonol	Taxifolin	OH	H	OH	OH	H

Isoflavones		A_5	A_7	R_4
	Genistein	OH	OH	OH
	Genistin	OH	Oglc	OH
	Daidzein	H	OH	OH
	Daidzin	H	Oglc	OH
	Biochanin A	OH	OH	OCH_3
	Formononetin	H	OH	OCH_3

Figure 1.6: Chemical structure of flavonoids retrieved from Gülçin (2012).

The structure that fits the following three features would be effective in radical scavenging: (1) the *O*-dihydroxy structure in the B ring, (2) the 2, 3 double bond in conjugation with 4-oxo function in the C ring, and (3) the 3- and 5-OH group with 4-oxo function in the A and C rings. Quercetin is the perfect example to demonstrate the criteria for effective radical scavenging (Rice-Evans et al., 1996) (Figure 1.7).

Figure 1.7: The chemical structure of quercetin.

Studies on the anti-inflammation, anti-allergenic, anti-carcinogenic, antihypertensive, anti-arthritic qualities and the inhibition of tumour promotion by phenolic compounds have been published to demonstrate their biological and pharmacological activities (Rice-Evans et al., 1997; Ma et al., 2006; Siedle et al., 2007). Phenolic compounds are not only important in plants but also in animals. Phenolic compounds are present everywhere in the human diet: they are the constituents of fruits and vegetable, and are present in large amounts in beverages such as red wine, tea, coffee and beer. Approximately 1g of flavonoids is consumed in a balanced daily diet (Di Carlo et al., 1999).

Table 1.2: Examples of the therapeutic application of antioxidants (Augustyniak et al., 2010).

Antioxidant	**Field of application**	**References**
N-acetylcysteine	Protection against ototoxicity after the administration of aminoglycoside antibiotics such as gentamycin, preservation of endothelial function in patients with end-stage renal failure, and treatment of chronic liver diseases, chronic obstructive pulmonary disease, pulmonary fibrosis, over-doses of acetaminophen, improvement of bronchial mucous fluidity	Tepel et al. (2000); Scholze et al. (2004); Tepel (2007) Day (2008); Rahman and MacNee (2012)
Coenzyme Q_{10}	Protection against adriamycin-induced cardiotoxicity, improvement of effects of coronary artery bypass graft surgery, therapy of hypertrophic cardiomyopathy, improvement of redox balance in Down's syndrome, and prophylaxis of migraine	Conklin (2005); Littarru and Tiano (2005); Sandor et al. (2005); Adarsh et al. (2008); Tiano et al. (2008)
Ibedenone	Attenuation of the effects of Friedreich ataxia	Pandolfo (2008)
Carnitine	Improvement of sperm quality after infection	Vicari et al. (2006)
Glutathione	Treatment of chronic liver diseases and cataracts	Suzuki (2009)
α-Tocopherol	Amelioration of cardiac complications in diabetes, treatment of infertility, increase in pregnancy rate, and some positive effects in Alzheimer's disease	Suleiman et al. (1996); Hill (2008); Kamat et al. (2008)
Melatonin	Attenuation of oxidative stress in newborns	Gitto et al. (2008)
Resveratrol	Amelioration of cardiac complications in diabetes	Hill (2008)

(Derivatized) Superoxide dismutase	Arthritis (application to synovial fluid), oxidative stress in premature babies	Aaseth et al. (1998); Suzuki (2009)
Edaravone	Treatment of cerebral infarctions	Lee et al. (2007a)
Nitroxides	Potential protection against ionizing radiation, cancer prevention and treatment, control of hypertension and weight, protection from damage resulting from ischaemia/reperfusion injury	Soule et al. (2007)
Probucol	Prevention of coronary artery disease in patients with familial hypercholesterolemia and reduction of post-angioplasty re-stenosis	Tardif et al. (1997); Yamashita et al. (2008)
AGI-1067	Reduction of re-stenosis	Tardif et al. (2003)
Ebselen	Protection against brain function deterioration in patients with cerebral infarctions or subarachnoid haemorrhages	Yamaguchi et al. (1998)

1.7 Introduction to Mushrooms

Fungi are widespread, non-photosynthetic microorganisms whcih play an important central role in the functioning of the ecosystem, recycling energy and nutrients, and influencing the composition of a plant community (Newbound et al., 2010). Fungi are also important food sources which provide nutrients such as nitrogen, minerals (Ca, Mg and K), and vitamins. In many different cultural groups and nationalities, people collect wild edible mushroom as food sources. There are about 12,000 recognized fungi considered as mushrooms, and approximately 1000 species of edible mushrooms are recognized from 110 countries (Aida et al., 2009; Newbound et al., 2010).

Around the world, about 35 species of mushrooms are cultivated commercially and *Agaricus bisporus*, *Lentinus edodes*, *Pleurotus spp*., *Auricularia auricula-judae*, *Flamulina velutipes* and *Volvariella volvacea* are world-wide the most commercially available mushrooms (Aida et al., 2009). Over 200 species have been collected from the wild and used for various traditional medical purposes, mostly in the Far East (Sanchez, 2004). For instant, *Cordyceps spp*., *Tramete versicolor* and *Ganoderma spp*. have been used in traditional Chinese medicine from historical times for a wide range of ailments (Ying, 1987; Ko and Leung, 2007; Newbound et al., 2010). *L. edodes* is a leading cultivated mushroom in China. Its production was $1,397.0 \times 10^3$ tons in 1997, which is equal to 35.6% of the total national mushroom production (Chang, 1999). *L. edodes* was first cultivated in China between 1000 and 1100AD, and other less popular mushrooms even developed in China between 600 and 1800AD (Sanchez, 2004).

1.7.1 *Health benefits of mushrooms*

In 1980, the term ‘functional food’ was first coined in Japan (Siro et al., 2008). According to a definition published by the US Academy of Science, functional foods are those which “encompass potential health products” including “any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains” (Smith et al., 2002). In China, mushrooms have been used not only as a source of food but also as a medicinal resource for over 2000 years (Cheung et al., 2008). Since the 1980s, the term ‘medicinal mushroom’ has gained great attention after at least 270 species of mushrooms are known to possess various therapeutic

properties, and so can be considered as functional foods providing health benefits beyond the traditional nutrients they contain. For example, *Cordyceps spp*., *G. lucidum, Phellinus linteus* and *Pleurotus spp.* have been found to possess antitumour properties *in vivo* (Ohmori et al., 1986; Ying, 1987; Han et al., 1999; Shin et al., 2003; Cao and Lin, 2004).

Mushrooms are low in calories, sodium, fat and cholesterol while containing high percentages of protein, carbohydrate, fibre, vitamins and minerals, and this makes them a very good dietary food, and they not only contribute to a well-balanced diet but also lead to significant health improvement (Aida et al., 2009).

1.7.2 *Current trends in mushroom use*

For their nutritional and culinary values, mushrooms are considered as functional foods and are used as tonics and medicines. Some traditional mushrooms such as *Ganoderma spp*. and *Cordyceps spp*. have been used by human for their medicinal and healing properties for many centuries. In Oriental medicine, mushrooms have long been part of prescriptions for treating atherosclerosis and other ailments (Cheung et al., 2008). According to the Food and Agriculture Organization of the United Nations (2009), in China, mushroom production had increased by about 65% during the pervious decade and more than 1.5 million metric tons were produced in 2007, followed by lower rates of production in the United States and Canada. The continuously increasing production of mushrooms and the increasing number of cultivated mushrooms being introduced into the market indicate high consumer demand and increasing awareness of the health benefits of mushrooms (Marshall and Nair, 2009).

1.7.3 *The history of herbs and mushrooms*

The term 'ethnobotany' was coined by Haoshberger in 1896. The definition of ethnobotany is "plants used by primitive and aboriginal people" (Nakanishi, 2005; Gurib-Fakim, 2006). In about 100BC, Shen Nong's experience and work on herbs were eventually recorded in a book, the 'Shen Nong Ben Cao Jing', *The Herbal Classic of the Divine Plowman* (Chan, 1995). It is the earliest Chinese book on the use of herbs. In 1505AD, Liu Wan-Tai re-edited the book as 'Pen Taso Ching'. In 1578, Li Shizhen wrote the *Compendium of Materia Medic*a ('Ben Cao Gang Mu') which comprised 52 volumes, and mentioned 1892 herbs and 1000 prescriptions based on

Shen Nong's Herbal. This book has made a great contribution to herbal therapies in China (Nakanishi, 2005; Gurib-Fakim, 2006). In Eastern Asian countries, especially in China, cultivating mushrooms for medicinal use has a long history, starting around 600AD (Aida et al., 2009).

In medicinal mushrooms, *Cordyceps spp.* are components of the Fuzheng Huayu recipe, a preparation containing herbs such as *Salvia miltiorrhizae*, *Cordyceps spp.,* and seeds of *Prunus persica* (L.) Batsch, formulated on the basis of Chinese medicine theory in treating liver fibrosis (Ng and Wang, 2005; Liu et al., 2009). *P. linteus* has been used to prevent aliments such as gastroenteric dysfunction, diarrhoea and haemorrhage in China for centuries (Zhu et al., 2008). *Ganoderma spp.* is employed traditionally in Chinese medicine for treating hepatopathy, arthritis, hypertension, neurasthenia and chronic hepatitis (Liu et al., 2010). In traditional Chinese medicine, according to the *Compendium of Materia Medica*, *T. versicolor* is considered to be a useful treatment for dispelling heat, removing toxins and strengthening the physique (Chu et al., 2002). *Wolfiporia extensa* is often used in traditional Chinese herb prescriptions in combination with other crude drugs such as Banxia-Houpu for the treatment of diuretic conditions and depression (Luo et al., 2000; Yi et al., 2009). A number of mushroom species recorded in the Pharmacopoeia of the People's Republic of China are *Cordyceps spp*., *Ganoderma spp*., *P. linteus*, and *W. extensa* (China Pharmacopoeia, 2005).

1.7.4 *Biosynthesis of phenolic compounds from mushrooms or fungi*

The shikimate pathway is found in prokaryotes, microbial eukaryotes and plants studies (Herrmann and Weaver, 1999). The shikimic acid pathway is the primary process which produces the aromatic amino acids, phenylalanine, tyrosine and tryptophan (Figure 1.9). In fungi, the phenolic compounds are secondary metabolites which are derived from the shikimic acid pathway. Fungi have distinct pathway for the biosynthesis of the aromatic amino acids because the enzymes, 3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, 3-Dehydroquinate dehydratase, and Shikimate dehydrogenase, are structurally different form the isoenzyme, which is encoded by part of the aromatic cluster (Figure 1.8) (Herrmann, 1995).

Phosphoenol pyruvate

Erythros 4-phosphate

H_2O → P_i

3-Deoxy-D-arabino-heptulosonate 7-phosphate(DAHP) synthase

3-Dexoy-D-arabino-heptulosonate 7-phosphate (DAHP)

→ P_i

3-Dehydroquinate synthase

3-Dehydroquinate

→ H_2O

3-Dehydroquinate dehydratase

3-Dehydroshikimate

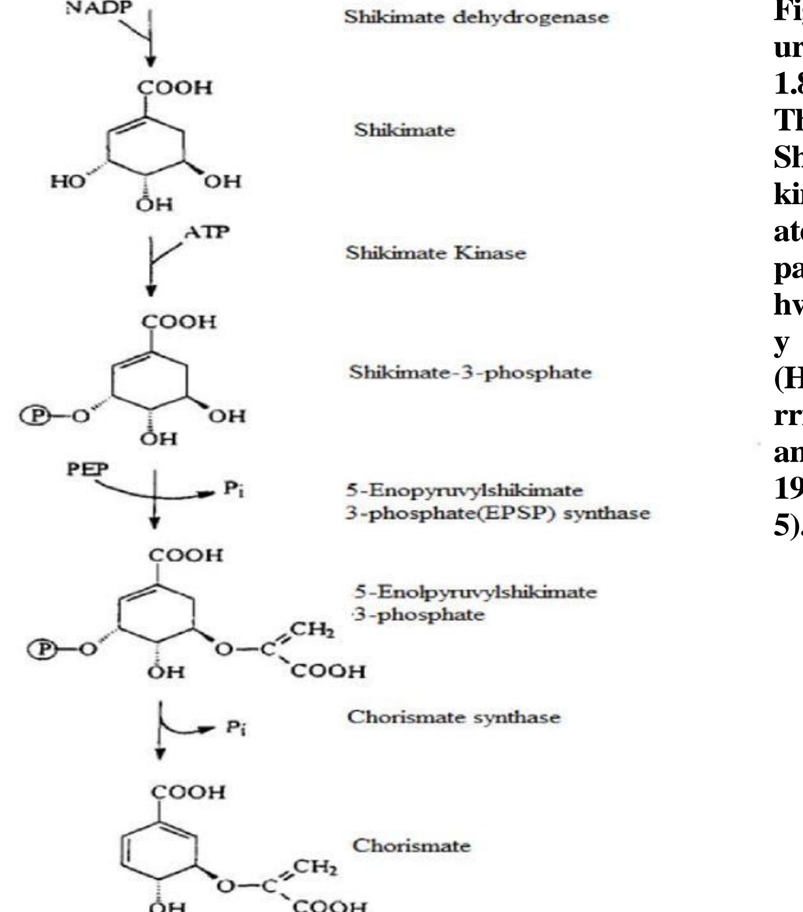


Figure 1.8: The Shikimate pathway (Herrmann, 1995).

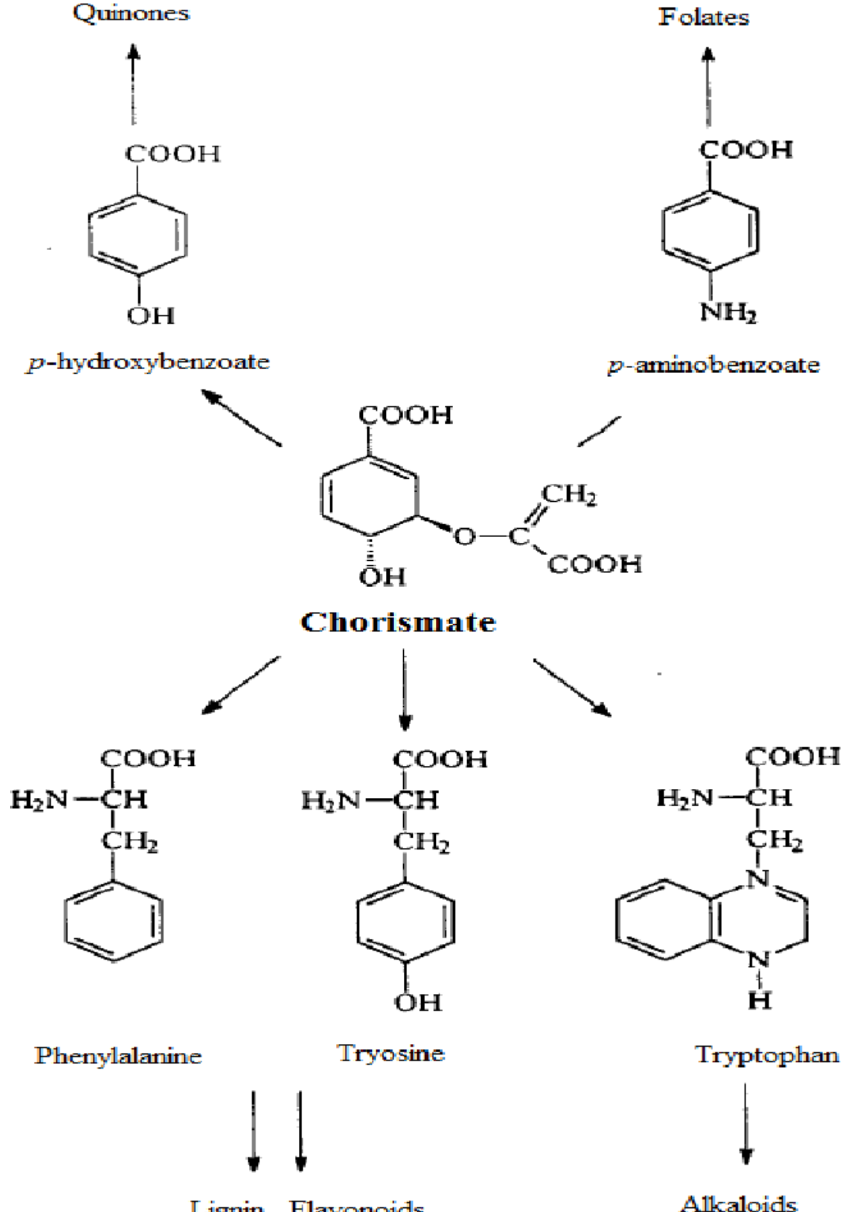


Figure 1.9: Chorismate as a precursor for primary and secondary metabolites (Herrmann, 1995).

The intermediates, chorismate, of the shikimate pathway are precursor of aromatic compounds including phenolic compounds. Through the shikimate pathway, phenolic compounds so derived in fungi are simple phenolic compounds. Depending on the carbon side chain, simple phenolic compounds are usually classified as C_6-C_3 including cinnamic acid and its derivatives, C_6-C_2 including phenylacetic acid and its derivatives, and C_6-C_1, benzoic acid and its derivatives.

Figure 1.10: Structure of simple phenolic compounds.

Studies of *Lentinus lepideus* have shown that the formation of cinnamic acid involves enzymatic conversion such as ammonia-lyases which convert phenylanine and tyrosine to cinnamic acid. To synthesize C_6-C_1 compounds, three possible pathways had been found. Studies on *Sporobolomyces roseus* have shown that C_6-C_3 compounds, such as cinnamic, *p*-coumaric and caffeic acid, are converted into C_6-C_1 compounds, such as protocatechuic acid, by â oxidation via benzoic and

p-coumaric, and caffeic acids. Another pathway is the forming of protocatechuic acid and gallic aicd from dehydroshikimic acid. The third steps of C_6-C_1 formation involves a stepwise degradation of C_6-C_3 compounds converting them into C_6-C_1 compounds via C_6-C_2 compounds, which has been demonstrated in *Polyporus tumulosus* (Cheung et al., 2008). The C_6-C_3 compounds are also intermediates in flavonoid biosynthesis, and the most important phenolic compounds with general character are in the diphenylpropane (C_6-C_3-C_6) skeleton. In fungi, flavonoids have been also been found from *Aspergillus candidus* and *Phallus impudicus*, namely, chlorflavonin and dihydrochalcone respectively (Cheung et al., 2008; Hanson, 2008) (Figure 1.11).

Chlorflavonin Dihydrochalcone

Figure 1.11: The structure of flavonoids isolated from fungi (Hanson, 2008).

1.7.5 *Therapeutic aspects of mushrooms*

Medicinal and culinary mushrooms have been studied for their medicinal and therapeutic properties such as their antitumour, antiviral, antibacterial and antidiabetic, effects, their effect on cardiovascular disorders, and immunomodulation (Ions and Birmingham, 1992; Mizuno, 1996; Liu et al., 1999; Kim et al., 2004).

1.7.5.1 *Hypocholesterolaemic effect*

In 1966, in a ten-week study during which rats were fed with a high-fat, high-cholesterol diet supplemented with 5% dried weight of *L. edodes*, the plasma cholesterol level of the animals was decreased significantly (Tokuda, 1966). A more recent study showed that eritadenine (the adenosine derivate of lentinacin), isolated from *L. edodes* extract, is the active compound of the hypocholesterolaemic effect and is capable of reducing the serum cholesterol level in mice (Cheung et al., 2008). Eritadenine also accelerates the excretion of cholesterol and its metabolic decomposition and affects the metabolism of phospholipids and fatty acid in rats. Therefore, an eritadenine supplemented dietary may decreased phosphatidylcholine biosynthesis, suppressed Ä6-destraurase activity (Shimada Y, 2003) and lowered cholesterol by decreasing the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) (Cheung et al., 2008). In other studies, animals were supplied with 2% and 4 % of *Pleurotus ostreatus* in a hyperlipidemic diet which was found to prevent cholesterol and triglyceride accumulation in both the blood and liver

(Bobek et al., 1991). The active cholesterol lowering agents isolated from *P. ostreatus* are well-known as lovastatin and its analogues 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. In a four week feeding experiment, two semisynthetic hypercholesterolaemic diets (1.5% cholesterol, 5% fat) each containing 5% dried powder of *A. auricula-judae* and *Tremella fuciformis* also had the ability to decrease serum total cholesterol (17% and 19%, respectively) and LDL cholesterol (24% and 31%, respectively) in rats (Cheung, 1996b).

1.7.5.2 *Hypoglycaemic effect*

The active components of the hypoglycaemic effect of mushrooms are include polysaccharides and lectins (Kiho et al., 1995; Mori et al., 1998). The mushroom bioactive components such as endopolymers extracted from *Cordyceps militaris*, *L. edodes*, and *P. linteus* have been found to have a potential hypoglycaemic effect on streptozotocin (STZ)-induced diabetic rats. Fed with 50mg/kg (body weight) of endopolymers extracts for seven days, the rat's plasma glucose was reduced by 18% after oral administration of endopolymers of *L. edodes* and *P. linteus* and 14% by *C. militaris* (Kim et al., 2001). Although â-glucans isolated from *Agrocybe cylindracea*, *Grifola frondosa* (the major components were galactose, glucose, fucose and mannose) and *Hericium erinaceus* (the major components of the extract were D-threitol, Darabinitol and palmitic acid) are different from one another, all the isolated polysaccharides displayed remarkable hypoglycaemic activity on diabetic model mice (Cheung et al., 2008). In another studying, in which STZ -induced diabetic rats were fed with 20% (dried weight) of *G. frondosa*, the initial fasting blood glucose of the control group was 225 mg/dL and the maximum blood glucose was 419 mg/dL (milligrams per decilitre), and the initial fasting blood glucose and maximum blood glucose of the diabetic rats were 170 mg/dL and 250mg/dL, respectively (Horio and Ohtsuru, 2001). Using intraperitoneal (IP) injection, two *Agrocybe cylindracea* crude polysaccharides extracts (HN1, high molecular weight, and HN2, low molecular weight) reduced the plasma glucose STZ- induced diabetic mice to 38% and 70% to control, respectively (Kiho et al., 1994a). Moreover, STZ-induced diabetic mice fed with the methanolic extract of *H. erinaceus* for successive days showed the ability to decrease the blood glucose by about 13% to 18% and by 19% to 26% than the non-fed group at dose rates of 100 and 200mg/kg respectively from day 5 through to day 20

(Wang et al., 2005). Lectins isolated from an edible mushroom (*A. bisporus* and *Agaricus campestris*) have also been found to enhance insulin sensitivity in isolated Langerhans islets in rats, and non-lectin components isolated from *A. campestris* present insulin-releasing and insulin-like activity (Cheung et al., 2008).

1.7.5.3 *Antitumour effect*

Letinan, a polysaccharide isolated from *L. edodes*, has shown an antitumour effect on Sarcoma 180-implanted mice at doses of 1mg/kg with no sign of toxicity (Chihara et al., 1970). Also, active polysaccharides isolated from *P. linteus* have significantly prolonged the survival rate of B16F-implanted mice and inhibited tumour growth in NCI-H23-implanted mice by giving IP injections at a dose rate of 100mg/kg and active polysaccharides did not induce direct toxicity in cancer cells (Han et al., 1999). A compound 3 β -hydroxy-lanosta-8,24-dien-21-al,inotodiol isolated from *Inonotus obliquus* exhibited inhibition ability of about 23.96% and 33.71% of tumour growth at concentrations of 0.1mg and 0.2mg per mouse per day in Balb/c mice bearing Sarcoma-180 cells, compared with the control group (Chung et al., 2010). *Agaricus subrufescens* is one of the more newly discovered mushrooms. The ammonium oxalate-soluble extract from *A. subrufescens* exhibited the most tumoricidal activity on Balb/c mice receiving intradermal injections of Meth-A tumour cells by intratumoral administration at a concentration of 5mg (Ebina and Fujimiya, 1998). Shin et al. (2003) studied IP injection of ethanol extracts of *Cordyceps sinensis*, a traditional medicinal mushroom, and *Paecilomyces japonica* for nine consecutive days at concentrations of 50mg/kg and 100mg/kg. The inoculated Scarcoma-180 tumour cells in mice showed a significant decrease in tumour weights (40.4% and 42.3% at 50mg/kg and 47.7% and 49.5% at 100mg/kg, respectively) and volumes (39.5% and 28.1% at 50mg/kg and 47.6% and 46.7% at 100mg/kg, respectively). The life span of inoculated mice also had a significant increase with the median survival times (32.3 and 33.9 days for *C. sinensis* at concentrations of 50mg/kg and 100mg/kg respectively, and 29.4 and 35.1 days for *P. japonica* at concentrations of 50mg/kg and 100mg/kg respectively, compared with a control group with 22.4 days) (Shin et al., 2003).

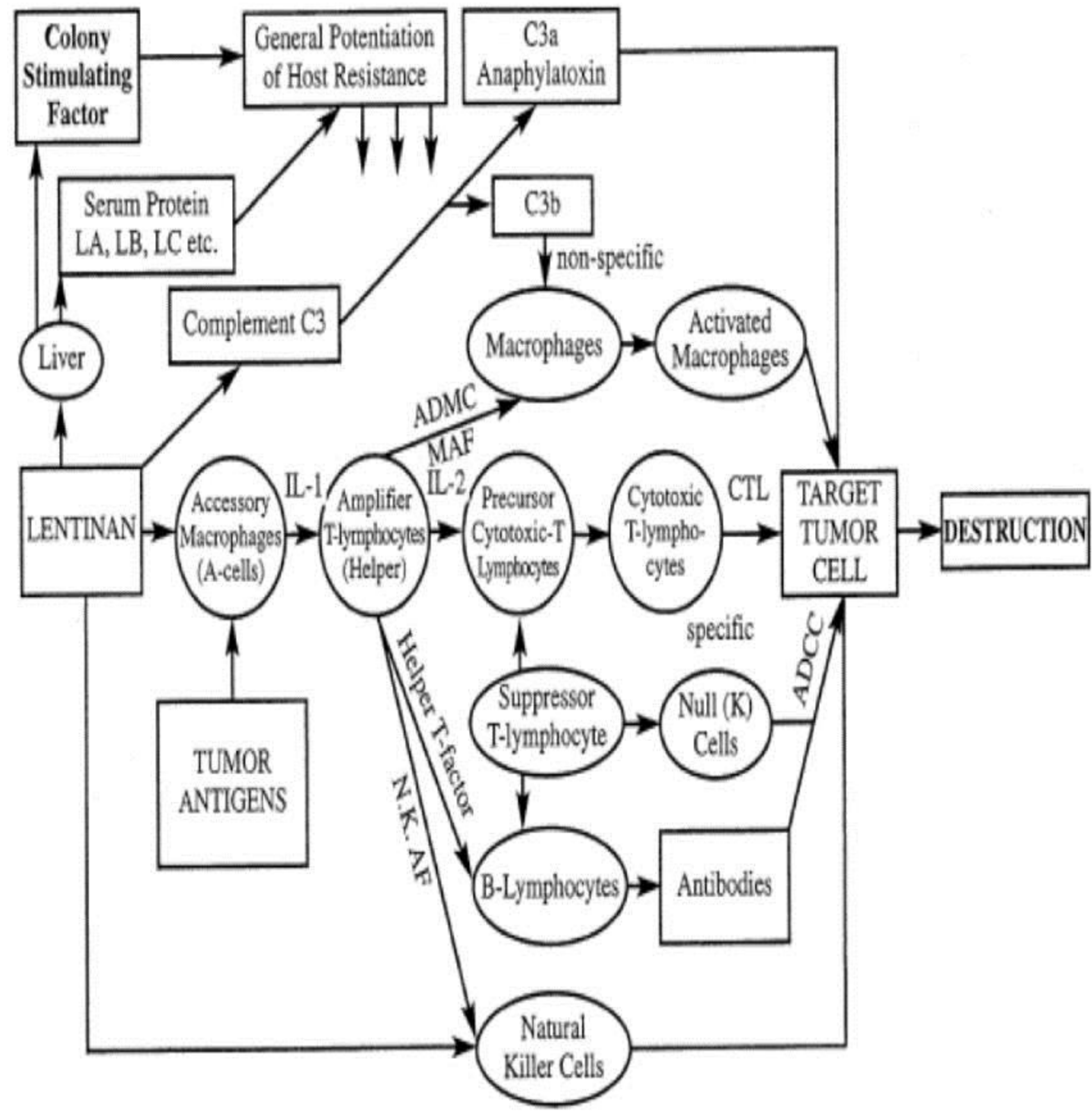


Figure 1.12: Possible pathways of lentinan action (Wasser, 2002).

1.7.5.4 *Immunomodulation*

Mushroom polysaccharides are regarded as biological response modifiers which exert their antitumour action mostly via activation of different immune responses in the host organism (Wasser and Weis, 1999). After administration of anti-lymphocyte serum, the antitumour activity of mushroom polysaccharides is lost in mice. This result suggests that the antitumour action of polysaccharides requires an intact T-cell and that the activity is mediated through a thymus-dependent immune mechanism. In addition, pre-treatment with antimacrophage agents (such as carrageenan) results in the antitumour activity of lentinan and polysaccharides being inhibited (Wasser,

2002).

Secretions of active oxygen and production of cytokines in peritoneal macrophages are also activated by lentinan and it can increase peritoneal macrophage cytotoxicity against metastatic tumours. The antitumour ability may be linked with hormonal factors, which play an important role in tumour growth (Wasser, 2002). Studies of *Ganoderma atrum* have shown that *G. atrum* polysaccharide-induced tumour apoptosis through the mitochondrial pathway by altering B*c*l-2 family proteins increases reactive oxygen species generation, loses mitochondrial membrane potential ($\Delta\psi_m$), and releases cytochrome *c* from the mitochondria into cytosol (Li et al., 2011).

It has been reported that the extract of *C. sinensis* has the ability to enhance the antibody response as judged by plaque-forming cells against T-dependent and T-independent antigens (Ng and Wang, 2005). Yamaguchi et al. (1990) reported that in tumour-bearing mice the *C. sinensis* extract restored the phagocytic activity of macrophages after tumour transplantation and lengthened the survival period. Studies of *Cordyceps scarabaecola* have shown that the water extracts have potential intestinal immune system modulating activity and the methanolic extracts manifest intermediate activity (Yu et al., 2003). Shin et al. (2001); Shin et al. (2003) demonstrated that the ethanolic extract of *P. japonica* stimulates phagocytosis and macrophage acid phosphatase activity.

1.7.5.5 *Antiviral, antibacterial and antiallergic*

Ganoderma annulare showed antifungal activity against *Trichophyton mentagrophytes* and *G. lucidum* presented active as an antiviral agent against human immunodeficiency virus type 1 (HIV-1) (El-Mekkawy et al., 1998; Smania et al., 2003). In addition, antiviral activity against influenza virus type A has been found in *Ganoderma pefeifferi* (Mothana et al., 2003). The ability to inhibit allergic reaction has also been found in mushrooms. For instance, extracts of *Hypsizygus marmoreus*, *F. velutipes* and *Pholiota nameko* showed anti-allergenic effects in mice and *G. lucidum* inhibited histamine release from rat cells (Tasaka et al., 1988; Sano et al., 2002).

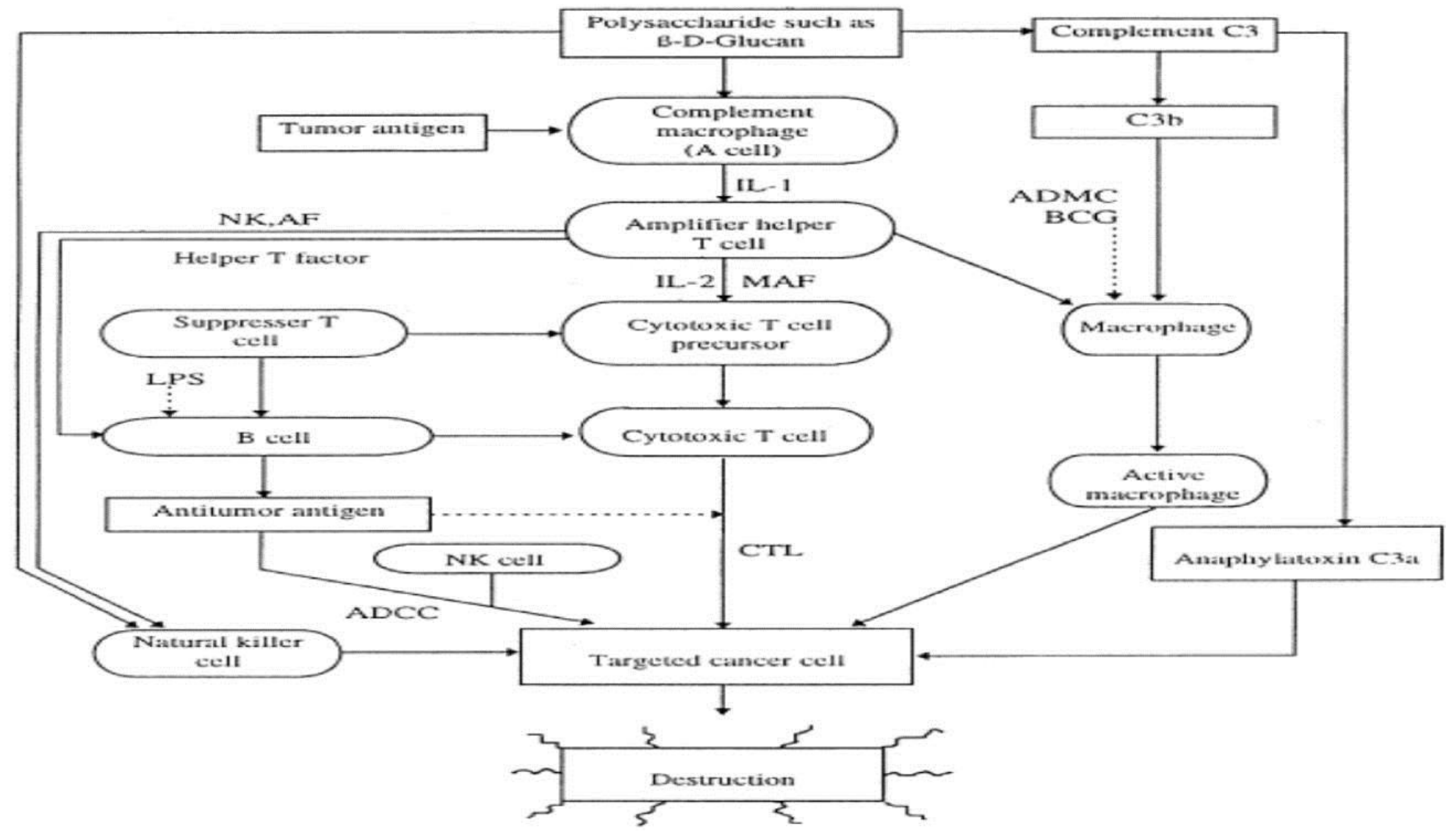


Figure 1.13: Possible immune mechanisms: â-D-glucan biological response modifier (Wasser, 2002).

Table 1.3: General compounds and effects of selected mushrooms.

Species	Active principle/constituents/extracts	Function	References
Agaricus bisporus	Lectins	Hypoglycemic	Ahmad et al. (1984)
Agaricus subrufescens	Beta (1,3)-D-glucan, Beat (1-4)-D-glucan	Antitumor	Wisitrassameewong et al. (2012)
Agrocybe aegerita	Lectins	Antitumor	Zhao et al. (2003a)
Auricularia auricula-judae	Polysaccharides	Hypoglycemic	Yuan et al. (1998)
Boletus edulis	Lectins	Antiviral(HIV-1)	Zheng et al. (2007)
Coprinus comatus	Comatin	Hypoglycaemic	Ding et al. (2010)
Cordyceps militaris	Cordycepin and cordycepic acid	Antitumour,immunostimulating	Zhou et al. (2009)
Trametes versicolor	Coriolan and polysaccharide-peptide	Antitumor	Stošić-Grujičić et al. (2011)
Ganoderma amboinense	Lanostanoid triterpenes	Antitumor	Li et al. (2005)
Ganoderma atrum	Ganoderma atrum polysaccharide (PSG-1)	Antitumor	Li et al. (2011)
Ganoderma lucidum	Triterpenes, Ganodeic acid	Antitumor,antiviral	El-Mekkawy et al. (1998)
Ganoderma sinense	protein-bound polysaccharide	Immunostimulator	Han et al. (2012)
Grifola frondosa	D-fractions (polysaccharides)	Antiumor	Kodama et al. (2002)

Species	Active principle/constituents/extracts	Function	References
Hericium erinaceus	Hericenones and amyloban	Ameliorative effect in Alzheimer's dementia	Wasser (2011)
Lentinula edodes	β-1,3-glucan (lentinan)	Antitumor and immunomodulating	Israilides et al. (2008)
Phellinus linteus	Proteoglycan	Antiinflammatory	Kim et al. (2003)
Pleurotus citrinopileatus	Polysaccharide	Antitumor	Zhang et al. (1994)
Pleurotus eryngii	Ethanolic extracts	Antiallergic	Han et al. (2011)
Pleurotus ostreatus	Polysaccharide	Immunomodulation	Sun and Liu (2009)
Wolfiporia extensa	Dehydrotumulosic acid, dehydrotrametenolic acid, and pachymic acid	Hypoglycemic	Perera and Li (2011)
Sarcodon imbricatus	Methanolic extract	Antimicrobial	Barros et al. (2007b)
Tremella fuciformis	Glucuronoxylomannan	Hypoglycemic	Kiho et al. (1994b)
Volvariella volvacea	β-gulcan	Hypocholesterolemic	Cheung (1996a)

Table 1.4: Selected medicinal mushrooms in traditional use and possible toxicity in China and Taiwan.

Species	Traditional use	Possible toxicity	References
Cordyceps militaris	Help the body to build up its strength and endurance, to alleviate fatigue, to ameliorate the functions of the upper respiratory tract and lungs.	No human toxicity has been reported, and animal models failed to find an LD50 (median lethal dose) injected i.p. in mice at up to 80 g/kg per day, with no fatalities after seven days.	Yu et al., (2004); Coates (2005)
Trametes versicolor	Use for dispelling heat, removing toxins, strengthening physique, increasing energy and spirit, and enhancing the host's immune function	The LD50 of crude CV extract administered orally in mice was greater than 18 g/kg. No death, toxic symptoms, or obvious haematological and pathophysiological changes were observed after a 3-month dosing period. No mutagenic and cytotoxic effects were detected with high doses of *T. versicolor* extract.	Chu et al., (2002)
Ganoderma spp,	Anti-cancer and immunomodulatory activities, and used for tonics and remedies for ailments such as cough, asthma, bronchitis and hepatitis	*G. lucidum* report toxicity in adult's and children's peripheral blood mononuclear cells.	Ions and Birmingham (1992); Paterson, (2006); Gill and Rieder (2008); Hsu et al., (2008)

Phellinus linteus	To treat stomach ache, arthritis and cancer of stomach, oesophagus and lung	May bring forth through interacting with such drugs as immunoregulatory antibodies or cytokines and potential risk of *P. linteus* intervention was implicated by the finding that administration of PL extract worsened benign prostatic hyperplasia in rats.	Hur et al., (2004) ; Zhu et al., (2008)
Wolfiporia extensa	Used as a diuretic, sedative, tonic and antitumour agent	Single-dose toxicity testing demonstrated that the LD_{50} of *W. extensa* was more than 50 g/kg, in mice. Repeated-dose toxicity testing, 5 g/kg or 10 g/kg orally administered to rats once daily for 14 days, demonstrated no changes in general symptoms, food consumption, water ingestion, and body weight	Plotnikoff (2005); Wu et al., (2005)

1.7.5.5 *Potential use of mushrooms*

Several pharmaceutical preparations have been developed based on specific compounds of mushrooms such as the polysaccharides lentinan (from *L. edodes*), krestin (from *T. versicolor*) and maitake D-fraction (from *G. frondosa*) (Cheung et al., 2008). In Taiwan, according to the Department of Health, cancer, cardiovascular disease and diabetes were the top three leading causes of death in 2011 (Yuan, 2012). In Taiwan, several types of selected culinary-medicinal mushroom product are available on the market today, such as *A. subrufescens*, *C. militaris*, *T. versicolor*, *G. lucidum* and *H. erinaceus*. However, only *C. militaris* and *G. lucidum* products have been licensed by Department of Health, Executive Yuan, R.O.C (Taiwan) and bear a label stating that a person using them for a specified health use may expect to obtain a health benefit through consumption (Department of Health Taiwan).

1.7.5.6 *Toxicity*

Approximately, there are 50 to 100 mushrooms known to be poisonous to humans (Berger and Guss, 2005). Based on the principal toxins present in the variety ingested, clinically, mushroom toxins are divided into several groups. The principal groups are cyclopeptide, orellanine, monomethylhydrazine, disulfiram-like, hallucinogenic-indoles, muscarinic, isoxazole and GI-specific irritants (Table 1.4) (Berger and Guss, 2005). *A. bisporus* and *Boletus edulis* are frequently involved in human poisoning. Between January 1995 and December 2009, the Swiss toxicological information centre received 160 calls about *A. bisporus*, 305 calls about *B. edulis* poisoning and 24 calls about *P. ostreatus* (Schenk-Jaeger et al., 2012).

Table 1.5: Toxic mushrooms classification (Berger and Guss, 2005).

Toxic group	Mushrooms	Clinical presentation
Cyclopeptide	*Amanita spp., Conocybe filaris, Galerina spp., Lepiota helveola*	Nausea, vomiting, Profuse abdominal pain, bloody diarrhoea, hematuria, hepatic enzymes rising, hepatic and renal failure, cardiomyopathy, encephalopathy, convulsions, coma, death
Orellanine	*Amanita smithiana; Cortinarius splendens; Cortinarius spp.*	Nausea, vomiting, diarrhoea, anorexia, abdominal cramps, ARF, thirst, decreased urine output.
Monomethylhydrazine	*Gyromitra spp., Helvella spp., Paxina spp., Sarcosphaera coronaria*	Nausea, vomiting, diarrhoea, abdominal cramps, delirium, seizures, coma, methemoglobinaemia
Disulfiram-like reaction	*Clitocybe claviceps; Coprinus atramentarius*	Headache, nausea, vomiting, flushing of face and trunk, hypotension, tachycardia, chest pain, apprehension
Muscarinic	*Boletus spp., Clitocybe spp., Inocybe spp.,*	Cholinergic syndrome: lacrimation, bradycardia, miosis, diaphoresis, salivation, bronchorrhea, bronchospasm,
Hallucinogenic-indoles	*Conocybe cyanopus, Gymnopilus spp., Panaeolus spp., Psilocybe spp., Psilocybe cubensis; Stropharia spp.,*	Hallucinations, unmotivated laughter, Euphoria, agitation, compulsive behaviour, dysphoria, fever/seizures in children
Isoxazoles	*Amanita spp., Panaeolus campanulatus*	Lethargy, stupor, alternating mania, delirium, hallucinations
GI-specific irritants	*Boletus spp., Russula emetica, Tricholoma spp.*	Nausea, vomiting, diarrhoea, abdominal cramps

AFR: Acute renal failure.

1.8 Aims and Objective

Mushroom extracts, therefore, have multiple and enormous therapeutic implications via a variety of mechanisms as discussed above. Mushrooms may be used directly as part of the diet to promote human health because of the synergistic effects of the bioactive compounds which are present in them. Mushrooms are ingredients widely used in the preparation of traditional Chinese medicine in China and Taiwan. Although research has so far focused on the therapeutic effects of speciality mushrooms, there is relatively little information about their antioxidant

properties and possible use in oxidative stress (Jayakumar et al., 2006). As an ingredient of traditional Chinese therapies, medicinal mushrooms are prepared in aqueous solution, and also most culinary mushrooms are prepared for consumption using aqueous methods (Chevallier, 1996). However, it remains unclear whether the beneficial effects of aqueous extracts of mushrooms are derived from their antioxidant properties. Therefore, the aim of this present study is to evaluate the antioxidant activities of medicinal and culinary mushroom extracts using crude extracts prepared according to traditional practices. Because the role of free radicals has been implicated in a large number of diseases as mentioned above, antioxidant activity is important in exploiting the potential therapeutic aspect of mushrooms. Proof of their antioxidant activity may also help to explain the mechanism of their therapeutic action.

The objective of this study, therefore, is to evaluate the antioxidant properties of commercially available and cultivated culinary and medicinal mushrooms in Taiwan. The hypothesis is that the health benefits of mushrooms may be due to their potent antioxidant compounds. The first aim of the study is to screen mushroom extracts (hot and cold water extracts) for their potential antioxidant activity by using Folin-Ciocalteu reducing capacity (FC), 2, 2'-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation (TEAC), ferric reducing antioxidant power (FRAP) and scavenging of 2, 2'-diphenyl-1-picrylhydrazyl radical (DPPH) antioxidant activity assays to evaluate the relative level of antioxidant activity of these selected mushrooms. The selected mushrooms are used in Taiwan in culinary purposes, or are traditionally used to treat various disorders in which free radicals are thought to be involved, and by this means to provide comparable data. Since mushrooms accumulate a variety of secondary metabolites such as phenolic compounds, polyketides, terpene and steroids, the selected mushrooms were also investigated in terms of their antioxidant activity in relationship to their total phenolic contents.

The phenolic compounds are one of the major group of primary antioxidants available for dietary as well as culinary purposes, consumption of mushrooms may provide potential antioxidant compounds, and the biological properties of potential antioxidant compounds depend on their bioavailability. The second aim, therefore, is

to investigate the *in vitro* availability of potential antioxidant compounds of mushrooms; the *in vitro* enzymatic digestion process will be employed to predict the release of potential antioxidant compounds of mushroom extracts within the human gastrointestinal tract (GI) tract system. The purpose of the present work was to evaluate potential antioxidant compounds released from traditional medicinal and culinary mushrooms using *in vitro* enzymatic digestion steps that mirror the human GI tract to observe whether GI condition could affect the antioxidant properties of mushroom extracts and to determine their stability during *in vitro* digestion. The objective was to investigate whether *in vitro* availability of those mushrooms could be used as a step prior to evaluating the antioxidant capacity.

To improve the understanding of the potential antioxidant activity of mushroom extracts, the third aim is to use a cellular model to obtain biologically relevant data on the bio-accessibility of potential antioxidants in mushrooms. In this study, the potential antioxidant compounds of mushroom extracts will be investigated using 2′-7′-dichlorofluorescin diacetate (DCFH-DA) as a fluorescent probe to estimate the cellular antioxidant activity and to compare the correlation between cellular antioxidant activity, total phenolic contents and chemical assays (TEAC, FRAP and DPPH). Since there is no published data which provide the cellular antioxidant activity of mushroom extracts, using cell culture is an important step as it provides more biologically relevant data than data obtained from chemical-based antioxidant activity assays. This combination of several chemistry-based antioxidant assays, *in vitro* enzymatic digestion steps and cellular antioxidant activity assays could provide a more reliable assessment of antioxidant activity.

Chapter 2 Materials and Methods

2.1 Materials and Chemicals

2.1.1 *Chemicals*

The chemicals used in this study were sodium acetate trihydrate ($CH_3COONa.3H_2O$), acetic acid (CH_3COOH), 2,4,6-tripyridyl-*s*-triazine (TPTZ), hydrochloric acid (HCl), 2,2´-azobis (2-amidinopropane) dihydrochloride (ABAP), ferrous sulphate,ferric chloride ($FeCl_3.6H_2O$), sodium chloride (NaCl), sodium dihydrogen phosphate (NaH_2PO_4 ^+H_2O), disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$), potassium persulphate($K_2S_2O_8$), 2,2´-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2- carboxylic acid) , Folin-Ciocalteu (FC), sodium carbonate anhydrous and gallic acid, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), 2´, 7´-dichlorofluorescin diacetate (DCFH-DA), glutaraldehyde, gentamic hepes, insulin, hydrocortisone, methylene blue, Hank's Balanced Salt Solution (HBSS), trypan blue and quercetin dehydrate, pancreatin and pepsin and were purchased from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) and acetic acid were obtained from Fisher Scientific. Foetal Bovine Serum (FBS), L-glutamine, PBS and penicillin/streptomcyin were purchased from PAA Laboratories Ltd. All other solvents were purchased from VWR International (Poole, Dorset, UK).

2.1.2 *Mushrooms*

All 29 mushrooms used in this study were purchased from China, Hong Kong, and from Nantou County and Chiayi County, Taiwan. The local mushroom farms, Taiwan, are all certificated with CAS label (Certified Agricultural Standards), and mushroom species are examined by Taiwan agricultural research institute, the Council of Agriculture, Taiwan. The mushrooms purchased from Peninsula farm with the support of The Chinese university and the funding from Innovation and Technology Commission, Hong Kong. The mushrooms from China were purchased from Liannan edible fungi research institute with the support with Guangzhou Agriculture technology research institute. The samples were freeze-dried before milling for use in assays. Since culinary and medicinal herbs are prepared for consumption using aqueous infusions/decoctions in traditional methods (Triantaphyllou, 2001), the

mushroom were prepared for measurement by aqueous extraction.

2.1.3 *Hot-water extract*

1g of each dried powdered mushroom was prepared by soaking in 20 mL de-ionized water (1:20 w/v) in a conical flask for thirty minutes then heating for thirty minutes in a boiling water bath. The extracts were filtered through Whatman No.1 filter paper and the filtrates were centrifuged at 4000 x *g* (gravity) for ten minutes to remove insoluble materials. The filtrates were freeze-dried.

2.1.4 *Cold-water extract*

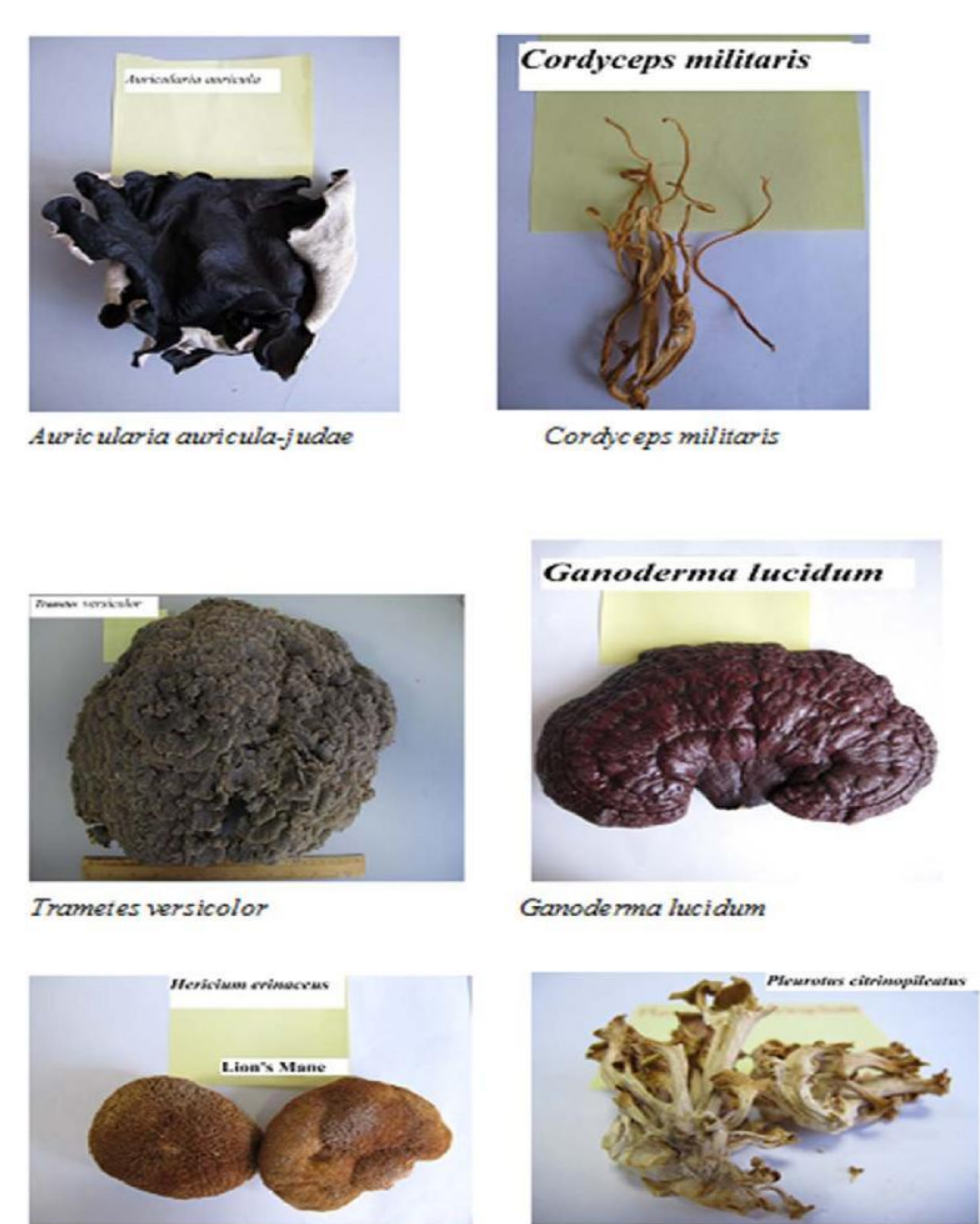
1g of each dried powdered mushroom was prepared by soaking in 20 mL de-ionized water (1:20 w/v) and shaking at room temperature at 150 rpm for twenty-four hours. The extracts were filtered through Whatman No.1 filter paper and the filtrates centrifuged at 4000 x *g* (gravity) for ten minutes to remove insoluble materials. The filtrates were freeze-dried.

The antioxidant activity of the extracts was determined using TEAC, FRAP and DPPH assays. The results of the TEAC and DPPH assays were expressed as Trolox equivalent per gram dry weight (d.w.), and the results of the FRAP assay were expressed as ferrous ion equivalent per gram dry weight (d.w.).

2.1.5 *Selection of the mushrooms for in vitro enzymatic digestion and cellular antioxidant assay*

Based on the previously performed antioxidant assays (described in detail in section 2.1), mushrooms were categorised into three groups as having low, moderate or high antioxidant activities. According to Council of Agriculture, Executive Yuan , Taiwan (2007) , *A. auricula-judae* , *H. erinaceus* , and *Pleurotus citrinopileatus* are commonly consumed in Taiwan (Yuan, 2007). Medicinal mushrooms known only for their medicinal properties are decidedly non-edible because of their coarse texture and bitter taste (Editorial Board of Zhong-Hua-Ben-Cao (China Herbal), 1999). Two mushrooms were selected per group – one medicinal mushroom and one culinary mushroom. *C. militaris* (a traditional medicinal mushroom) and *P. citrinopileatus* (a culinary mushroom) have been categorised into group A (high antioxidant activity), *T. versicolor* (a traditional medicinal mushroom) and *H. erinaceus* (a culinary mushroom) into group B (moderate antioxidant activity) and *G. lucidum* (a traditional medicinal mushroom) and *A. auricula-judae* (a culinary mushroom) assigned to group C (low

antioxidant activity) (Figure 2.1). These selected mushrooms were subjected to *in vitro* enzymatic digestion to investigate activity during the simulated human GI tract digestion and cellular antioxidant activity assay.



Hericium erinaceus *Pleurotus citrinopileatus*

Figure 2.1: Pictures of selected mushrooms for digestion and cellular assays.

Table 2.1: The origin of selected mushrooms.

Species	Orders	Families	Origin
Agaricus bisporus	Agricales	Agricaceae	Peninsular farm,HK
Agaricus subrufescens	Agricales	Agricaceae	Nantou, Taiwan
Agaricus subrufescens	Agricales	Agricaceae	Peninsular farm,HK
Agrocybe cylindracea	Agricales	Strophariaceae	Peninsular farm,HK
Agrocybe cylindracea	Agricales	Strophariaceae	Nantou, Taiwan
Auricularia auricula-judae	Auriculariales	Auriculariaceae	Chiayi, Taiwan
Auricularia auricula-judae	Auriculariales	Auriculariaceae	Nantou, Taiwan
Boletus edulis	Boletales	Boletaceae	Nantou, Taiwan
Coprinus comatus	Agaricales	Agricaceae	Nantou, Taiwan
Cordyceps militaris	Hypocreales	Clavicipitaceae	Chiayi, Taiwan
Trametes versicolor	Polyporales	Polyporaceae	Natou, Taiwan
Ganoderma amboinense	Polyporales	Ganodermataceae	Chiayi, Taiwan
Ganoderma atrum	Polyporales	Ganodermataceae	Yunnan, China
Ganoderma lucidum	Polyporales	Ganodermataceae	Yen-Ten farm, Taiwan
Ganoderma sinense	Polyporales	Ganodermataceae	Lianan Yao autonomous county, China
Grifola frondosa	Polyporales	Meripilaceae	Nantou, Taiwan

Hericium erinaceus	Russulales	Hericiaceae	Nantou, Taiwan
Hericium erinaceus	Russulales	Hericiaceae	Lianan Yao autonomous county, China
Lentinula edodes	Agaricales	Marasmiaceae	Nantou, Taiwan
Phellinus linteus	Hymenochaetales	Phellinus	Natou, Taiwan
Pleurotus citrinopileatus	Agaricales	Pleurotaceae	Nantou, Taiwan
Pleurotus citrinopileatus	Agaricales	Pleurotaceae	Peninsular farm, HK
Pleurotus eryngii	Agaricales	Pleurotaceae	Nantou, Taiwan
Pleurotus eryngii	Agaricales	Pleurotaceae	Peninsular farm ,HK
Pleurotus ostreatus	Agaricales	Pleurotaceae	Lianan Yao autonomous county, China
Wolfiporia extensa	Polyporales	Fomitopsidaceae	Lianan Yao autonomous county, China
Sarcodon imbricatus	Thelephorales	Bankeraceae	Yunnan, China
Tremella fuciformis	Tremellales	Tremellaceae	Nantou, Taiwan
Volvariella volvacea	Agaricales	Pluteaceae	Peninsular farm, HK

Table 2.2: Mushroom growing Substrates.

Species	Origin	Subtracts
Agaricus bisporus	Peninsular farm,HK	Unknown
Agaricus subrufescens	Nantou, Taiwan	Rice straw , lime, urea and rice bran
Agaricus subrufescens	Peninsular farm,HK	Unknown
Agrocybe cylindracea	Peninsular farm,HK	Unknown
Agrocybe cylindracea	Nantou, Taiwan	foliage tree sawdust, cottonseed hull, oat, corn starch, tea seed hull powder and calcium carbonate
Auricularia auricula-judae	Chiayi, Taiwan	foliage tree sawdust, rice bran, calcium carbonate, and sucrose
Auricularia auricula-judae	Nantou, Taiwan	Unknown
Boletus edulis	Nantou, Taiwan	Unknown
Coprinus comatus	Nantou, Taiwan	Sawdust, rice bran, barley powder, and calcium carbonate
Cordyceps militaris	Chiayi, Taiwan	Rice powder, silkworm chrysalis pupa powder Vitamin B, sucrose
Trametes versicolor	Natou, Taiwan	Unknown
Ganoderma amboinense	Chiayi, Taiwan	Sawdust (*Acacia confuse*), calcium carbonate, and rice bran

Ganoderma atrum	Yunnan, China	Unknown
Ganoderma lucidum	Yen-Ten farm, Taiwan	Sawdust (*Acacia confuse*), calcium carbonate, and rice bran
Ganoderma sinense	Lianan Yao autonomous county, China	Unknown
Grifola frondosa	Nantou, Taiwan	foliage tree sawdust, rice bran
Hericium erinaceus	Nantou, Taiwan	foliage tree sawdust, rice bran, lime
Hericium erinaceus	Lianan Yao autonomous county, China	Unknown
Lentinula edodes	Nantou, Taiwan	foliage tree sawdust, rice bran, corn starch, and barely powder
Phellinus linteus	Natou, Taiwan	Unknown
Pleurotus citrinopileatus	Nantou, Taiwan	Unknown
Pleurotus citrinopileatus	Peninsular farm, HK	Unknown
Pleurotus eryngii	Nantou, Taiwan	Unknown
Pleurotus eryngii	Peninsular farm ,HK	Unknown
Pleurotus ostreatus	Lianan Yao autonomous county, China	Unknown
Wolfiporia extensa	Lianan Yao autonomous county, China	Unknown
Sarcodon imbricatus	Yunnan, China	Unknown
Tremella fuciformis	Nantou, Taiwan	Unknown
Volvariella volvacea	Peninsular farm, HK	Unknown

2.2 Antioxidant assays

2.2.1 *The Chemical assays*

During the past two decades, there have been many investigations into the antioxidant ability of plant materials or foodstuffs using several methods and strategies (Prior et al., 1998; Sánchez-Moreno, 2002). Because of the relationship between free radicals and human diseases, and the relevance that plant-derived antioxidants may have in mammals, a large number of investigations of the potential antioxidant capacities of traditional medicinal herbs, due to their long history of use and effects, have been conducted. For example, the rhizome of *Salvia miltiorrhiza* Bunge (Lamiaceae) (DanShen) in China is administered as a traditional Chinese medicine to treat diseases that are caused by oxidative stresses, for example ischemia and inflammation. In the Indian traditional medicine system (Ayurveda), *Phyllanthus emblica* L has been used for thousands of years in the treatment of several diseases or used as a rejuvenator to slow the process of ageing and related disorders. *S. miltiorrhiza* and *P. emblica* are known to scavenge free radicals (Scartezzini and Speroni, 2000; Auddy et al., 2003; Zhu et al., 2004).

In general, most of the methods used to study antioxidants are based on generating free radicals in the presence of the antioxidant that potentially inhibits the oxidation of substrates (Sánchez-Moreno, 2002). Further, the mechanisms of oxidants and antioxidants are much more complex than one simple chemical assay. Different oxidants have different chemical and physical features that may cause antioxidants to respond to different oxidants or radicals with different redox mechanisms. Therefore, a single antioxidant assay cannot provide adequate data on the antioxidant ability of analytes. In order to elucidate the total antioxidant ability of analytes, using different antioxidant assay methods to evaluate the total antioxidant ability of the same sample is needed (Frankel and Meyer, 2000; Ronald et al., 2005).

To determine the antioxidant ability of foods and biological antioxidants, based on simplicity, cost-effectiveness and easy accessibility, there are four frequently used methods: scavenging of 2, 2′-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$) or trolox equivalent antioxidant capacity (TEAC assay), ferric reducing antioxidant power (FRAP assay), scavenging of 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$ assay) and Folin-Ciocalteu reducing capacity (FC assay). A large number of antioxidant capacity evaluations of plant

material have been published based on these antioxidant assays (Triantaphyllou, 2001; Zheng and Wang, 2001; Hinneburg et al., 2006; Stratil et al., 2006; Wong et al., 2006a; Stratil et al., 2007; Wojdylo et al., 2007). Taking this into account, in this study, the levels of antioxidant activity of selected medicinal and culinary mushrooms were evaluated using FC, TEAC, FRAP and DPPH assays.

2.2.2 *Scavenging of 2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation (ABTS$^{\bullet+}$)*

A modification of the TEAC assay was carried out using the method of Re *et al.* (1999). The long-lived radical cation chromophore 2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation (ABTS $^{\bullet+}$) was generated by reacting ABTS stock solution with potassium persulfate. Specifically, 7 mM of ABTS stock solution was mixed with 2.45mM potassium persulfate at a ratio 9:1 (v/v) (both solutions were prepared in deionised water) and the mixture was allowed to stand in the dark at room temperature for twelve hours before use. The ABTS$^{\bullet+}$ working solution is stable for up to five days if refrigerated. For the study of mushroom antioxidant capacity, the ABTS $^{\bullet+}$ solution was diluted with 5mM phosphate buffer solution (PBS), pH 7.4, to an absorbance of 0.7 (± 0.2) at 734nm and equilibrated at 30°C. The assay was performed in a 96-well microplater reader. The protocol was as follows: 10μL (0.05mg/ μL) of mushroom extracts or Trolox standard solution were mixed with 290μL of ABTS$^{\bullet+}$ radical cation solution and incubated for six minutes at 37°C. The reduction of ABTS$^{\bullet+}$ radical cation by adding compounds that contain antioxidants was measured by the change of absorbance of ABTS$^{\bullet+}$ radical cation at 734nm (Figure 2.1). A standard solution of Trolox was prepared at a range of 0.1 to 0.5mM in ethanol (HPLC grade, BDH). The quantification of TEAC equivalent was calculated by reference to the Trolox solution on a molar basis (Re et al., 1999) (Figure 2.2). Additional dilution was needed if the TEAC value measured was over the linear range of the standard curve. A corresponding blank was treated in exactly the same manner as the other samples. All determinations were performed in triplicate (*n*=3) (Re et al., 1999).

antioxidant

K_2SO_5

ABTS$^{\cdot-}$ (λ_{max} = 734 nm)

ABTS^{2-} (colorless)

Figure 2.1: ABTS reaction (Huang et al., 2005).

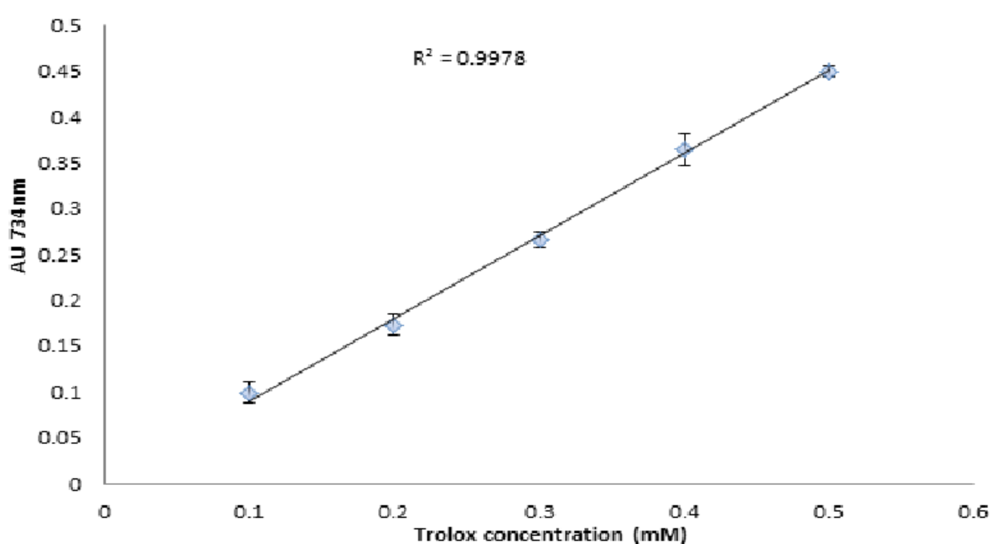


Figure 2.2: TEAC Standard Calibration Curve. Each value is expressed as mean ± standard deviation (n=3).

2.2.3 *Ferric reducing antioxidant power (FRAP assay)*

The FRAP assay was first described by Benzie and Strain (1996). The method is based on measuring the reduction of the ferric 2, 4, 6-tripyridyl-*s*-triazine complex (Fe^{3+}-TPTZ) to the intensely blue ferrous form (Fe^{2+}-TPTZ) (Figure 2.3). The assay in this current study was carried out according to the method of Benzie and Strain (1996) with minor modification. Briefly, Fe^{3+}-TPTZ was dissolved into 40mM hydrochloric acid to a 10mM of Fe^{3+}-TPTZ solution, and the working solution was mixed with 2.5mL of 10mM Fe^{3+} -TPTZ solution with 25mL of 300mM sodium acetate buffer of pH 3.6 (3.1g sodium acetate an 16mL acetic acid per litre), plus 2.5mL of 20mM ferric chloride ($FeCl_3.6H_2O$). Then 2mM of ferrous sulphate standard solution was diluted to a range from 0-1000μM in deionised water. An aliquot of 10μL (0.05mg/μL) of mushroom extract (or ferrous sulphate standard solution or water for blank) was mixed with 300μL of FRAP working solution and incubated at 37°C for four minutes. FRAP absorbance values were measured at 593nm over the incubation period solution using a 96-well microplate reader and relating it to a ferrous iron standard (Figure 2.4). Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve. A corresponding blank was treated in exactly the same manner as the other samples. All determinations were performed in triplicate ($n = 3$) (Benzie and Strain, 1996).

Figure 2.3: FRAP Reaction (Huang et al., 2005).

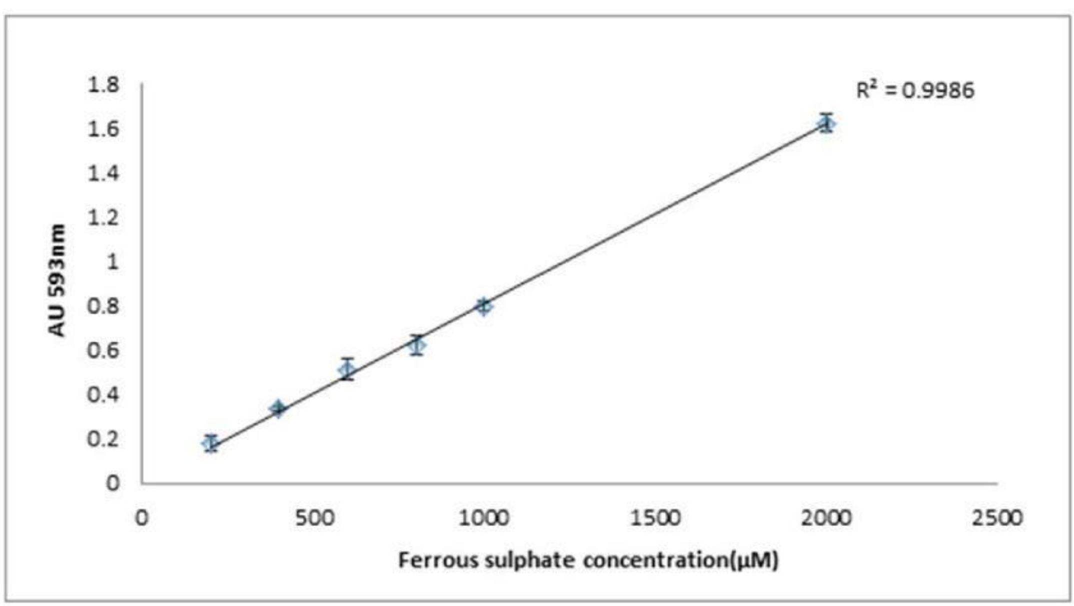


Figure 2.4: FRAP Standard Calibration Curve. Each value is expressed as mean ± standard deviation (n=3).

2.2.4 ***Scavenging of 2, 2-diphenyl-1-picrylhydrazyl radical*** **(*DPPH*● *assay*)**

A modification of the 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH●) method of Brand-Williams *et al*. (1995) and Thaipong *et al*. (2006) was adopted (Figure 2.5). A concentration of 0.6mM of DPPH● stock solution was prepared in methanol (HPLC grade, BHD) and stored at -20°C prior to analysis. The DPPH● working solution was obtained by mixing 30mL stock solution with 80mL methanol to obtain the final concentration of 0.16mM. The solution was stored in a flask covered with aluminium foil, and kept in the dark at 4°C between the measurements. 10μL (0.05mg/μL) of trolox standard or mushroom extracts and 300μL of methanolic solutions of DPPH● were mixed with 30μL deionised water as a diluter. The mixture was incubated for thirty minutes at 30°C in the dark. The decrease in absorbance was measured at 517nm, using a 96-well microplate reader against a water or methanol blank. A blank sample containing the same amount of water or methanol and DPPH solution was prepared freshly. The absorbance values of the mushroom extracts were interpolated in a trolox calibration curve, and the antioxidant status of the test sample was expressed on a molar basis (Figure 2.6). Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve. A corresponding blank was treated in exactly the same manner as the other samples. All determinations were performed in triplicate (n = 3) (Brand-Williams et al., 1995; Thaipong et al.,

2006).

Figure 2.5: DPPH assay (Molyneux, 2004).

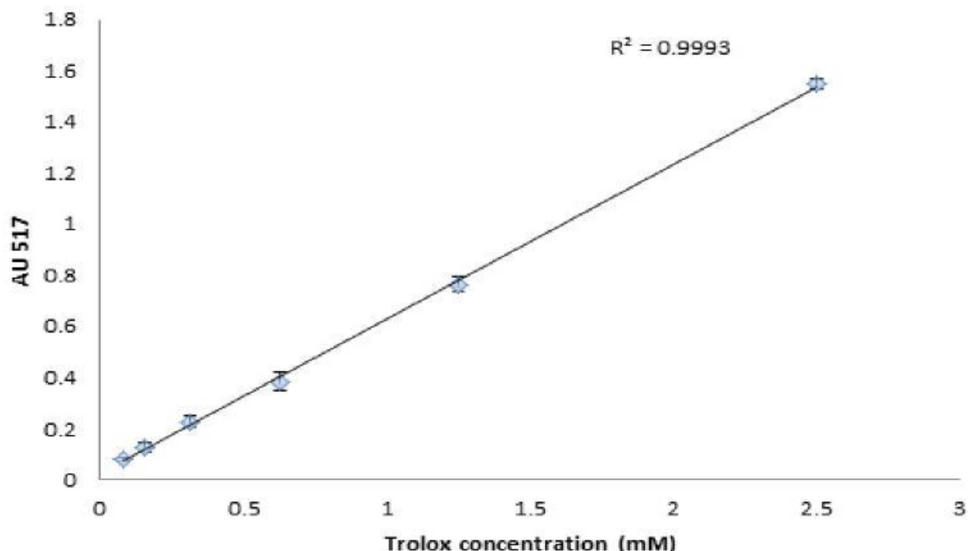


Figure 2.6: DPPH Standard Calibration Curve. Each value is expressed as mean ± standard deviation (n=3).

2.2.5 *Folin-Ciocalteu reducing capacity* (*FC assay*)

The total phenolic contents of the mushroom extracts were assessed using the Folin-Ciocalteu phenol reagent as described by Zang Q. *et al.* (2006) with slight modification. Before the analysis, a commercial Folin-Ciocalteu phenol reagent was diluted with deionised water in 1:10 (v/v). 10μL of serial dilution of standard solution or each mushroom extract was pipetted into a 96-well micro plate and 130μL of

diluted Folin-Ciocalteu phenol reagent added and mixed well. After five minutes, 100µL of 7.5% sodium carbonate solution was added. The absorbance at 750nm was measured thirty minutes after incubation at 40°C. Gallic acid was used as a reference standard at 100mg/L, and serial dilution was carried out accordingly at 100, 50, 25, 12.5, 6.25, 3.125, 1.57 and 0.78µg ml^{-1} (Figure 2.7). The total phenolic contents were expressed as gallic acid equivalent (GAE) (Zhang et al., 2006). Additional dilution was needed if the FC value measured was over the linear range of the standard curve. A corresponding blank was treated in exactly the same manner as the other samples. All determinations were performed in triplicate (*n* = 3).

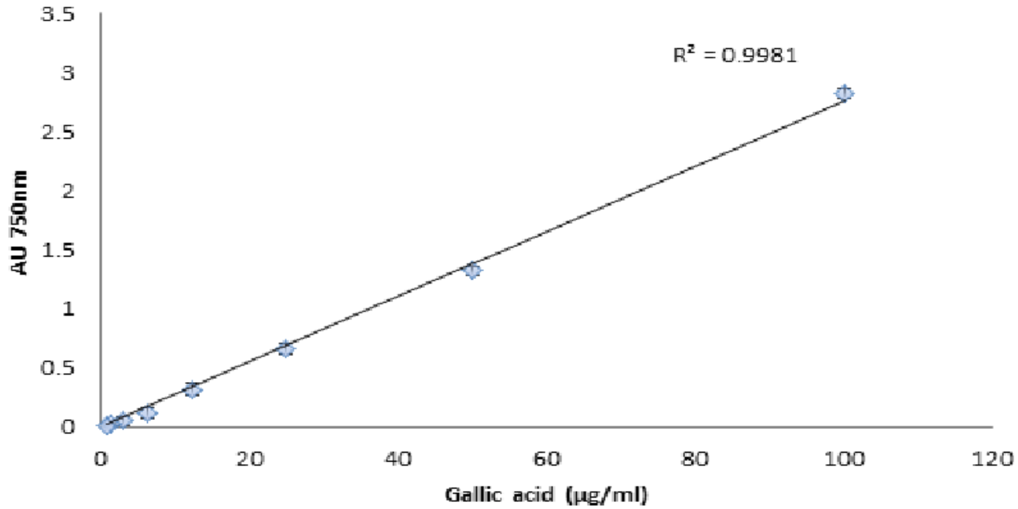


Figure 2.7: Phenolic Content Standard Calibration Curve. Each value is expressed as mean ± standard deviation (n=3).

2 .3 *In vitro* enzymatic digestion assay

The GI tract is constantly exposed to harmful substances since humans may consume millimoles of various oxidation products per day (Halliwell and Gutteridge, 2007; Corona et al., 2009; Halliwell, 2009). Vitamin C, vitamin E, carotenoids and phenolic compounds have often been suggested to have the ability to delay or prevent cancer development by being absorbed from the GI tract into the plasma and from there into the tissue, where they might decrease levels of oxidative DNA damage. High levels of phenols existing in the stomach, small intestine and colon could

therefore conceivably exert antioxidant and other protective effects there (Halliwell et al., 2000).However, the biological properties of these compounds are dependent on their bio-availability. In nature, these antioxidant compounds are normally present in foods and beverages in various chemical forms such as polymeric complexes. To access gut absorption, adequate degrading is required (Scalbert and Williamson, 2000; Troncoso, 2001; Serrano et al., 2005). The bio-availability of some phenolic compounds has been studied in humans by measuring their concentration in plasma and urine post-ingestion (Scalbert and Williamson, 2000). Some of these antioxidant compounds can be inert and digested as they pass along the human GI tract, a process that could perhaps degrade the antioxidant and pharmacological properties of the parent molecules by pre-absorption (Ryan and Prescott, 2010).

A number of studies have shown that some phenolic compounds are present in human plasma and other biological fluids after absorption (Troncoso, 2001). Studies have also shown that hesperetin and naringenin, flavanones present in orange juice, can be detected in plasma with maximal concentration after the ingestion of the orange juice (Gil-Izquierdo et al., 2003). Another study has shown that the absorption of orally administered quercetin was 24%, while the absorption of quercetin glycoside from onion was 52%, 17% for querctein-3-rutinoside and 24% for quercetin aglycone (Hollman et al., 1995). It is therefore important to determine how the digestion process affects the potential antioxidant compounds and their stability.

There has been a recent increase in research into understanding the fate of molecular, physicochemical and physiological processes during the consumption of food products which are believed to benefit human health (McClements et al., 2008). In order to extract the nutrients required for maintaining health, the human body has developed a complex system to break down foods. To simulate these physiological conditions, *in vitro* human GI tract delivery systems have been designed to release bioactive compounds at a specific location during digestion (Hur et al., 2011).

Several applications of *in vitro* biochemistry models have been designed to provide rapid representative sampling at any time point instead of the complex, multi-stage process of human and animal studies which are technically difficult, ethically challenging and expensive to perform (Gil-Izquierdo et al., 2001; Pérez-Vicente et al., 2002; Nagah and Seal, 2005; Wickham et al., 2009). Many of the

in vitro models involve changes in pH and activity of proteolytic enzymes by acidification with hydrochloric acid, the addition of gastric enzymes followed by a varying delay simulating gastric residence time, neutralisation with sodium carbonate or hydroxide, and the addition of pancreatic enzymes and bile salts all the while stirring at 37°C (Wickham et al., 2009). Since it is well known that antioxidant activity can be strongly influenced by many factors (such as the antioxidant concentration, temperature, pH of the medium, and existing chemicals which may act either as positive or negative synergism), it would be interesting to evaluate the release of potential antioxidant compounds from mushroom extracts and possible chemical transformation due to the enzyme and pH condition of the stomach and small intestine (hydrolysis and other chemical transformations).

After all these digestive treatments, the surviving antioxidants have to be absorbed and pass through the intestinal enterocyte barrier to reach the lymph or the blood. The ingested food matrix can considerably limit or enhance the absorption process. Mushrooms are rich in indigestible compounds such as polysaccharides which human intestinal enterocytes might not be able to absorb (Cheung et al., 2008; Soler-Rivas et al., 2009). Therefore, the current study was designed to investigate the antioxidant availability of culinary and medicinal mushrooms after *in vitro* enzymatic digestion.

2.3.1 *In vitro enzymatic digestion procedure*

The *in vitro* enzymatic digestion steps were based on the method described by Aura *et al.* (1999) and modified by Nagah and Seal (2005). Briefly, 1mL of mushroom extract was placed in a 50mL plastic, screw-topped tube and five glass marbles added; 19mL of deionised water was added and the tubes were incubated in a shaking water bath at 37°C and at 90 stroke/min^{-1}. After ten minutes, 10 mL of 0.05M HCl was added to each tube (simulating pH conditions in the stomach). After twenty minutes incubation, one mg pepsin dissolved in 0.5ml of HCl was added. Then after further 20 minute incubation, 1mL of 0.5M NaOH was added (simulating pH conditions in the small bowel). Finally, after 20 minutes, 5mL of pancreatin was added (from 3g of pancreatin dissolved in 20mL of deionised water, the tubes were centrifuged for ten minutes at 1500g and then 15mL of supernatant sufficient for three tubes, removed) was added and incubated for twenty minutes. Each extract was run in

triplicate. Aliquots of 1mL were taken from each tube immediately before each addition and stored at -20°C for antioxidant analysis. A corresponding blank was treated in exactly the same manner as the other samples for each experiment to detect if the reagents themselves had any antioxidant activity.

2.4 Cellular antioxidant activity assay

The representative dietary antioxidant examples such as vitamins, carotenoids, phenolics and flavonoids have been evaluated for their antioxidant ability using different measurement methods, many of which are chemical-based assays (Velioglu et al., 1998; Cai et al., 2004; Ozgen et al., 2006; Wojdylo et al., 2007). These methods do not address the uptake, distribution and metabolism of antioxidants in the cells. The lack of *in vivo* relevant results to predict the antioxidant activity is a critical issue (Wolfe and Liu, 2007).

Natural antioxidants have been assumed to lead to decreased oxidative damage in key molecules as has already been stated. Many of the conclusions about antioxidant activity are based on chemical experiments, which are not necessarily reflected in the biological system (Wolfe and Liu, 2008). With different antioxidant properties, every available chemical-based assay has its specific benefits and drawbacks because each reaction is based on detecting the antioxidant interference caused by chemical reactions (Xu and Chang, 2010). For example, pure antioxidant compounds do not show any clear correlation between TEAC and FRAP values and the number of electrons that an antioxidant can react with oxidants. Ascorbic acid, α-tocopherol, glutathione and uric acid have similar TEAC values (Huang et al., 2005), however, normally glutathione donates one electron while the others donate more electrons. Quercetin and kaempferol have very different TEAC values although they have a similar chemical structure (Huang et al., 2005). FRAP results suggest that 1mol of ascorbic acid can reduce 2mol of Fe(III) and that 1mol of bilirubin can reduce 4mol Fe(III) (Huang et al., 2005). In contrast, both vitamin C and bilirubin are two-electron reductants (Huang et al., 2005). Genistein and daidzein have shown an effective effect on scavenging the ABTS free radicals in a TEAC assay but poor reducing power in a FRAP assay. Further, both of them had no activity in cellular antioxidant assays

(Huang et al., 2005).

Living cells are protected from oxidative damage through several defence mechanisms such as the enzymatic conversion of ROS into less toxic substances and through detoxification by reaction with antioxidants (Cotgreave et al., 1988; Dean et al., 1993; Aruoma, 1996). Lipids are recognized as important compounds in various metabolic pathways and also important compounds of the cell membrane. When biological membranes are attacked by free radicals, the rheology of membrane lipid is changed, and it will affect several biological events. Exposing red blood cells to oxidative stress showed that inhibition of lipid peroxidation could provide little protection against protein modification (Lissi et al., 1991). These biological functions of lipid membrane are susceptible to ROS damage. Therefore, lipid peroxidation of membrane functions and the protective role of antioxidants in membranes have motivated this investigation. With rapidly increasing evidence suggesting that oxidative stress-induced damage is involved in many pathologies, a number of approaches have been developed to measure cellular oxidative stress, such as electron spin resonance (Ramos et al., 1992), luminol (LM) and lucigenin (LC) (Faulkner and Fridovich, 1993) and dihydroethidium (DHE) (Zhao et al., 2003b). Using *in vitro* experiments with cultured mammalian or bacterial cells to measure antioxidant activity can reflect the real activity of potential antioxidant compounds incorporated into the cell. For analysis of reactive oxygen species (ROS), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) is widely applied in cell culture. DCFH-DA has been employed as a fluorogenic probe and provides a convenient and sensitive way to detect intracellular oxidants (Wang and Joseph, 1999; Mody et al., 2001; Girard-Lalancette et al., 2009).

DCFH-DA was first used for measuring hydrogen peroxide levels in a cell free system by adding alkali to remove the diacetate moiety (Keston and Brandt, 1965). It has been extensively used in the detection of oxidant production during the respiratory burst in inflammatory cells, or in cell lysates (Tarpey et al., 2004). Because it is highly sensitive to ROS, widely available and easy to use, DCFH-DA has been used as a potential probe for studying ROS generation (Hempel et al., 1999; Afzal et al., 2003). When non-fluorescent DCFH-DA compounds are taken up by cells, the diacetate moiety will be hydrolysed enzymatically by intracellular cellular esterases and

converted to non-fluorescent DCFH. The polar DCFH compounds will be retained within cells. When oxidants are present, DCFH is oxidized to highly fluorescent compounds (DCF) which can be measured with excitation at 485nm and emission at 538nm. When antioxidant compounds are able to penetrate into the cells and scavenge oxidants, the oxidization of the DCFH reaction will be quenched. Therefore, the intracellular DCF fluorescence can be used as an index to quantify the cellular antioxidant activity (Karadag et al., 2009) (Figure 2.8).

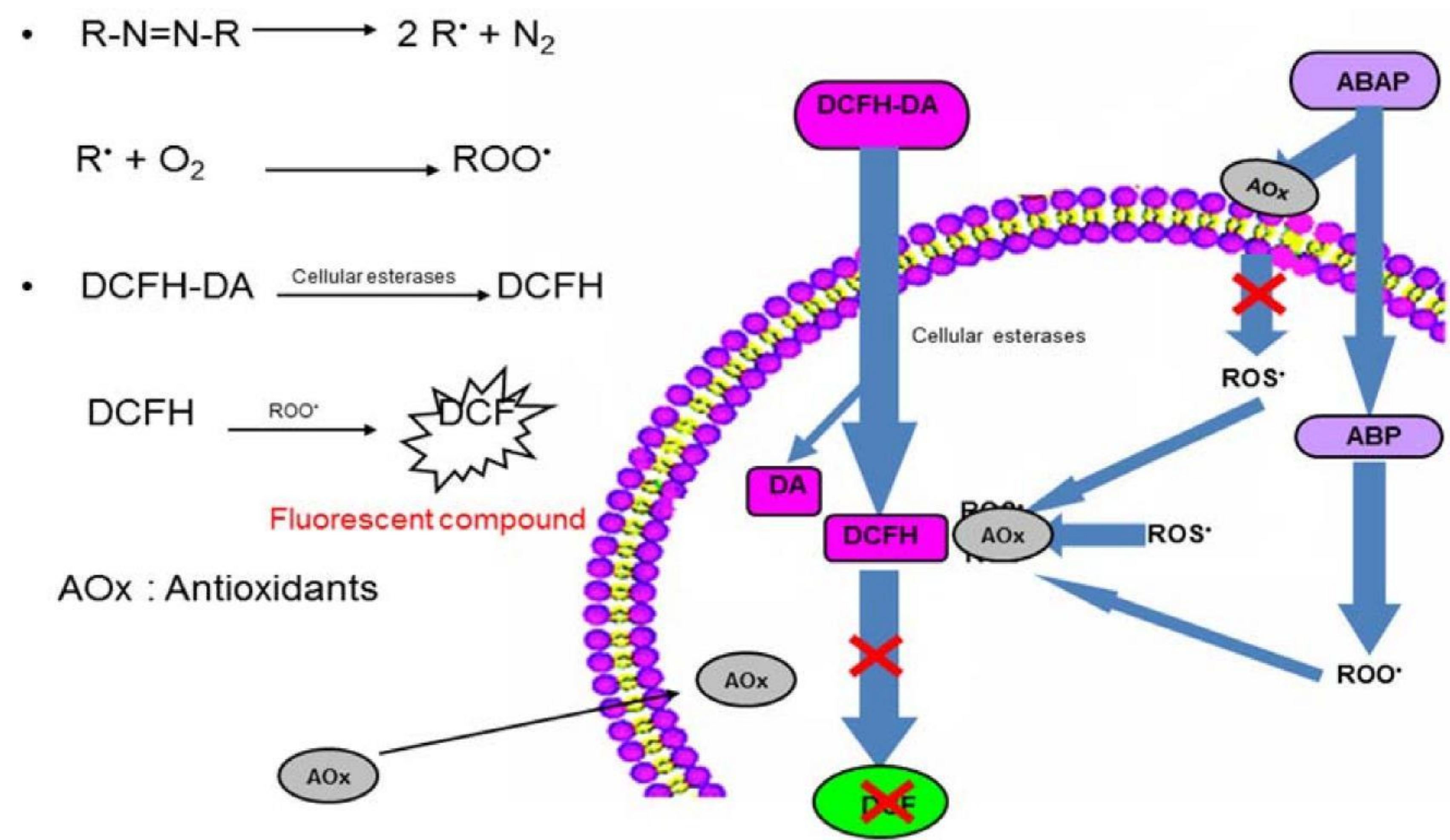


Figure 2.8: Schematic of DCFH-DA (Wolfe and Liu, 2007).

2.4.1 *Cell Culture*

Human hepatocellular carcinoma (Huh7) cells were grown in a growth medium (WME supplemented with 5% FBS, 10mM of hepes, 2mM of L-glutamine, 5μg/mL of insulin, 0.05μg/mL of hydrocortisone, 50units/mL of penicillin, 50μg/mL streptomycin, and 100 μg/mL gentamicin) and were maintained at 37°C and 5% CO_2. Cells used in this study were between passages 75 to 85.

2.4.2 *Cytotoxicity*

Cytotoxicity was determined by a methylene blue assay. Huh7 cells were seeded at 4×10^4 cells/well on a 96-well microplate in 100μL of growth medium and incubated for twenty-four hours at 37°C. The medium was removed, and the cells were washed with PBS. Treatment of the mushroom extracts or antioxidant compounds in 100μL treatment medium (WME supplemented with 2mM L-glutamine and 10mM hepes) was applied to the cells, and the plates were incubated at 37°C for twenty-four hours. The treatment medium was removed, and the cells were washed with PBS. A volume of 50μL/well methylene blue staining solution (98% HBSS, 0.67% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37°C for one hour. The dye was removed, and the plate was immersed in deionized water three times, or until the water was clear. The water was tapped out of the wells, and the plate was allowed to air-dry briefly before 100μL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a bench-top shaker for twenty minutes to allow uniform elution. The absorbance was measured at 570 nm with blank subtraction using a plate reader.

2.4.3 *Cellular antioxidant activity*

The *in vitro* cellular antioxidant activity assays were based on the method described by Wolfe and Liu (2007) with minor modified. Huh7 cells were seeded at a density of 6×10^4 cells/well on a 96-well microplate in 100μL of growth medium/well. The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Twenty-four hours after seeding, the growth medium was removed and the wells were washed with PBS. Triplicate wells were treated for one hour with 100μL mushroom extract plus 25μM 2′, 7′-dichlroofluoresin diacetate (DCFH-DA) dissolved in treatment medium. When a PBS wash was utilized, the wells were then washed with 100μL of PBS. Then 1mM of ABAP was applied to the

cells in 100µL of HBSS, and the 96-well microplate was placed into a fluorescent microplate reader at 37°C. Emission at 538 nm was measured with excitation at 485 nm every five minutes for one hour. Each plate included triplicate control and blank wells; control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with dye and HBSS without oxidant. In each experiment, quercetin was used as a standard, and cellular antioxidant activities were expressed as micromoles (µmol) of quercetin equivalents (QE) per gram of dried weight (d.w.) (Wolfe and Liu, 2007). The quercetin was in serial concentrations of 10, 8, 6, 4, 2 and 1µmol (Figures 2.9).

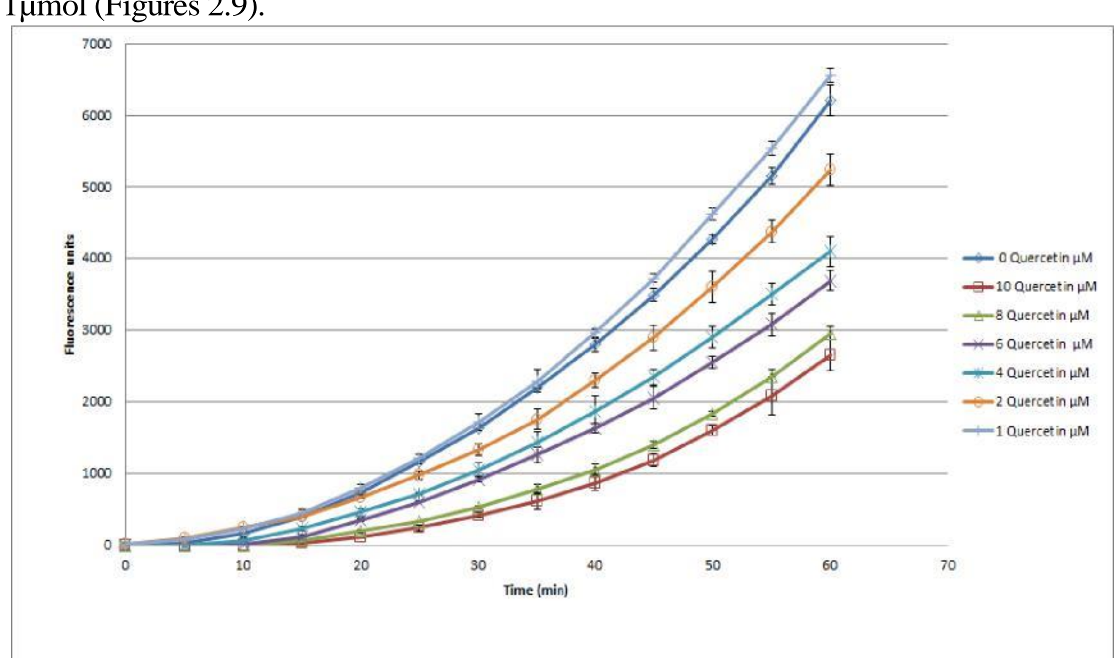


Figure 2.9: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by quercetin. Each value is expressed as mean ± standard deviation (n=3

2.4.4 *Quantification of cellular antioxidant activity*

After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus time was integrated to calculate the cellular antioxidant activity at each concentration of mushrooms as follows:

Cellular antioxidant activity unit = $100 - (\int SA / \int CA) \times 100$

where ∫ SA is the integrated area under the sample fluorescence versus time curve

and ∫CA is the integrated area from the control curve (Figure 2.10).

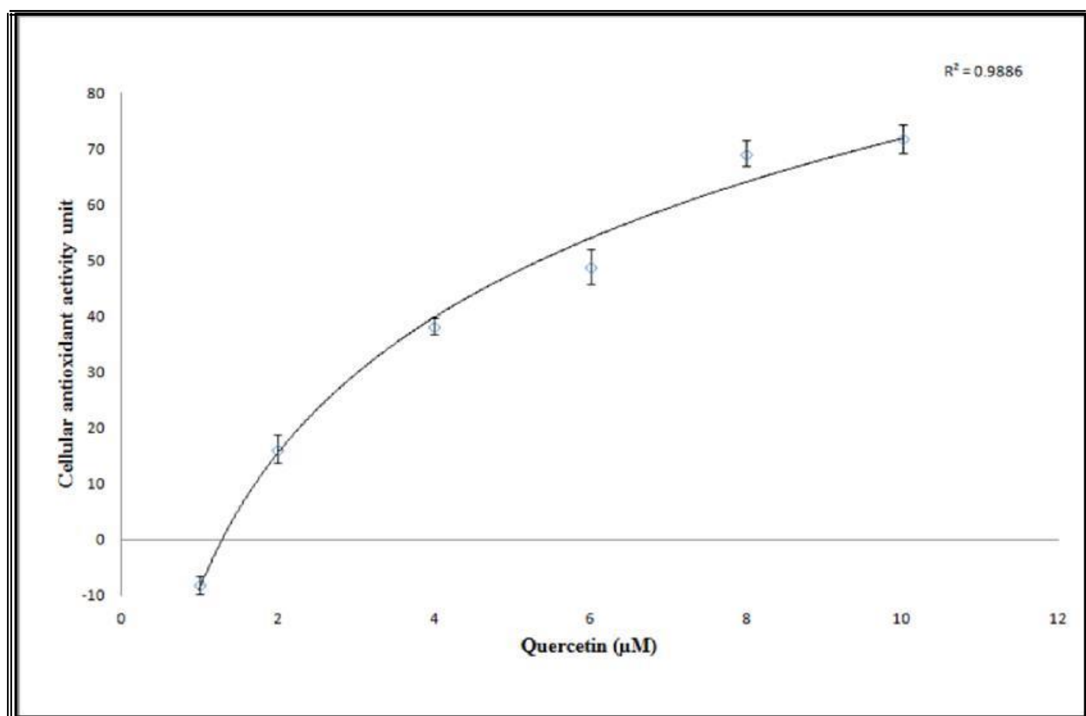


Figure 2.10: Dose-response curve for the inhibition of peroxyl radical-induced DCFH oxidation by quercetin. Each value is expressed as mean ± standard deviation (n=3).

2.5 Statistical analysis

All the results were presented as means ± standard deviation (SD). Analysis of variance was performed on the data taken from triplicates. Differences were detected by one-way analysis for variance (ANOVA) followed by multiple comparisons using the Fisher least significant difference test. The change in potential antioxidant compound release for each mushroom extract during the *in vitro* enzymatic digestion steps was assessed by the differences between ten minutes and ninety minutes of the enzymatic incubation step for each sample individually using paired-sample t-tests. All data were tested for distribution and if data were not a normal distribution, square root data transformation was employed before the correlation test. Correlations were obtained by Pearson correlation coefficient. Significance of differences between means was determined at the level of 0.05 ($p<0.05$). All the statistical tests were completed using Minitab 16 and Microsoft Excel.

Chapter 3 Results

3.1 Total phenolic contents

The amount of total phenolic contents, measured by Folin-ciocalteu methods, varied widely in mushroom materials and ranged from 203 to 7734µg GAE/g of d.w and 126 to 9741µg GAE/g of d.w in hot and cold-water extracts respectively (*see* Table 3.1).

In the hot-water extracts, the highest phenolic contents of the medicinal mushrooms tested were in *C. militaris* (7734µg GAE/g of d.w.), followed by *G. sinense*, *T. versicolor*, *P. linteus* , *Ganoderma amboinense* ,*G. lucidum*, *Ganoderma atrum*, *W. extensa* (109 ±7µg GAE/g of d.w.) in the hot-water extracts ($p< 0.001$) (*see* Tables 3.1 and 3.2 and Figure 3.1). In the cold-water extracts, the phenolic contents of the medicinal mushrooms tested were *C. militaris* (7313µg GAE/g of d.w.), *T. versicolor*, *G. amboinense*, *G. atrum*, *G. sinense*, *G. lucidum*, *P. linteus*, and *W. extensa* ($p< 0.001$) (Tables 3.1 and 3.2 and Figure 3.1).

In culinary mushrooms, the highest total phenol contents of the hot-water extracts of *V. volvacea* (5082µg GAE/g of d.w) had highest phenolic content, followed by *Agricus subrufescens*, *A. cylindracea*, and *A. bisporus*. In the cold-water extracts the highest levels of total phenol contents was found in the *P. citrinopileatus* (9741µg GAE/g of d.w), followed by *A. bisporus*, *A. cylindracea* and *A. subrufescens* ($p< 0.001$) (Tables 3.1and 3.2 and Figure 3.1).

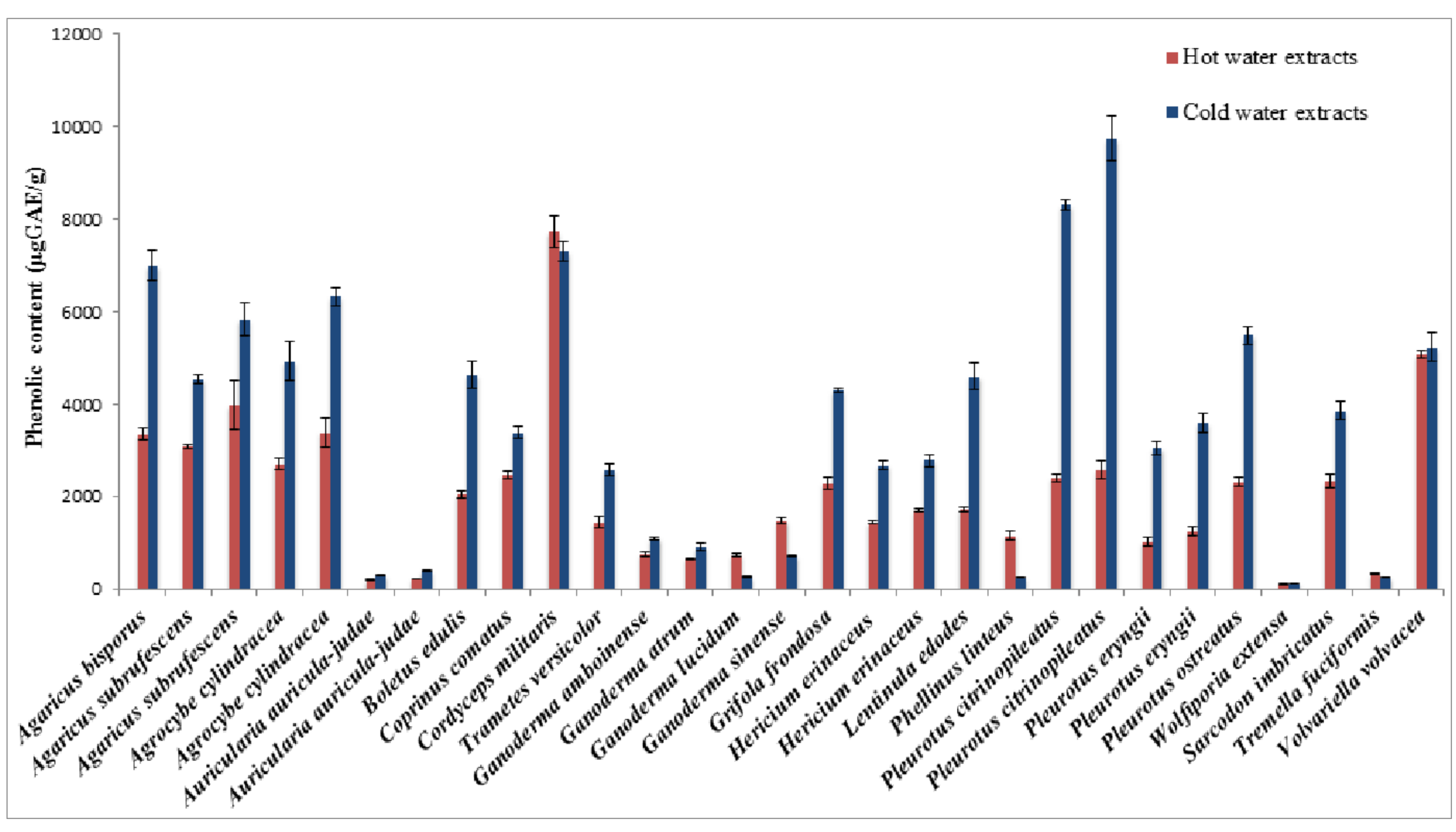


Figure 3.1: Total phenolic compounds assay of hot and cold water extracts. Each value is expressed as mean ± standard deviation (n=3).

3.2 Scavenging of 2, 2'-azinobis-(3—ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^{\bullet+}$)

The $ABTS^{\bullet+}$ free radical scavenging ability of the hot-water extracts ranged between 66mmol TE/g of d.w. and 0.67mmol TE/g of d.w., and that of the cold-water extracts ranged between 89mmol TE/g of d.w. and 0.2mmol TE/g of d.w. In both the hot- and cold-water extracts, the highest $ABTS^{\bullet+}$ free radical scavenging ability was found in *C. militaris* (66 and 89mmol TE/g of d.w. in hot- and cold-water extracts respectively, and the lowest $ABTS^{\bullet+}$ free radical scavenging ability was found in *W. extensa* (0.67 and 0.21mmol TE/g of d.w. in hot- and cold-water extracts respectively) ($p<0.001$). (*see* Tables 3.1 and 3.2, and Figure 3.2). The medicinal mushrooms with the highest TEAC value in hot-water extracts were *C. militaris* (66mmol of TE/g of d.w.), followed by *P. linteus*, *G. sinense*, *T. versicolor*, *G. amboinense*, *G. lucidum*, *G. atrum* , and *W. extensa*. On the other hand, in the cold-water extracts, the highest TEAC value of the medicinal mushrooms was found in *C. militaris* (89.5mmol of TE/g d.w), followed by *T. versicolor*, *G. amboinense*, *G. atrum*, *G. sinense*, *P. linteus*, *G. lucidum* and *W. extensa*. (*see* Tables 3.1 and 3.2, and Figure 3.2).

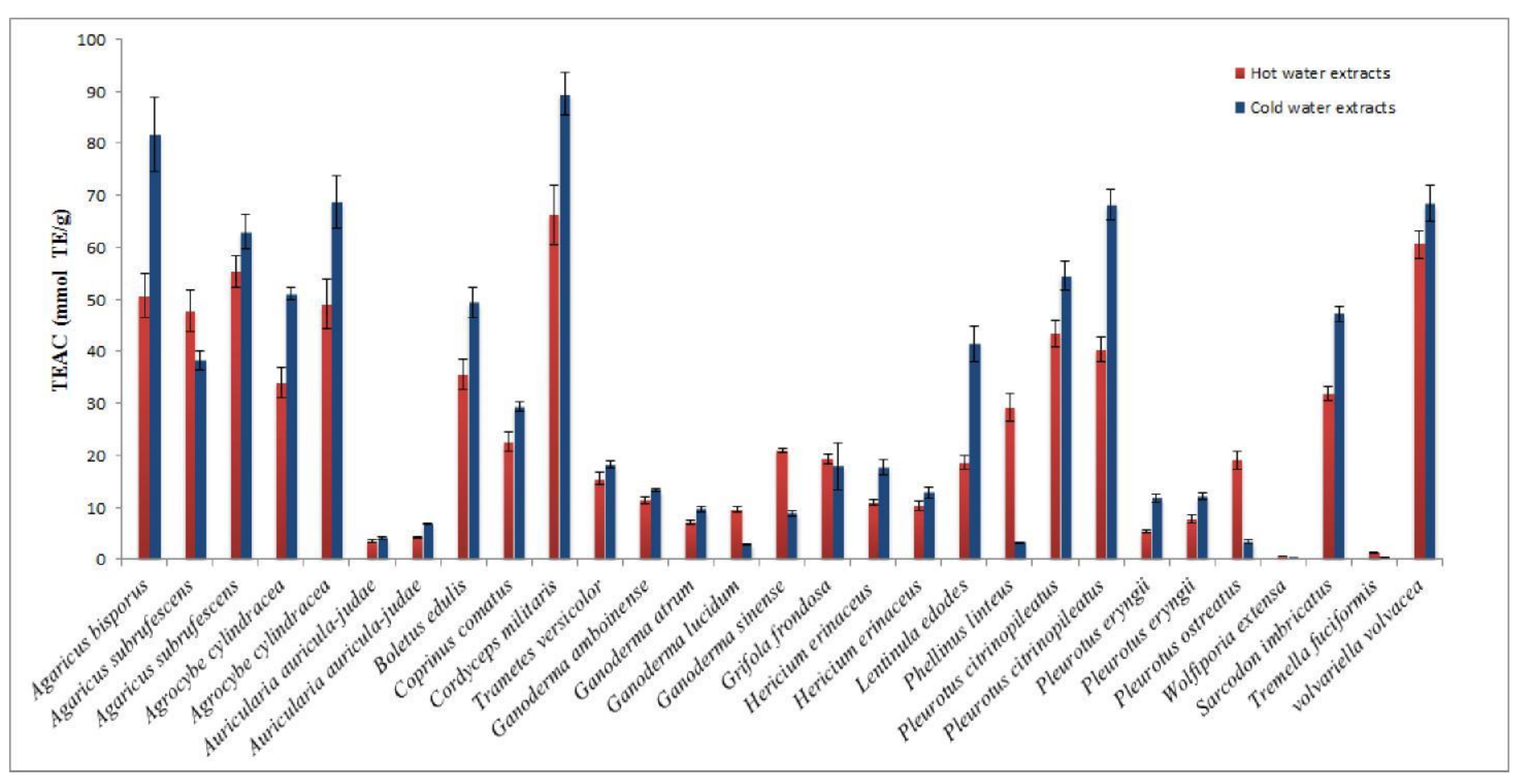


Figure 3.2: TEAC assay of hot- and cold-water mushroom extracts. Each value is expressed as mean ± standard deviation (n=3).

A statistically significant relationship was also observed between TEAC and the total phenolic contents of the medicinal mushrooms in both hot- and cold-water extracts with correlation coefficients equal to 0.937 (p=0.001) and 0.983 (p<0.001) respectively (*see* Figures 3.3 and 3.4).

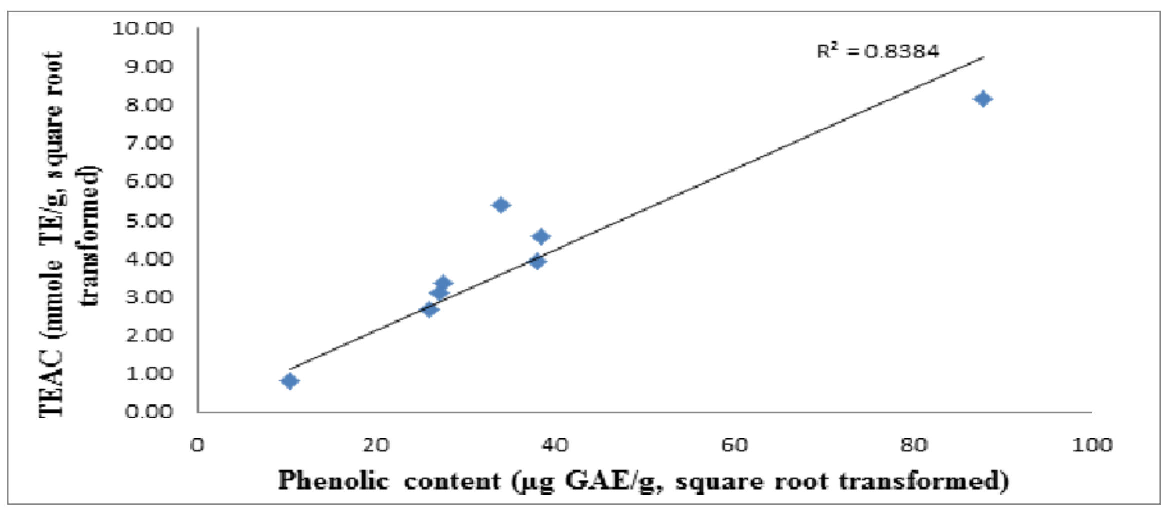


Figure 3.3: Correlation between phenolic content and TEAC of hot-water extractions of medicinal mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

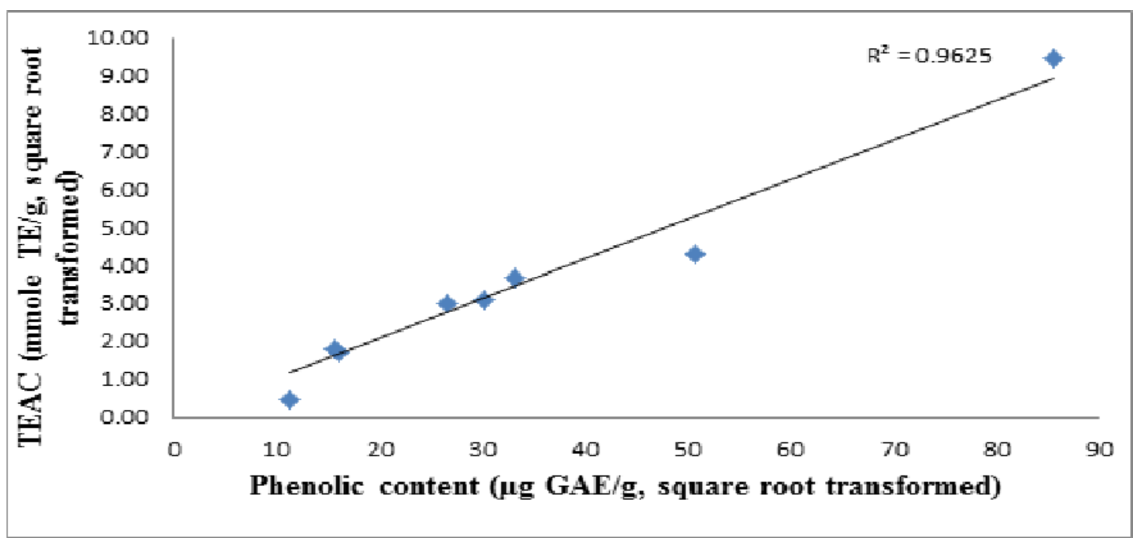


Figure 3.4: Correlation between phenolic content and TEAC of cold-water extractions of medicinal mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

The TEAC value for the culinary mushrooms in hot- and cold-water extractions ranged from 60mmol of TE/g d.w. to 1mmol of TE/g d.w. and 81mmol of TE/g d.w. to 0.2mmol of TE/g d.w. respectively (p<0.001). In hot-water extraction, the culinary mushrooms *V. volvacea* (60 mmol TE/g of d.w.) had highest TEAC value, followed by *A. subrufescens*, *A. bisporus*, *A. cylindracea*, *P. citrinopileatus* and lowest TEAC value was found in *W. extensa*. Whereas, in cold-water extraction, *A. bisporus* (81mmol TE/g of d.w.) had highest TEAC value, followed by *A. cylindracea*, *V. volvacea, P. citrinopileatus*, and lowest TEAC value was found in *W. extensa* and *Tremella funcifromis* (0.2mmol TE/g of d.w in both mushrooms). A positive relationship between the TEAC values and the phenolic contents of the culinary mushrooms was observed with coefficients of 0.861 (p<0.001) and 0.903 (p<0.001) in hot-water and cold-water extracts respectively (Figures 3.5and 3.6).

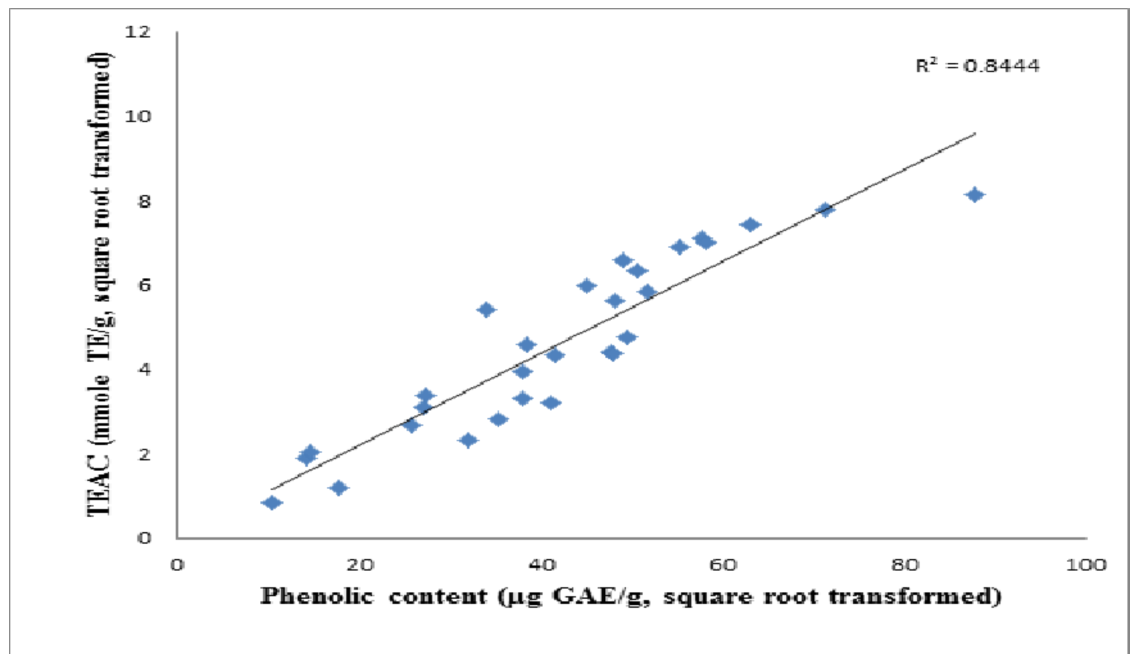


Figure 3.5: Correlation between phenolic content and TEAC of hot-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

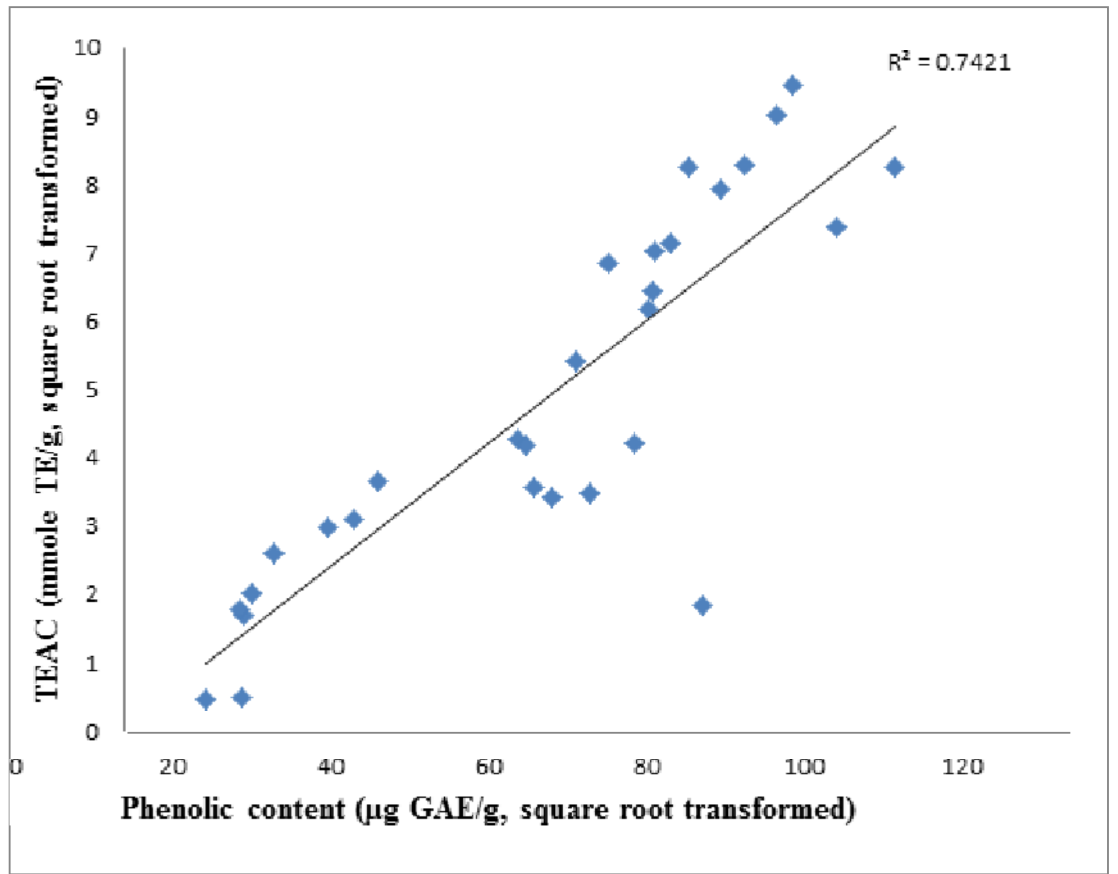


Figure 3.6: Correlation between phenolic content and TEAC of cold-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

3. 3 Ferrous reducing power

The FRAP value of the medicinal mushrooms in hot- and cold-water extraction were found within in the range from 14882µmol of Fe^{2+} /g of d.w. to 435µmol Fe^{2+} /g of d.w, and 39400µmol Fe^{2+} g of d.w. to 0.463µmol Fe^{2+} /g of d.w respectively (Tables 3.1 and 3.2 and Figure 3.7) (p<0.001).

In the hot-water extracts, the highest reducing ability was found in *G. sinense* (14882µmol of Fe^{2+} /g of d.w), followed by *C. militaris*, *G. amboinense*, *P. linteus*, *T. versicolor, G. atrum*, *G. lucidum*, *W. extensa*. In the cold-water extraction, *C. militaris* (39400µmol of Fe^{2+} /g of d.w.) had the highest FRAP value, followed by *T. versicolor*, *G. amboinense*, *G. atrum* , *G sinense*, *G. lucidum*, *P. linteus*, and *W. extensa* (Table 3.1 and 3.2 and Figure3.7).

The ferrous reducing antioxidant power (FRAP) of water extracts from the selected culinary mushrooms varied from 37196µmol Fe^{2+}/g of d.w. to 213µmol Fe^{2+}/g of d.w. and 47099µmol Fe^{2+}/g of d.w. to 275µmol Fe^{2+}/g of d.w. for the hot- and cold-water extracts respectively. In hot-water extracts of *A. bisporus* had the highest FRAP (37196µmol Fe^{2+} /g of d.w.), followed by *A. subrufescens*, *V. volvacea*, and *Sarcodon imbricatus*. The highest FRAP value of the cold-water extracts of the culinary mushrooms was found in *A. cylindracea* with 47099±1757µmol Fe^{2+} /g of d.w., followed by *P. citrinopileatus*, *A. subrufescens*, and *Boletus edulis* (p<0.001) (Tables 3.1 and 3.2 and Figure 3.7).

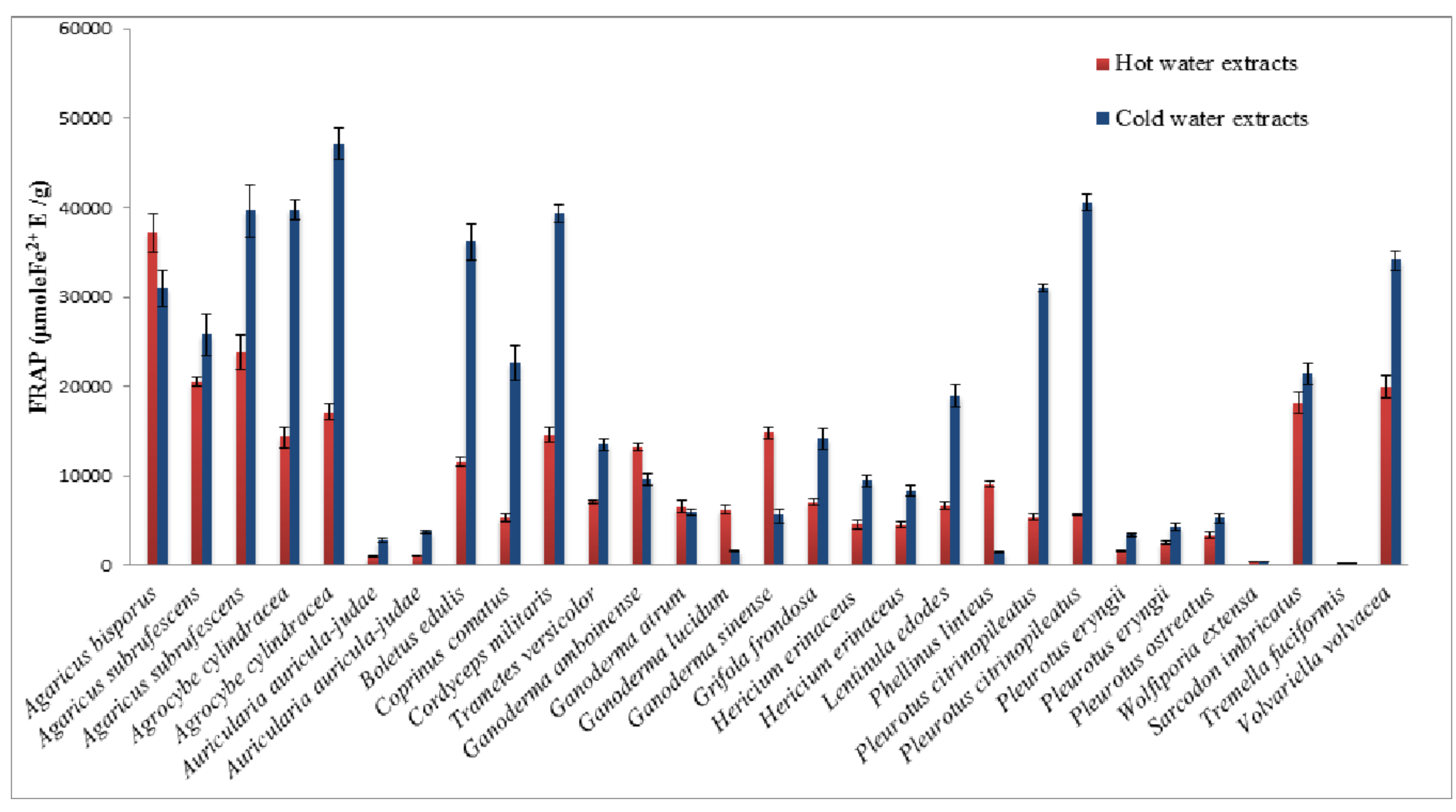


Figure 3.7: FRAP assay of hot- and cold-water mushroom extracts. Each value is expressed as mean ± standard deviation (n=3).

There was no statistically significant correlation between FRAP and total phenolic contents of the hot-water extractions of the medicinal mushrooms, for which the correlation coefficient was 0.67 (p=0.068) (Figure 3.8), whereas with the cold-water extractions, the FRAP value and total phenolic contents showed a significant correlation R= 0.98 (p<0.001) (Figure 3.9).

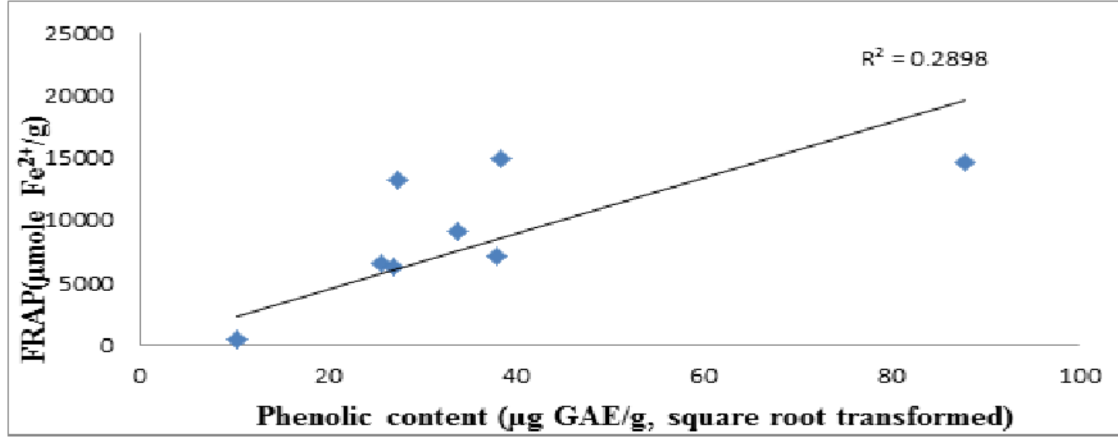


Figure 3.8: Correlation between phenolic content and FRAP of hot-water extractions of medicinal mushrooms (the phenolic data have been square root transformed). Each value is expressed as mean (n=3).

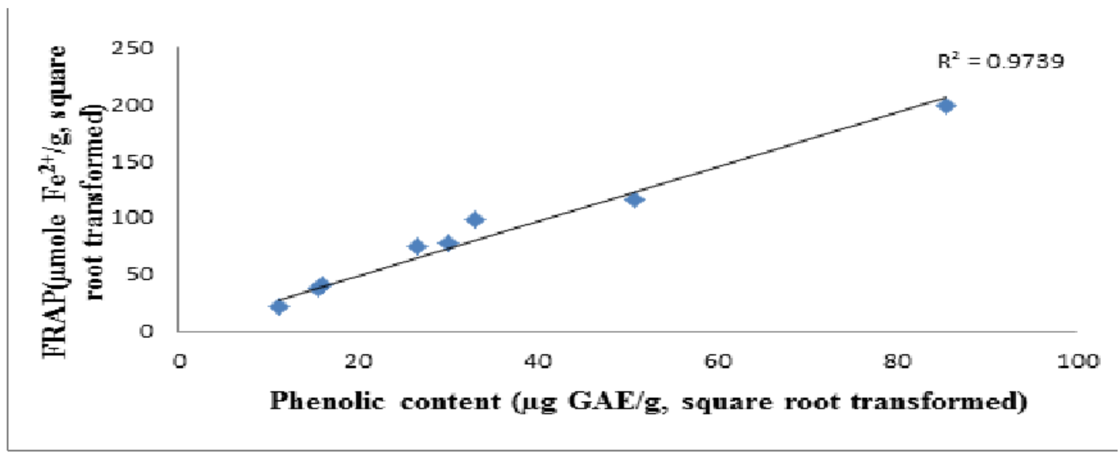


Figure 3.9: Correlation between phenolic content and FRAP of cold-water extractions of medicinal mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

A positive relationship was also found for both the hot- and cold-water extracts of the culinary mushrooms (R=0.72, $p<0.001$ and R= 0.86, $p<0.001$, respectively) (Figures 3.10 and 3.11).

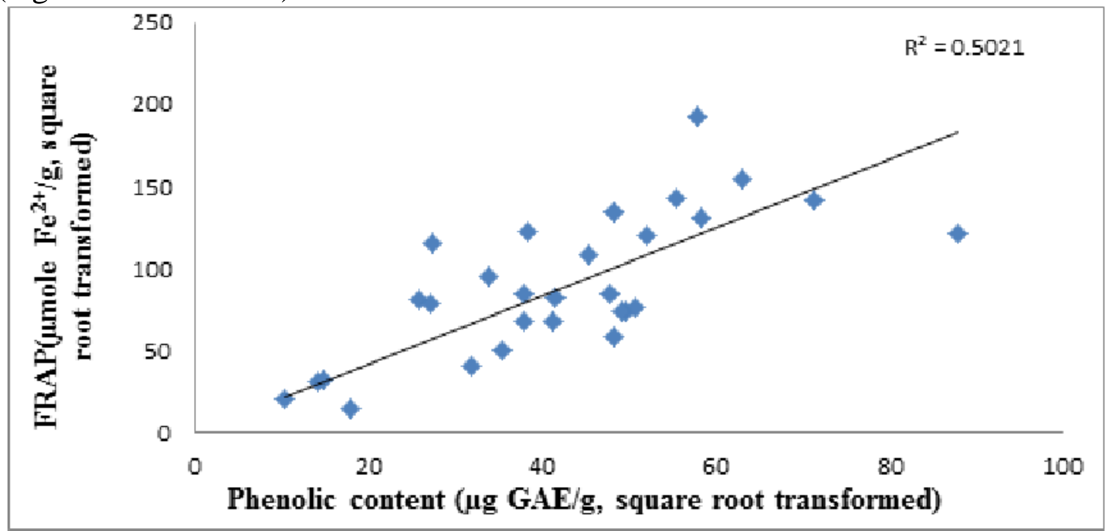


Figure 3.10: Correlation between phenolic content and FRAP of hot-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

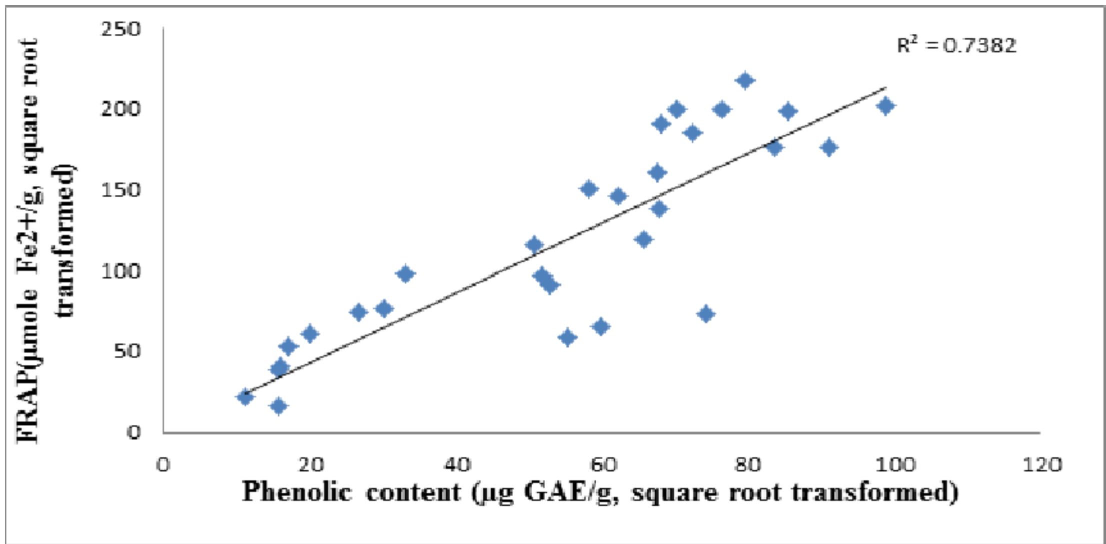


Figure 3.11: Correlation between phenolic content and FRAP of cold-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

3.4 Scavenging effect on 2, 2-diphenylhydrazyl radicals (DPPH)

The DPPH value of the medicinal mushrooms in hot- and cold-water extractions ranged from 4.5mmol TE/g of d.w. to 0.05mmol TE/g of d.w., and from 7.3mmol TE/g of d.w. to 0.02mmol TE/g of d.w. respectively. In hot-water extracts, *C. militaris* (4.5mmol TE/g of d.w.) had the highest DPPH values, followed by *P. linteus*, *G. sinense*, *T. versicolor* , *G. amboinense*, *G. atrum*, *G. lucidum*), and *W. extensa* in the hot-water extract (p<0.001) (Table 3.1 and 3.2 and Figure 3.12).

In the cold-water extracts, *C. militaris* also showed the highest DPPH value (7.3mmol TE/g of d.w.), followed by *G. atrum*, *T. versicolor*, *G. amboinense*, *G. sinense*, *P. linteus*, *G. lucidum*, and *W. extensa* (p<0.001) (Table 3.1 and 3.2 and Figure 3.12).

The DPPH values were found in the culinary mushrooms to be between 8.6mmol TE/g of d.w. to 0.5mmol TE/g of d.w. and 8.4mmol TE/g of d.w. to 0.18mmol TE/g of d.w. in the hot- and cold-water extracts respectively. *V. volvacea*, *A. bisporus*, *A. cylindracea*, *A. subrufescens*, and *P. citrinopileatus* had high DPPH values among the culinary mushrooms in both hot and cold-water extractions (Table 3.1 and 3.2 and Figure 3.12).

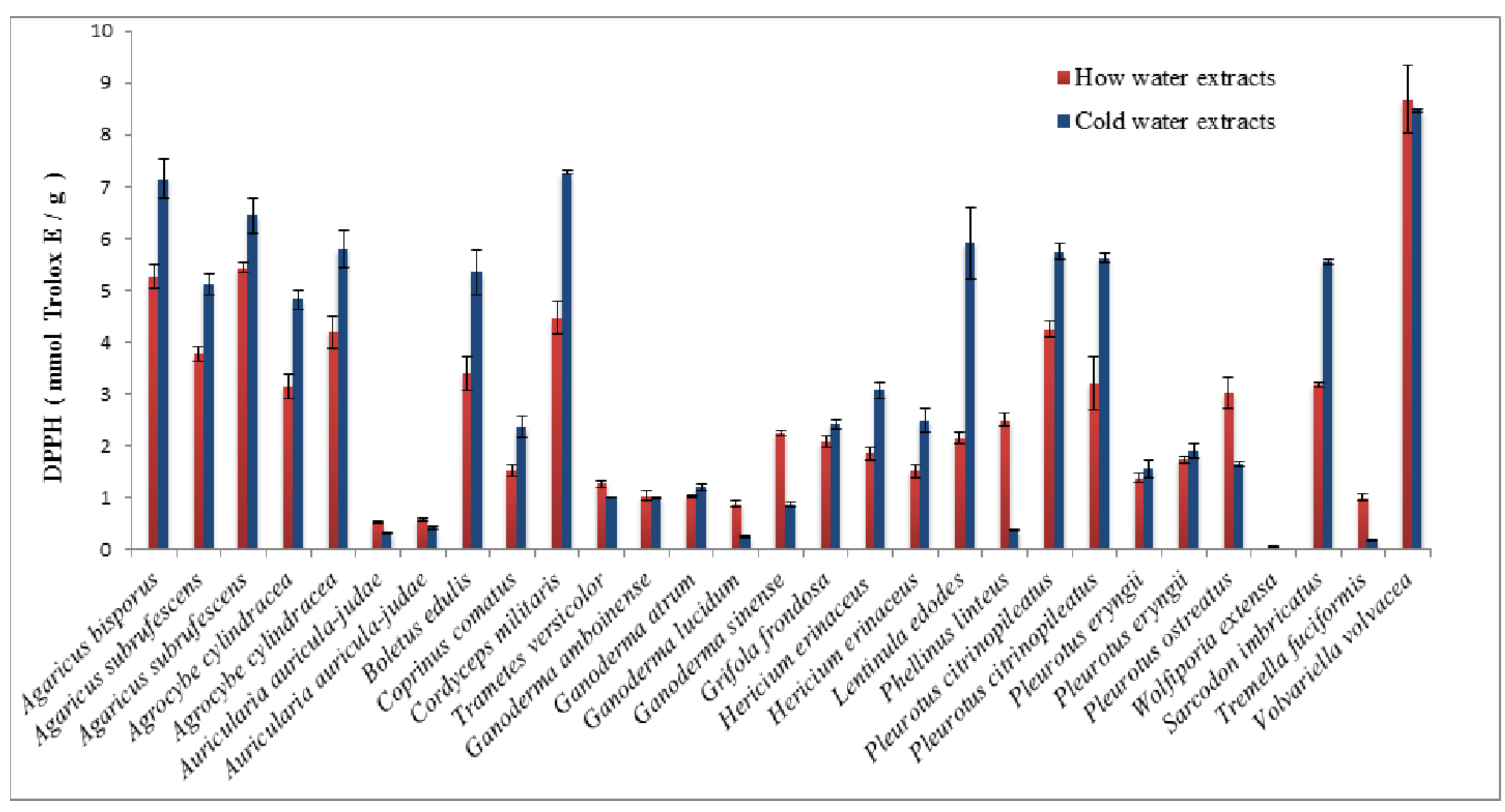


Figure 3.12: DPPH assay of hot and cold mushroom extracts. Each value is expressed as mean ± standard deviation (n=3).

A positive relationship between DPPH value and phenolic concentration was found in hot- and cold-water extractions with correlation coefficients of 0.94 (p=0.001) and 0.95 (p<0.001) respectively (*see* Figures 3.13 and 3.14).

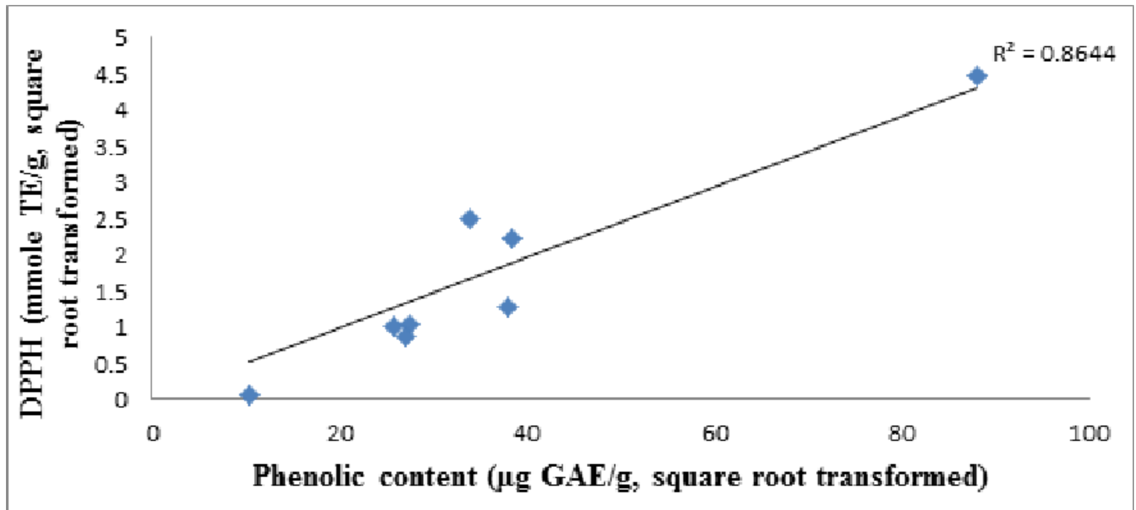


Figure 3.13: Correlation between phenolic content and DPPH of hot-water extractions of medicinal mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

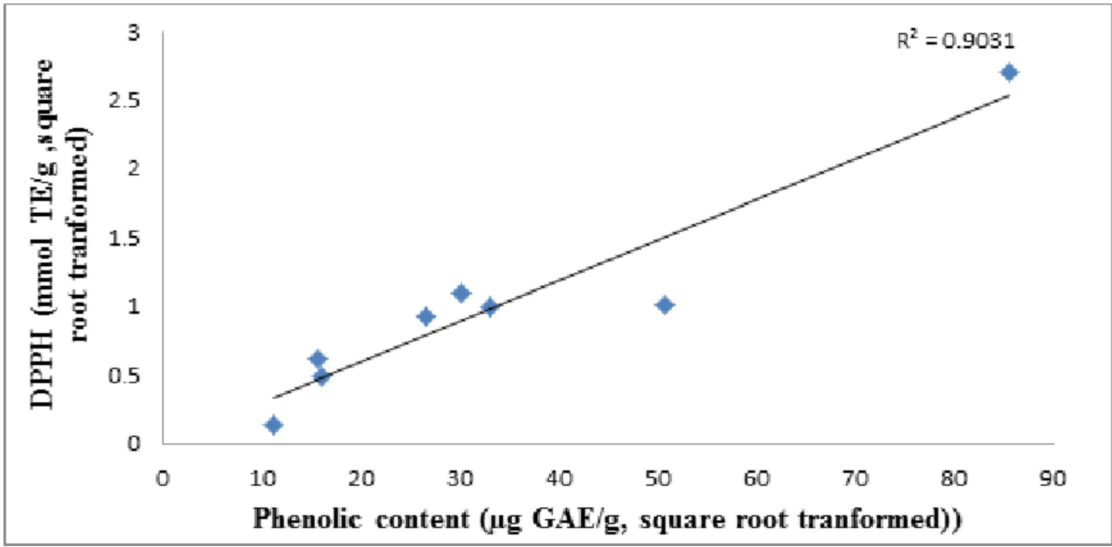


Figure 3.14: Correlation between phenolic content and DPPH of cold-water extractions of medicinal mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

With the DPPH method, in the culinary mushrooms, the strong correlation between antioxidant activity and total phenolic content was also found in hot-water extractions with a correlation coefficient of 0.888 (p<0.001). A positive correlation was also found between cold-water extraction and DPPH values, R= 0.903 (p<0.001) (*see* Figures 3.15 and 3.16).

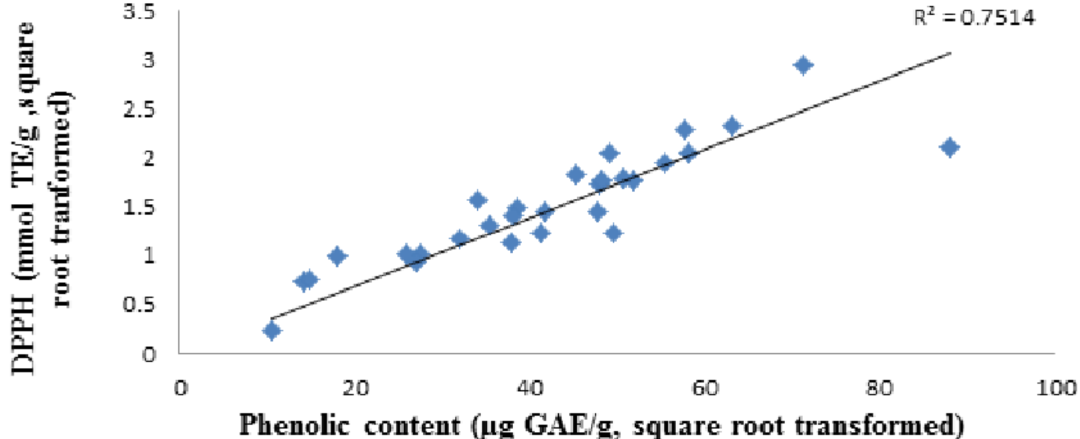


Figure 3.15: Correlation between phenolic content and DPPH of hot-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

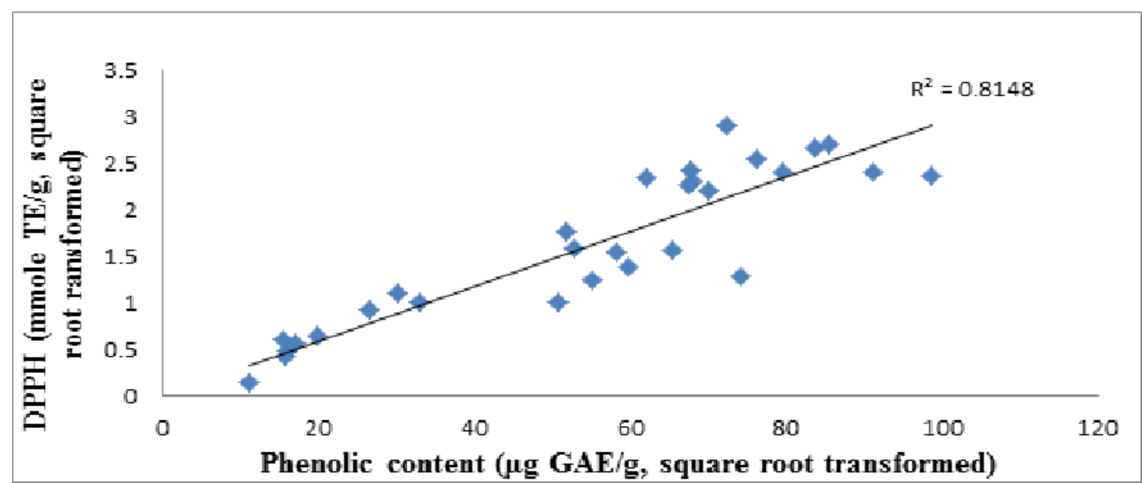


Figure 3.16: Correlation between phenolic content and DPPH of cold-water extractions of culinary mushrooms (all data have been square root transformed). Each value is expressed as mean (n=3).

Table 3.1: Hot-water extraction

Mushroom species	Phenolic content (μg GAE/g dried weight)	TEAC (mmol TE/g dried weight)	FRAP (μmol Fe^{2+}/g dried weight)	DPPH (mmol TE /g dried weight)
*Agaricus bisporus a**	3345±137	50.8±4.3	37196±2218	5.26±0.24
*Agaricus subrufescens b**	3073±39	47.7±3.9	20533±530	3.77±0.14
*Agaricus subrufescens a**	3980±371	55.4±3.1	23822±1884	5.44±0.10
*Agrocybe cylindracea a**	2697±135	34.1±2.9	14377±1192	3.15±0.24
*Agrocybe cylindracea b**	3390±323	49.1±4.7	17176±865	4.20±0.31
*Auricularia auricula-judae e**	203±10	3.6±0.2	984±38	0.53±0.02
*Auricularia auricula-judae b**	218±7	4.2±0.1	1046±46	0.58±0.03
*Boletus edulis e**	2049±80	35.7±2.9	11598.±534	3.38±0.33
*Coprinus comatus b**	2457±74	22.6±1.8	5377±367	1.52±0.10
*Cordyceps militaris d**	7734±342	66.2±5.6	14617±828	4.46±0.31
*Trametes versicolor b**	1441±131	15.5±1.2	7139±160	1.27±0.06
*Ganoderma amboinense d**	753±40	11.3±0.7	13232±494	1.04±0.08
*Ganoderma atrum e**	667±17	7.1±0.30	6589±630	1.02±0.02
*Ganoderma lucidum d**	735±23	9.6±0.57	6254±522	0.88±0.05

Ganoderma sinense c*	1478±67	20.9±0.4	14882±702	2.24±0.06
Grifola frondosa b*	2287±133	19.3±1.0	7073±345	2.09±0.10
Hericium erinaceus b*	1442±27	10.9±0.5	4616±451	1.85±0.12
Hericium erinaceus c*	1697±40	10.3±0.8	4576±293	1.52±0.12
Lentinula edodes b*	1731±46	18.7±1.2	6708±449	2.14±0.11
Phellinus linteus b*	1154±109	29.2±2.5	9096±347	2.50±0.13
Pleurotus citrinopileatus b*	2407±86	43.4±2.4	5411±336	4.25±0.15
Pleurotus citrinopileatus a*	2580±195	40.2±2.4	5734±118	3.20±0.52
Pleurotus eryngii b*	1021±84	5.4±0.3	1662±82	1.38±0.09
Pleurotus eryngii a*	1252±89	7.9±0.8	2542±132	1.74±0.06
Pleurotus ostreatus c*	2316±97	19.1±1.7	3403±395	3.02±0.29
Wolfiporia extensa c*	109±7	0.67±0.1	435±37	0.05±0.01
Sarcodon imbricatus e*	2330±138	31.9±1.4	18158 ±1216	3.17±0.05
Tremella fuciformis b*	321±11	1.4±0.1	213±48	1.01±0.06
Volvariella volvacea a*	5082±84	60.6±2.7	19913±1268	8.67±0.66

Values are mean ± standard deviation of triplicate analysis (n = 3). $p < 0.001$

Origin: *a**: Peninsular farm, HK, *b**: Nantou, Taiwan, *c**: Lianan Yao autonomous county, China, d*: Yen-Ten farm, Chay,I Taiwan ,*e**:Yunnan,China

Table 3.2: Cold-water extraction

Mushroom species	Phenolic content (µg GAE/g dried weight)	TEAC (mmol TE/g dried weight)	FRAP (µmol Fe^{2+} /g dried weight)	DPPH (mmol TE/g dried weight)
Agaricus bisporus a*	6998 ±324	81.6 ±7.2	30949±2068	7.15±0.38
Agaricus subrufescens b*	4548 ±107	38.2 ±1.7	25725±2342	5.11±0.21
Agaricus subrufescens a*	5830 ±360	62.9 ±3.3	39653±2929	6.44±0.34
Agrocybe aegerita a*	4926 ±411	51.1 ±1.2	39767±1094	4.81±0.17
Agrocybe aegerita b*	6335 ±196	68.8 ±5.0	47099±1757	5.80±0.36
Auricularia auricula-judae e *	290 ±18	4.1 ±0.3	2805±203	0.32±0.01
Auricularia auricula-judae b*	396 ±18	6.8 ±0.2	3775±159	0.42±0.03
Boletus edulis e*	4642 ±287	49.4 ±2.9	36213±2001	5.34±0.44
Coprinus comatus b*	3386 ±138	29.40 ±0.9	22598±1898	2.36±0.21
Cordyceps militaris d*	7313 ±206	89.53 ±4.2	39400±1009	7.27±0.03
Trametes versicolor b*	2575 ±128	18.27 ±0.7	13521±689	1.01±0.01
Ganoderma amboinense d*	1097 ±25	13.34 ±0.3	9589±682	1.00±0.01
Ganoderma atrum e*	907 ±75.0	9.58 ±0.5	5928±345	1.20±0.06
Ganoderma lucidum d*	259 ±10.0	2.90 ±0.1	1649±53	0.24±0.01
Ganoderma sinense c*	712 ±17	8.9 ±0.50	5588±767	0.87±0.06

Grifola frondosa b*	4300 ±45	17.8 ±4.6	14209±1170	2.42±0.1
Hericium erinaceus b*	2676± 90	17.7 ±1.5	9428±693	3.07±0.16
Hericium erinaceus c*	2783 ±130	12.7 ±1.1	8319±605	2.49±0.23
Lentinula edodes b*	4604±289	41.5 ±3.4	18934±1208	5.90±0.69
Phellinus linteus b*	244 ±5	3.2 ±0.1	1452±114	0.37±0.02
Pleurotus citrinopileatus b*	8306 ±102	54.6 ±2.7	31040±372	5.74±0.16
Pleurotus citrinopileatus a*	9741±474	68.3 ±2.9	40537±898	5.62±0.09
Pleurotus eryngii b*	3044 ±145	11.4 ±0.7	3383±153	1.56±0.16
Pleurotus eryngii a*	3590 ±208	12.2 ±0.7	4330±459	1.89±0.13
Pleurotus ostreatus c*	5497 ±193	3.4 ±0.4	5335±494	1.65±0.06
Wolfiporia extensa c*	126 ±6	0.2 ±0.01	463±12	0.02±0.001
Sarcodon imbricatus e*	3870 ±197	47.2 ±1.5	21380±1253	5.54±0.05
Tremella fuciformis b*	250 ±6	0.2 ±0.01	275±26	0.18±0.01
Volvariella volvacea a*	5226 ±306	68.4 ±3.5	34077±1143	8.46±0.03

Values are mean ± standard deviation of triplicate analysis (n = 3). $p < 0.001$

Origin: *a**: Peninsular farm, HK, *b**: Nantou, Taiwan, *c**: Lianan Yao autonomous county, China, d*: Yen-Ten farm, Chay,I Taiwan ,*e**:Yunnan,China

3.5 *In vitro* enzymatic digestion

3.5.1 *TEAC*

After pepsin digestion, *A. auricula-judae*, *C. militaris*, *G. lucidum* and *H. erinaceus* showed significant decreases ($p<0.05$) in both the hot- and cold-water extracts and *P. citrinopileatus* had no significant effect ($p=0.07$ and $p=0.059$ in the hot- and cold-water extracts respectively). *T. versicolor* showed significant increase after pepsin digestion in both the hot- and cold-water extracts ($p=0.041$ and $p=0.047$ in the hot- and cold-water extracts respectively).

After pancreatin digestion, the ability of ABTS$^{\bullet +}$ radical scavenging was a significant increase in *A. auricular-judae* ($p<0.01$ in cold extracts), *T. versicolor* ($p=0.13$ and $p< 0.01$ in hot- and cold-water extracts respectively), *G. lucidum* ($p=0.04$ and $p<0.01$ in hot- and cold-water extracts respectively), *H. erinaceus* ($p<0.01$ in hot-water extracts), and *P. citrinopileatus* ($p<0.01$ in both extracts, whereas there was no significant different in hot-water extract of *A. auricular-judae* ($p=0.07$), hot-water extract of *C. militaris* ($p=0.05$) and cold-water extract of *H. erinaceus* ($p=0.054$) after enzymatic digestion.

In contrast, the potential antioxidant compound release of *C. militaris* extracts showed a decrease in ABTS free radical scavenging ability in cold-water extracts ($p<0.01$) (Figures 3.17 to 3.22 and Table 3.3).

Table 3.3: Total antioxidant activity (mmol TE/g dried weight) of selected mushrooms measured by TEAC during the *in vitro* enzymatic digestion at 10 and 90 min. Values are means ± SD. Each value is expressed as mean ± standard deviation (*n*=3).

Mushroom	10 (min)	90 (min)	*p* (10 VS 90)
Auricularia auricula-judea (Hot water)	4.03±04	5.04±0.21	0.072
Auricularia auricula-judea (Cold water)	6.63±04	9.70±0.65	0.004
Cordyceps militaris (Hot water)	66.58±3.6	51.9±3.4	0.050
Cordyceps militaris (Cold water)	90.23±3.5	65.91±1.5	0.004
Trametes versicolor (Hot water)	15.09±0.6	22.13±1.4	0.013
Trametes versicolor (Cold water)	18.04±1.4	33.11±2.3	0.002
Ganoderma lucidum (Hot water)	9.22±0.3	11.86±1.0	0.041
Ganoderma lucidum (Cold water)	2.9±0.2	10.95±0.92	0.003
Hericium erinaceus (Hot water)	11.48±1	14.9±0.64	0.004
Hericium erinaceus (Cold water)	17.53±0.8	20.59±1.5	0.054
Pleurotus citrinopileatus (Hot water)	45.27±2	123.56±11.1	0.006
Pleurotus citrinopileatus (Cold water)	57.77±1.9	102.31±3.6	0.004

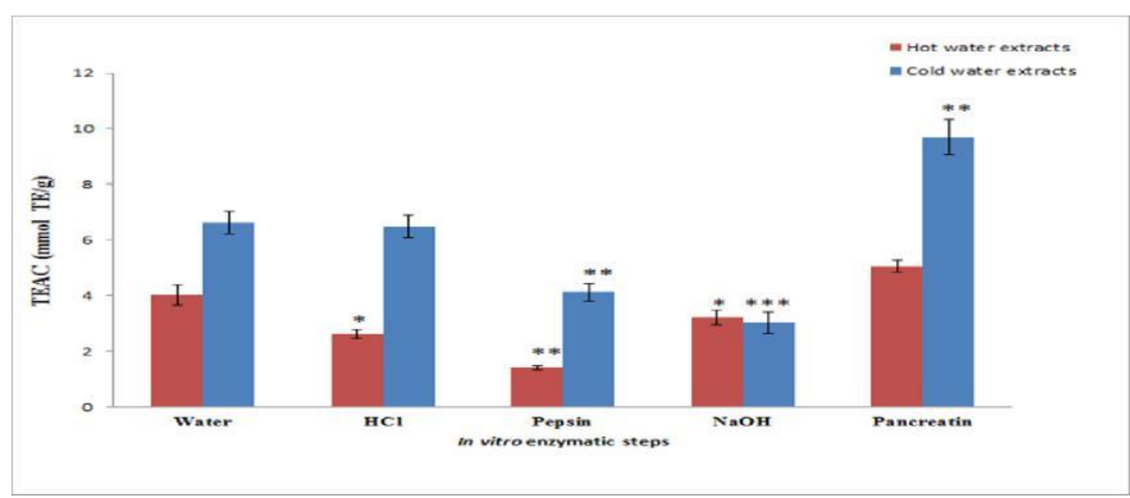


Figure 3.17: Total antioxidant capacity of *A. auricula-judae* extracts (hot-water and cold-water) measured by using the TEAC assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps (The symbols * (*p*<0.001), ** (*p* <0.01) and * (*p* <0.05) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).**

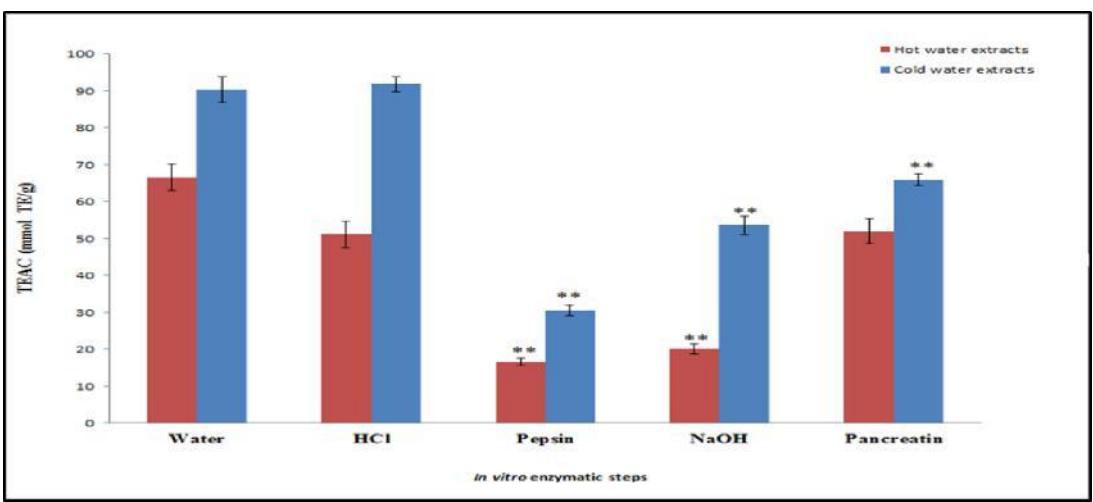


Figure 3.18: Total antioxidant capacity of *C. militaris* extracts (hot-water and cold-water) measured by TEAC (mmol Trolox equivalents) during the in vitro enzymatic digestion steps. The symbols ** (*p* <0.01) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).

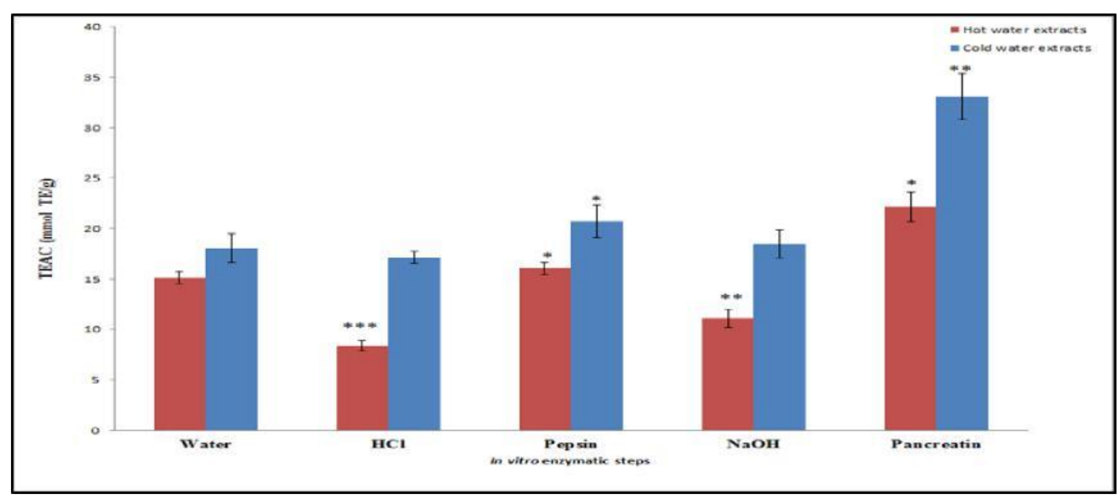


Figure 3.19: Total antioxidant capacity of *T. versicolor* extracts (hot-water and cold-water) measured by TEAC (mmol Trolox equivalents) during the in vitro enzymatic digestion steps (The symbols * (p <0.001), ** (p <0.01) and * (p <0.05) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).**

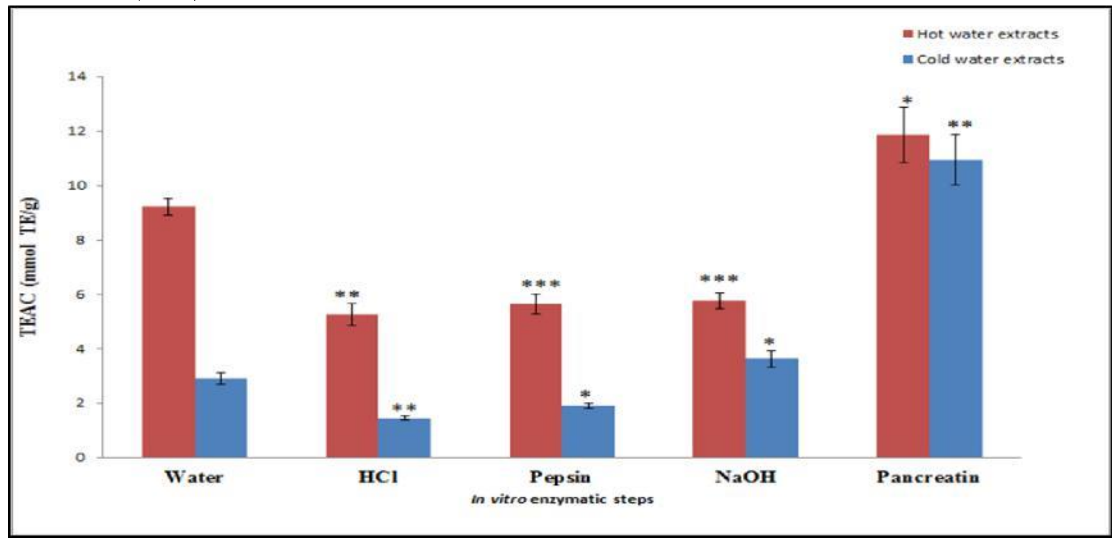


Figure 3.20: Total antioxidant capacity of *G. lucidum* extracts (hot-water and cold-water) measured by TEAC (mmol Trolox equivalents) during the in vitro enzymatic digestion steps The symbols * (p <0.001), ** (p <0.01) and * (p <0.05) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).**

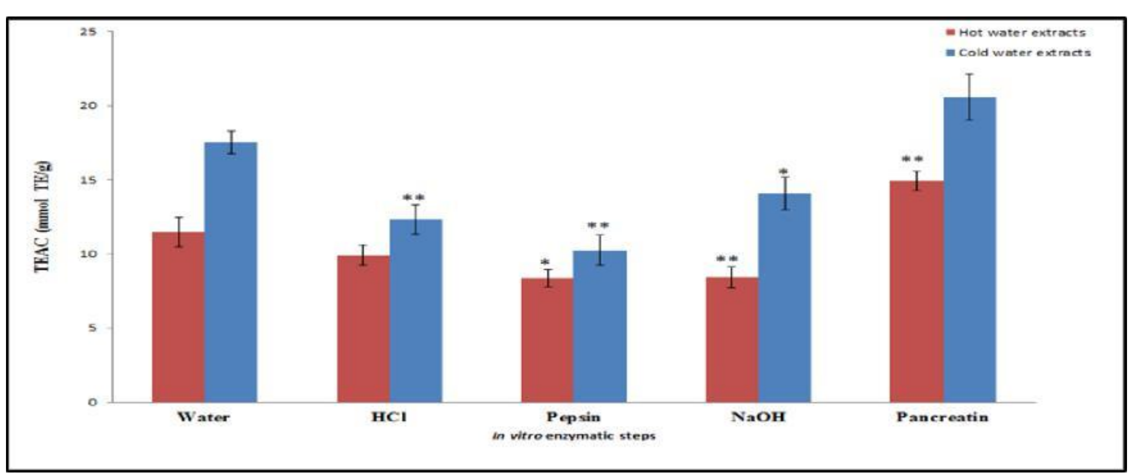


Figure 3.21: Total antioxidant capacity of *H. erinaceus* extracts (hot-water and cold-water) measured by using the TEAC assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps. The symbols ** (p <0.01) and * (p <0.05) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.) Each value is expressed as mean ± standard deviation (n=3).

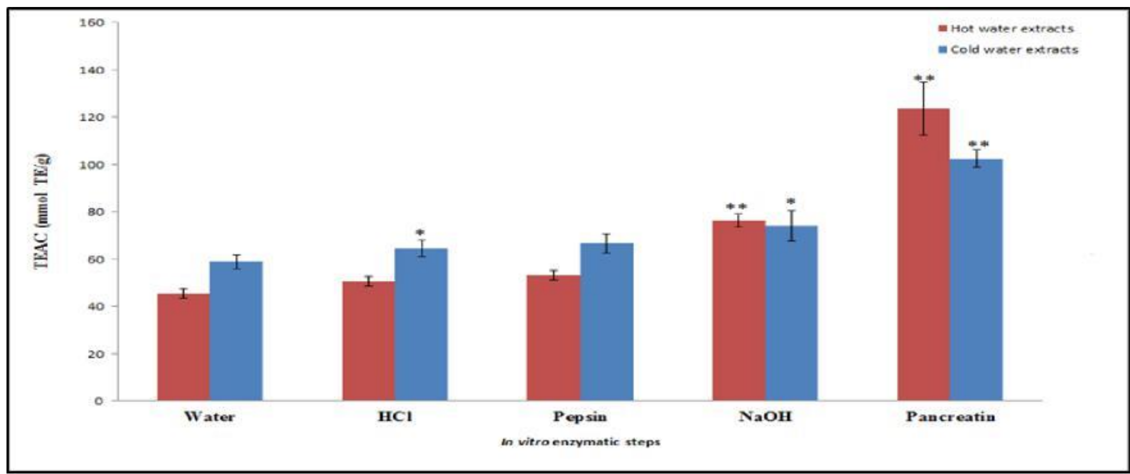


Figure 3.22: Total antioxidant capacity of *P. citrinopileatus* extracts (hot-water and cold-water) measured using the TEAC assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps The symbols ** (p <0.01) and * (p <0.05) denote significant differences from the ABTS scavenging values prior to the in vitro digestion steps.) Each value is expressed as mean ± standard deviation (n=3).

Overall, after ninety minutes of *in vitro* digestion, *P. citrinopileatus* (123.6 mmol TE/g of d.w. in the hot-water extract) showed the highest ATBS free radical scavenging ability, followed by *P. citrinopileatus* (the cold-water extract) > *C. militaris* (the cold-water extract) > *C. militaris* (the hot-water extract) > *T. versicolor* (the cold-water extract) > *T. versicolor* (the hot-water extract) > *H. erinaceus* (the cold-water extract) > *H. erinaceus* (the hot-water extract) > *G. lucidum* (the hot-water extract) > *G. lucidum* (the cold-water extract) > *A. auricula-judae* (9 the cold-water extract) > *A. auricula-judea* (the hot-water extract) (p<0.001) (Figure 3.23).

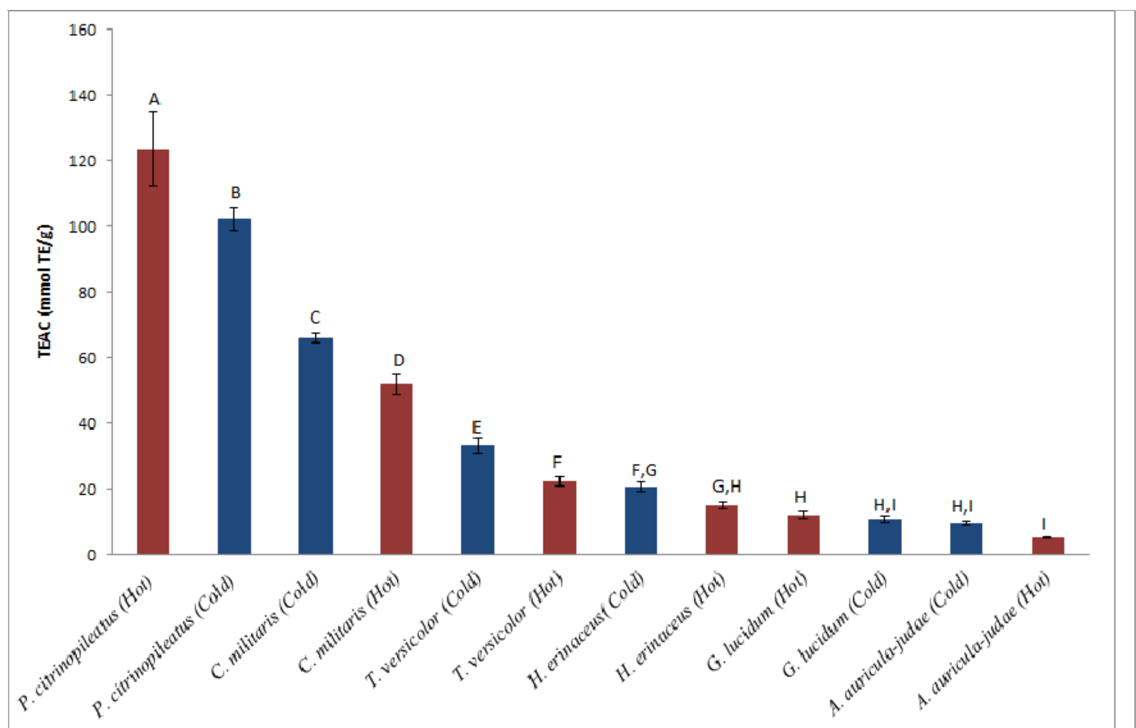


Figure 3.23: Total antioxidant capacity of selected mushroom extracts (hot-water and cold-water) measured by using the (mmol Trolox equivalent) after the in vitro enzymatic digestion steps. Bars with different letters are significantly different (p < 0.05). Each value is expressed as mean ± standard deviation (n=3).

3.5.2 *FRAP*

The pattern of potential redox compound release from the six samples during the *in vitro* enzymatic digestion process determined by using the FRAP assay is shown in Figures 3.24 to 3.29 and Table 3.4. After pepsin digestion, *C. militaris* (in both extracts) and *G. lucidum* (in the hot-water extract) showed significant decreases (p<0.05), and *A. auricula-judae* (in the hot-water extract), *T. versicolor* (in both extracts), *G. lucidum* (in the cold-water extract), *H. erinaceus* (in both extract) and *P. citrinopileatus* (in both extracts) showed significant increases (p<0.05). The cold-water extract of *A. auricula-judae* showed no significant effect after pepsin digestion (p=0.1).

A. auricula-judae (p<0.01 in both extracts), *T. versicolor* (p<0.01 in both extracts), *G. lucidum* (p<0.01 in both extracts), *H. erinaceus* (p<0.01 in both extracts) and *P. citrinopileatus* (p<0.01 in both extracts) all showed significant increases in ferric reducing ability after *in vitro* enzymatic digestion. On the other hand, the potential redox compounds release from *C. militaris* showed significant decreases in FRAP value in both extracts (p<0.01 and p<0.05 in the hot- and cold-water extracts respectively) (*see* Figures 3.24 to 3. 29 and Table 3.4).

Table 3.4: Total antioxidant activity (µmol Fe^{2+}/g dried weight) of selected mushrooms measured by FRAP during the in vitro enzymatic digestion at 10 and 90 min. Values are means ± SD. Each value is expressed as mean ± standard deviation (n=3).

Mushroom	10 (min)	90 (min)	*p* (10 VS 90)
Auricularia auricula-judae (Hot water)	1040±100	2784±148	0.003
Auricularia auricula-judae (Cold water)	3825±198	17229±1280	0.004
Cordyceps militaris (Hot water)	13818±581	8080±803	0.017
Cordyceps militaris (Cold water)	37692±762	29950±1201	0.003
Trametes versicolor (Hot water)	7788±702	82010±5632	0.002
Trametes versicolor (Cold water)	14465±1287	59218±5875	0.005
Ganoderma lucidum (Hot water)	5869±459	14469±744	0.004
Ganoderma lucidum (Cold water)	1559±67	4094±397	0.006
Hericium erinaceus (Hot water)	4720±360	9090±647	0.007
Hericium erinaceus (Cold water)	9399±675	17532±1586	0.004
Pleurotus citrinopileatus (Hot water)	5008±376	15905±868	0.001
Pleurotus citrinopileatus (Cold water)	30131±2305	54133±2710	0.001

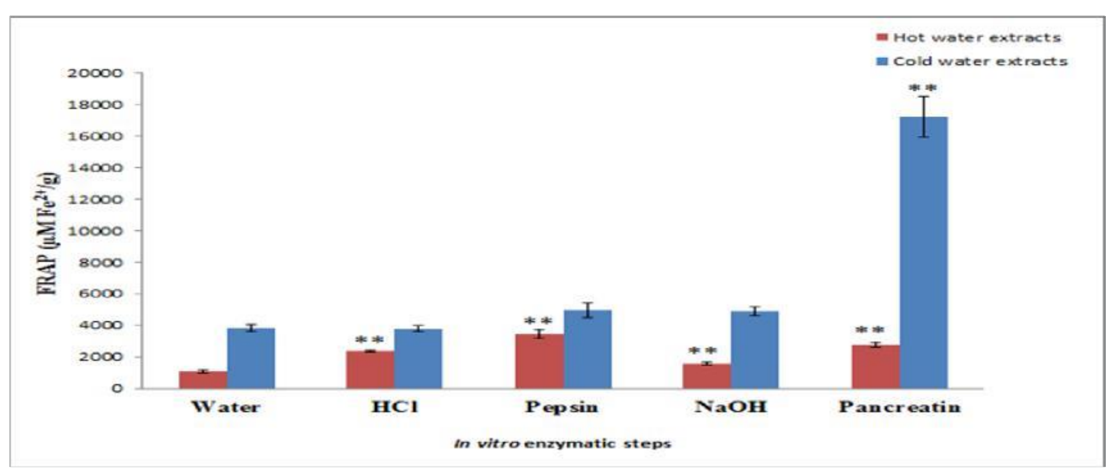


Figure 3.24: Total antioxidant capacity of *A. auricula-judae* extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) during the in vitro enzymatic digestion steps The symbols ** (p < 0.01) denote significant differences from the FRAP values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).

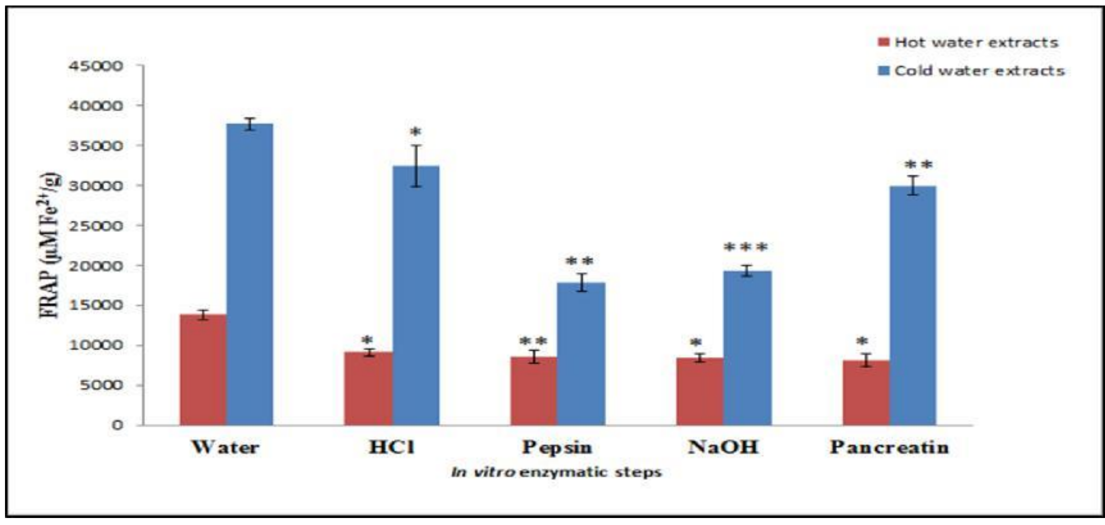


Figure 3.25: Total antioxidant capacity of *C. militaris* extracts (hot-water and cold-water) measured by FRAP (µmol ferrous ion equivalent) during the in vitro enzymatic digestion steps The symbols * (p <0.001), ** (p <0.01) and * (p <0.05) denote significant differences from the FRAP values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).**

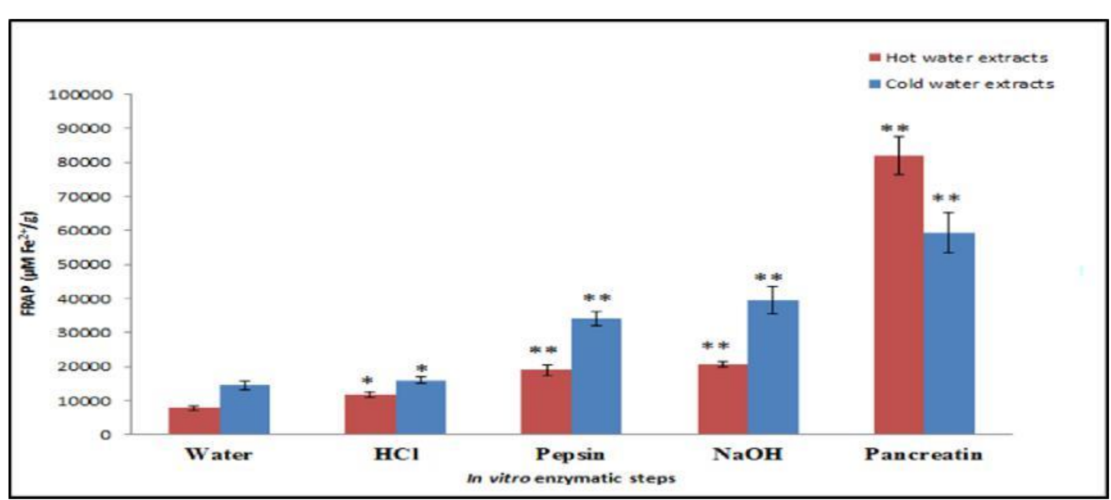


Figure 3.26: Total antioxidant capacity of *T. versicolor* extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) during the in vitro enzymatic digestion steps The symbols ** (*p* <0.01) and * (*p* <0.05) denote significant differences from the FRAP values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).

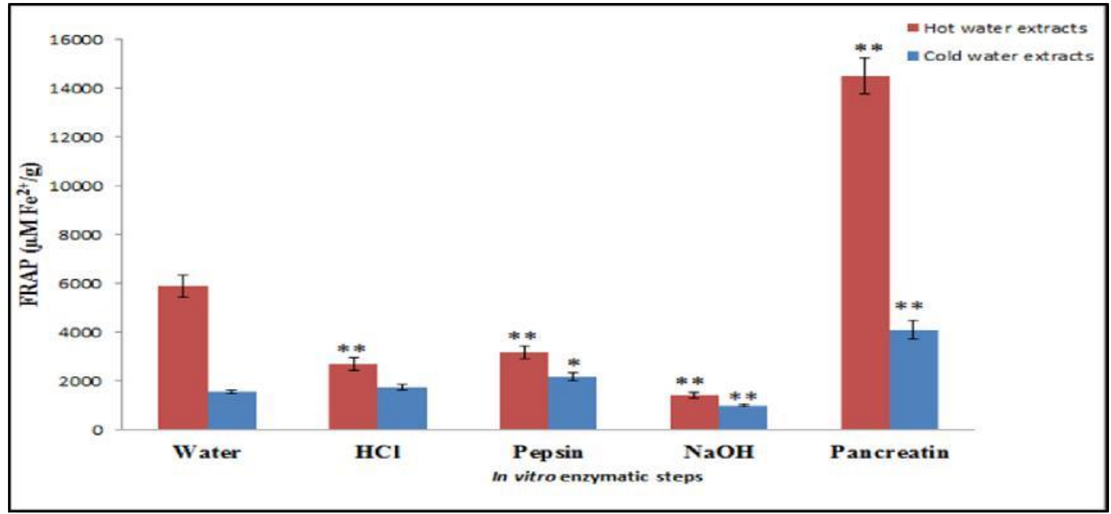


Figure 3.27: Total antioxidant capacity of *G. lucidum* extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) during the in vitro enzymatic digestion steps The symbols ** (*p* <0.01) and * (*p*<0.05) denote significant differences from the FRAP values prior to the in vitro digestion steps.) Each value is expressed as mean ± standard deviation (n=3).

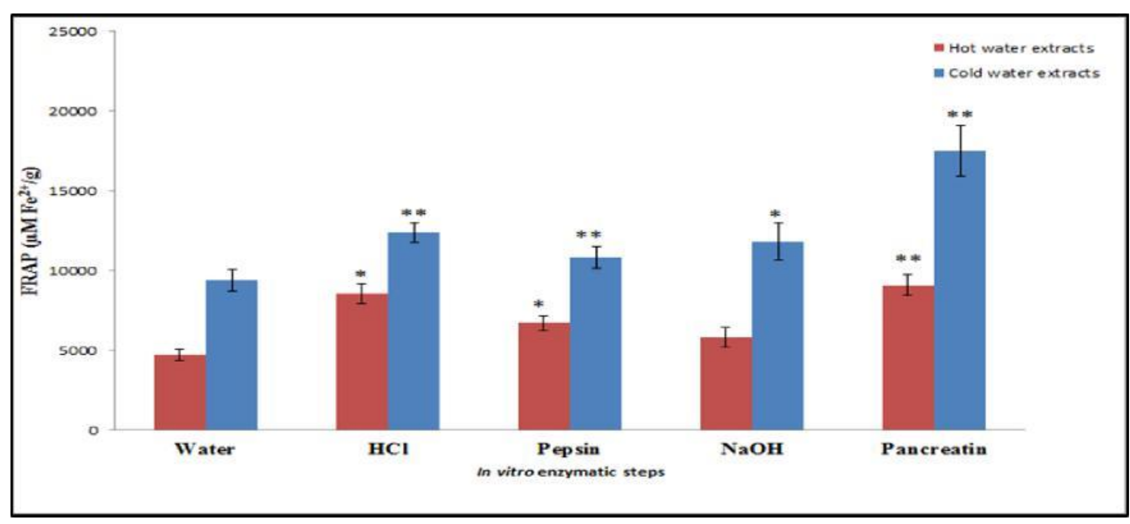


Figure 3.28: Total antioxidant capacity of *H. erinaceus* extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) during the in vitro enzymatic digestion stepsThe symbols ** (*p* <0.01) and * (*p* <0.05) denote significant differences from the FRAP values prior to the in vitro digestion steps.). Each value is expressed as mean ± standard deviation (n=3).

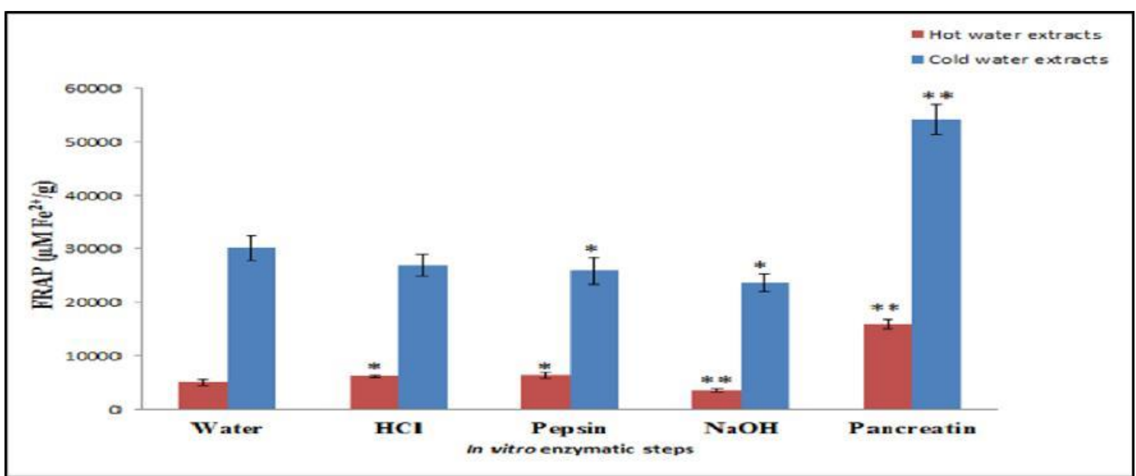
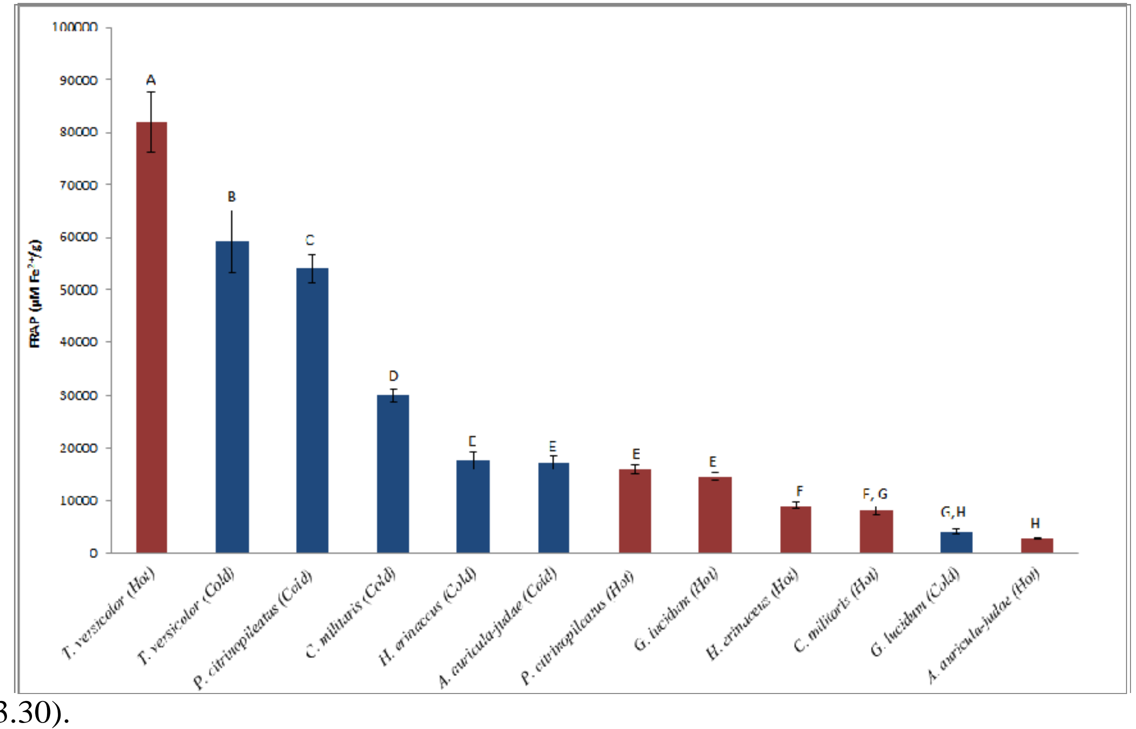


Figure 3.29: Total antioxidant capacity of *P. citrinopileatus* extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) during the in vitro enzymatic digestion stepsThe symbols ** (*p* <0.01) and * (*p* <0.05) denote significant differences from the FRAP values prior to the in vitro digestion steps.) Each value is expressed as mean ± standard deviation (n=3).

The hot-water extract of *T. versicolor* (82010 µmol Fe^{2+}/g of d.w.) showed the highest redox ability, followed by *T. versicolor* (the cold-water extract) > *P. citrinopileatus* (the cold-water extract) > *C. militaris* (the cold-water extract), *H. erinaceus* (the cold-water extract) > *A. auricula-judea* (the cold-water extract) > *P. citrinopileatus* (the hot-water extract) > *G. lucidum* (the hot-water extract) > *H. erinaceus* (the hot-water extract) > *C. militaris* (the hot-water extract) > *G. lucidum* (the cold-water extract) > *A. auricula-judea* (the hot-water extract) (p<0.001) (Figure



3.30).

Figure 3.30: Total antioxidant capacity of selected mushroom extracts (hot-water and cold-water) measured by using the FRAP assay (µmol ferrous ion equivalent) after the in vitro enzymatic digestion steps. Bars with different letters are significantly different (p <0.05). Each value is expressed as mean ± standard deviation (n=3).

3.5.3 *DPPH*

The pattern of potential antioxidant compound release from six mushroom samples during *in vitro* enzymatic digestion determined by using the DPPH assay is shown in Figures 3.31 to 3.36 and Table 3.5. After pepsin digestion, *C. militaris* (in both extracts), *G. lucidum* (in the cold-water extract), *H. erinaceus* (in both extracts) and *P. citrinopileatus* (in both extracts) showed significant decreases (p<0.05). The hot-water extract of *G. lucidum* and *A. auricula-judae* (in both extracts) showed no significant effect (p=0.5). *T. versicolor* (in both extracts) showed significant increases post-pepsin digestion in both extracts (p<0.05).

There was a significant increase in the total apparent antioxidant ability on scavenging DPPH free radicals for *A. auricula-judae* (p<0.01 in the hot- and cold-water extracts respectively), *T. versicolor* (p<0.01 in both extracts), *G. lucidum* (p<0.01 and p=0.047 in both extracts), *H. erinaceus* (p<0.05 in both extracts), and *P. citrinopileatus* (p<0.01 in both extracts) after the *in vitro* enzymatic digestion steps. The DPPH value of *C. militaris* showed a significant decrease after the ninety-minute digestion step (p< 0.01 in both extracts) (Figures 3.31 to 3.36 and Table 3.5).

Table 3.5: Total antioxidant activity (mmol TE/g dried weight) of selected mushrooms measured by DPPH during the *in vitro* enzymatic digestion at 10 and 90 min. Values are means ± SD. Each value is expressed as mean ± standard deviation (*n*=3).

Mushroom	10 (min)	90 (min)	*p* (10 VS 90)
Auricularia auricula-judae (Hot water)	0.5±0.023	2.43±0.115	0.001
Auricularia auricula-judae (Cold water)	0.41±0.01	1.86±0.104	0.001
Cordyceps militaris (Hot water)	4.58±0.043	1.07±0.044	< 0.001
Cordyceps militaris (Cold water)	7.05±0.363	5.55±0.280	0.005
Trametes versicolor (Hot water)	1.25±0.068	4.01±0.178	0.001
Trametes versicolor (Cold water)	0.97±0.084	1.96±0.103	<0.001
Ganoderma lucidum (Hot water)	0.85±0.072	1.57±0.066	0.004
Ganoderma lucidum (Cold water)	0.27±0.018	0.33±0.005	0.047
Hericium erinaceus (Hot water)	1.84±0.137	2.15±0.137	0.016
Hericium erinaceus (Cold water)	2.98±0.265	3.36±0.199	0.034
Pleurotus citrinopileatus (Hot water)	4.05±0.225	6.89±0.378	0.001
Pleurotus citrinopileatus (Cold water)	5.83±0.328	8.84±0.504	0.002

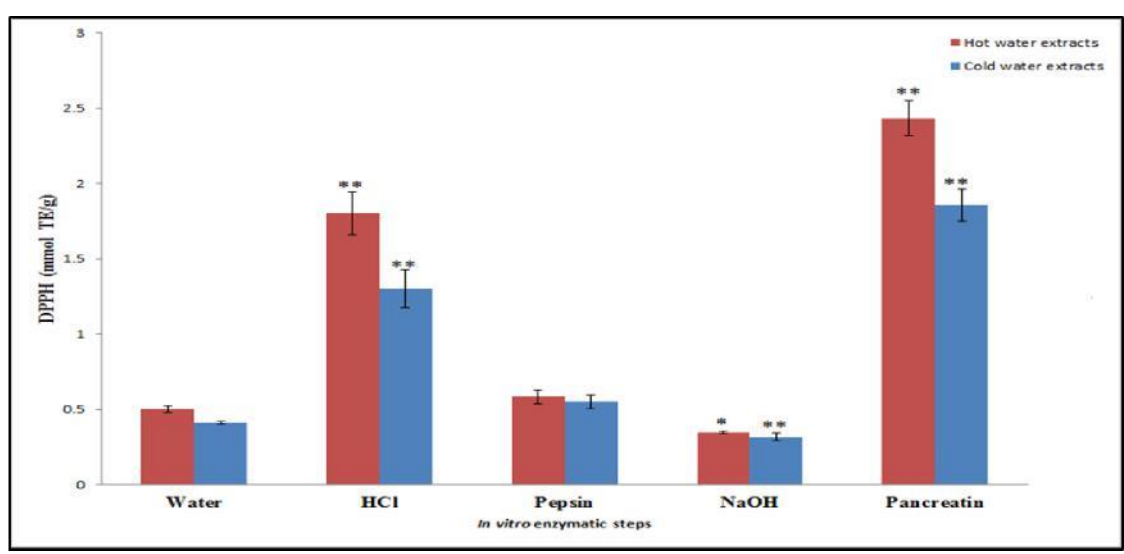


Figure 3.31: Total antioxidant capacity of *A. auricula-judae* extracts (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) during the *in vitro* enzymatic digestion stepsThe symbols ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the *in vitro* digestion steps). Each value is expressed as mean ± standard deviation (n=3).

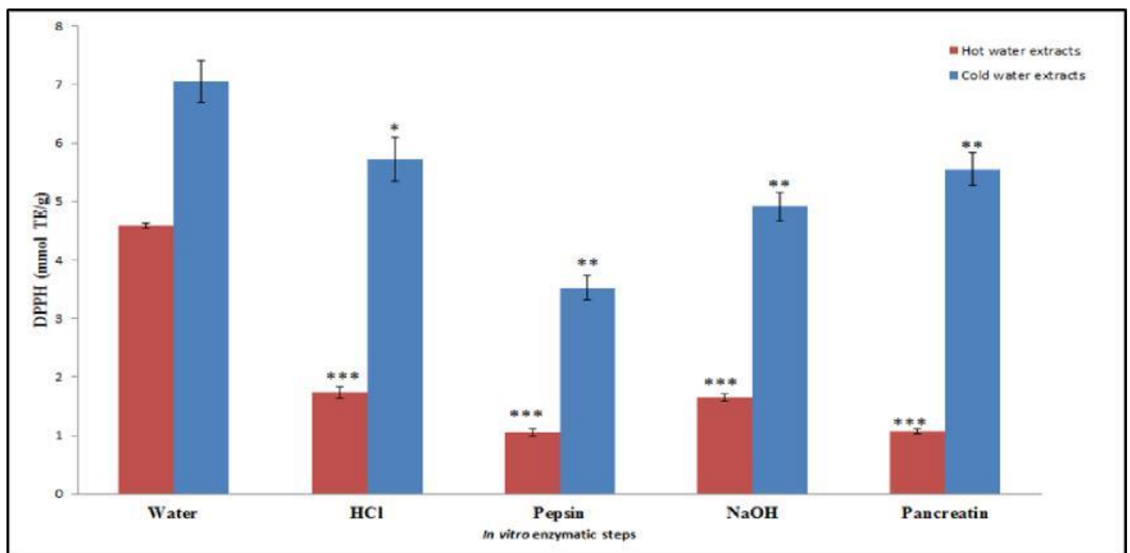


Figure 3.32: Total antioxidant capacity of *C. militaris* extracts (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps. The symbols * ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the in vitro digestion steps). Each value is expressed as mean ± standard**

deviation (n=3).

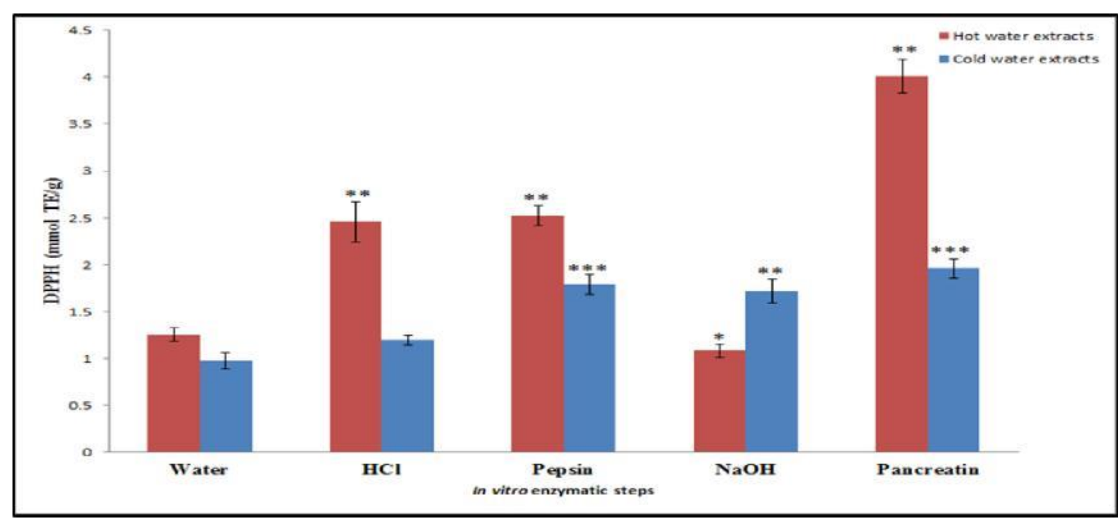


Figure 3.33: Total antioxidant capacity of *T. versicolor* extracts (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) during the *in vitro* enzymatic digestion steps The symbols * ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the *in vitro* digestion steps). Each value is expressed as mean ± standard deviation (n=3).**

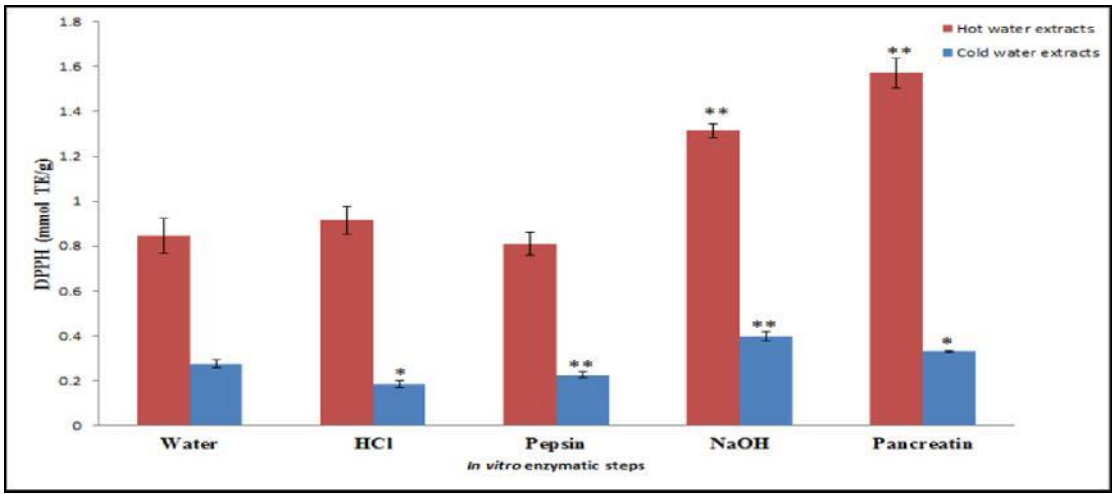


Figure 3.34: Total antioxidant capacity of *G. lucidum* extracts (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps The symbols ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the in vitro digestion steps). Each value is expressed as mean ± standard deviation (n=3).

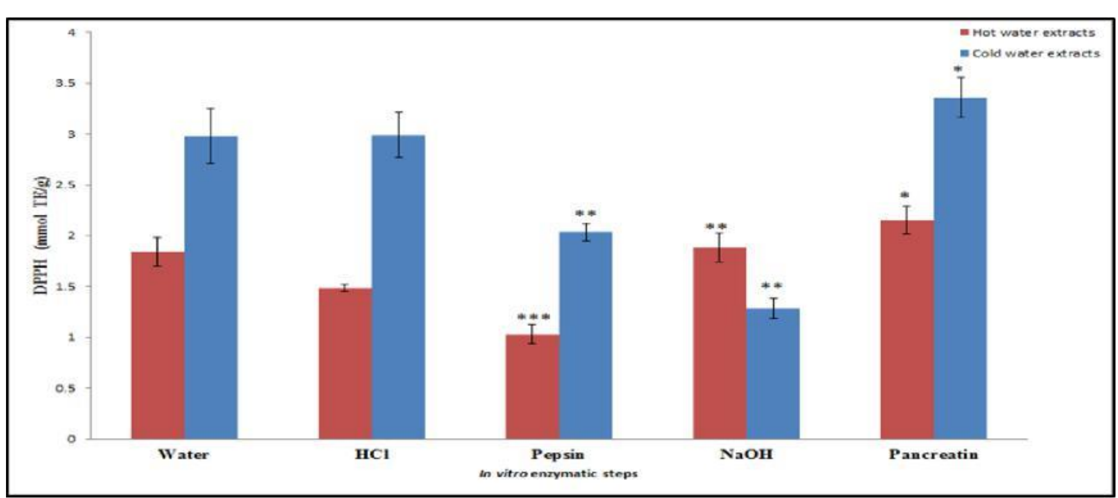


Figure 3.35: Total antioxidant capacity of *H. erinaceus* extracts (hot-water and cold-water) by using the DPPH assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps The symbols * ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the in vitro digestion steps). Each value is expressed as mean ± standard deviation (n=3).**

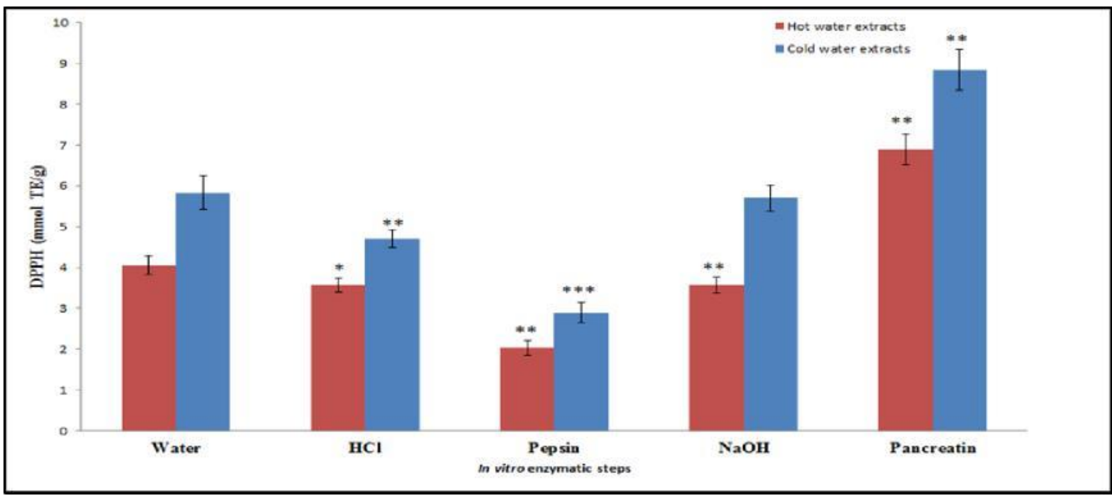


Figure 3.36: Total antioxidant capacity of *P. citrinopileatus* extract (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) during the in vitro enzymatic digestion steps The symbols * ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.05$) denote significant differences from the DPPH scavenging values prior to the in vitro digestion steps). Each value is expressed as mean ± standard deviation (n=3).**

Overall, after ninety minutes of *in vitro* digestion, *P. citrinopileatus* (8.8mmol TE/g of d.w. in the cold-water extract) showed the highest DPPH free radical scavenging ability, followed by *P. citrinopileatus* (the hot-water extract) > *C. militaris* (the cold-water extract) > *T. versicolor* (the hot-water extract) > *H. erinaceus* (the cold-water extract) > *A. auricula-judae* (the hot-water extract) > *H. erianceus* (the hot-water extracts) > *T. versicolor* (the cold-water extract) > *A. auricula-judae* (the cold-water extract) > *G. lucidum* (the hot-water extract) > *C. militaris* (the hot-water extract) > *G. lucidum* (the cold-water extract) ($p < 0.001$) (Figure 3.37).

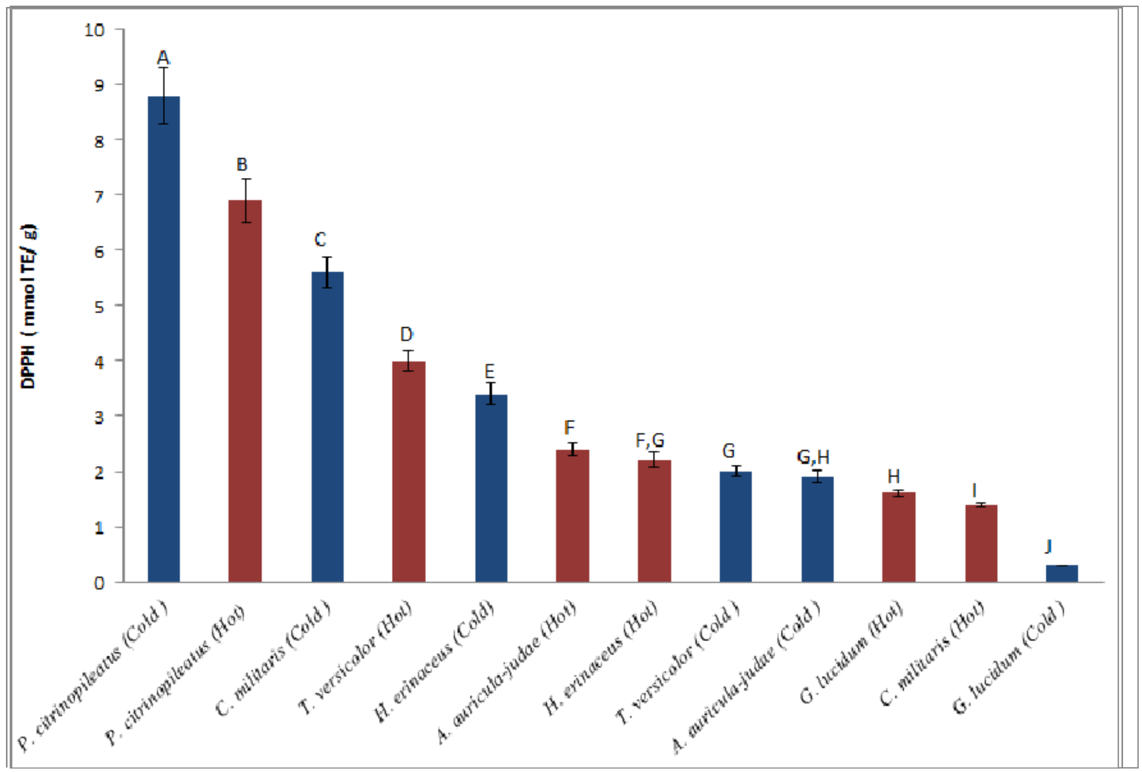


Figure 3.37: Total antioxidant capacity of selected mushroom extracts (hot-water and cold-water) measured by using the DPPH assay (mmol Trolox equivalent) after the in vitro enzymatic digestion steps. Bars with different letters are significantly different ($p < 0.05$). Each value is expressed as mean ± standard deviation ($n=3$).

3.6 Cytotoxicity

Concentrations of mushroom extracts which decreased the absorbance by > 10% when compared with the control were considered to be cytotoxic. According to the cytotoxicity assay (*see* Figures 3.38 to 3.49), the dosages of each mushroom extract for cellular antioxidant assay were: *A. auricula-judae* 1 mg /ml for both hot- and cold-water extracts, *C. militaris* 0.25 mg/ml and 0.6 mg/ml for hot- and cold-water extracts respectively, *T. versicolor* 0.5 mg/ml for both hot- and cold-water extracts, *G. lucidum* 0.48 mg/ml and 0.5 mg/ml for hot- and cold-water extracts respectively, *H. erinaceus* 5 mg/ml and 4 mg/ml for hot- and cold-water extracts respectively, and *P. citrinopileatus* 1.5 mg/ml and 2 mg/ml for hot- and cold-water extracts respectively.

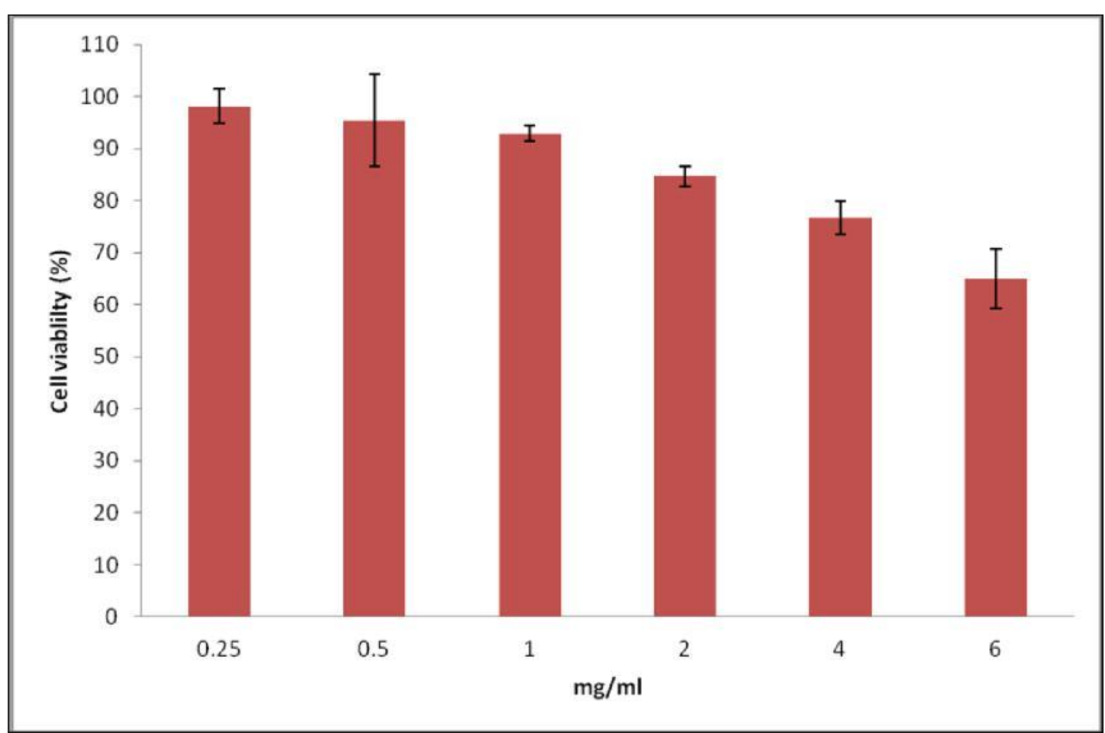


Figure 3.38: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *A. auricula-judae.* Each value is expressed as mean ± standard deviation (n=3).

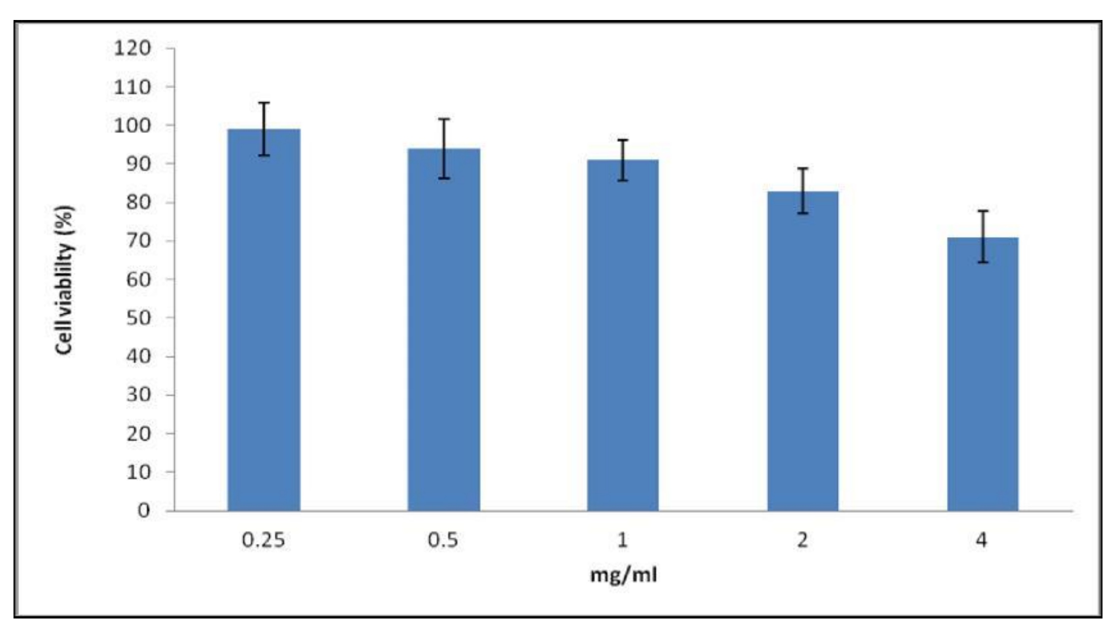


Figure 3.39: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *A. auricula-judae*. Each value is expressed as mean ± standard deviation (n=3).

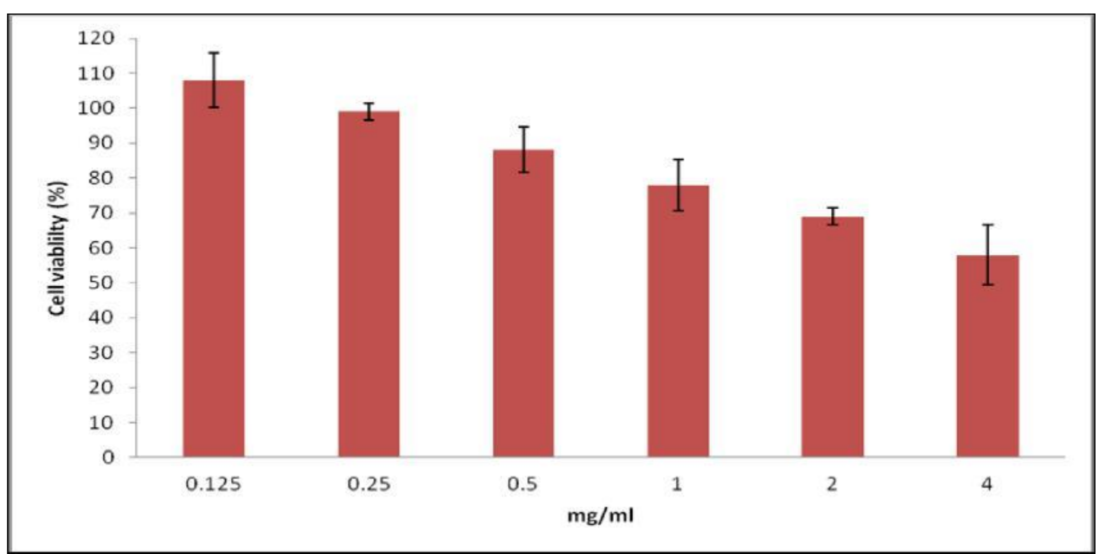


Figure 3.40: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *C. militaris*. Each value is expressed as mean ± standard deviation (n=3).

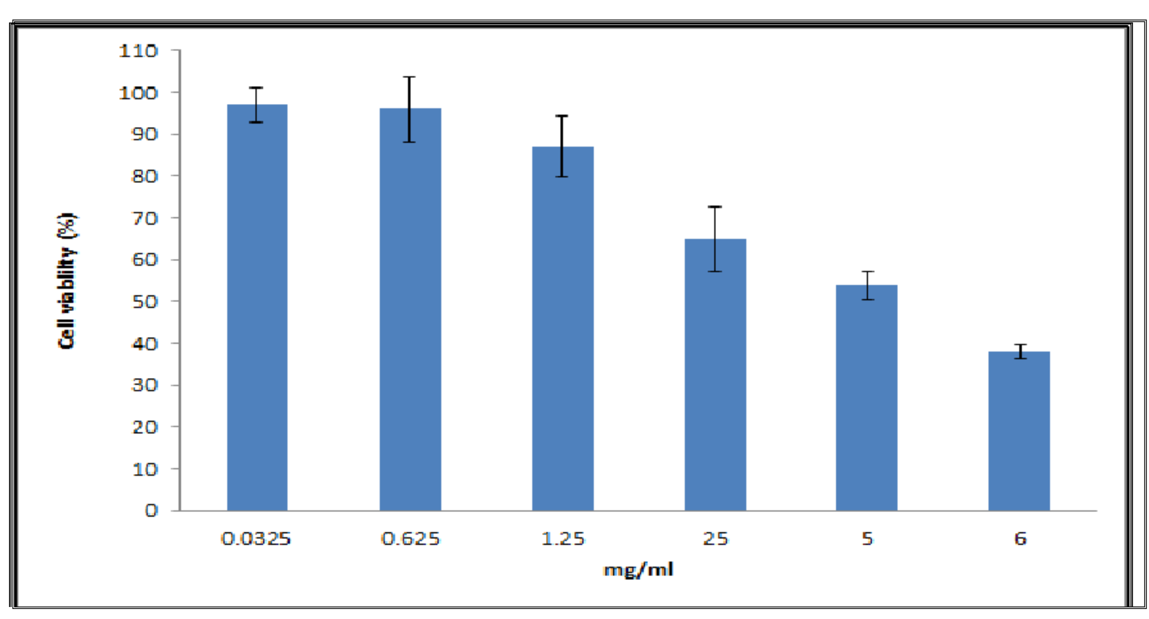


Figure 3.41: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *C. militaris*. Each value is expressed as mean ± standard deviation (n=3).

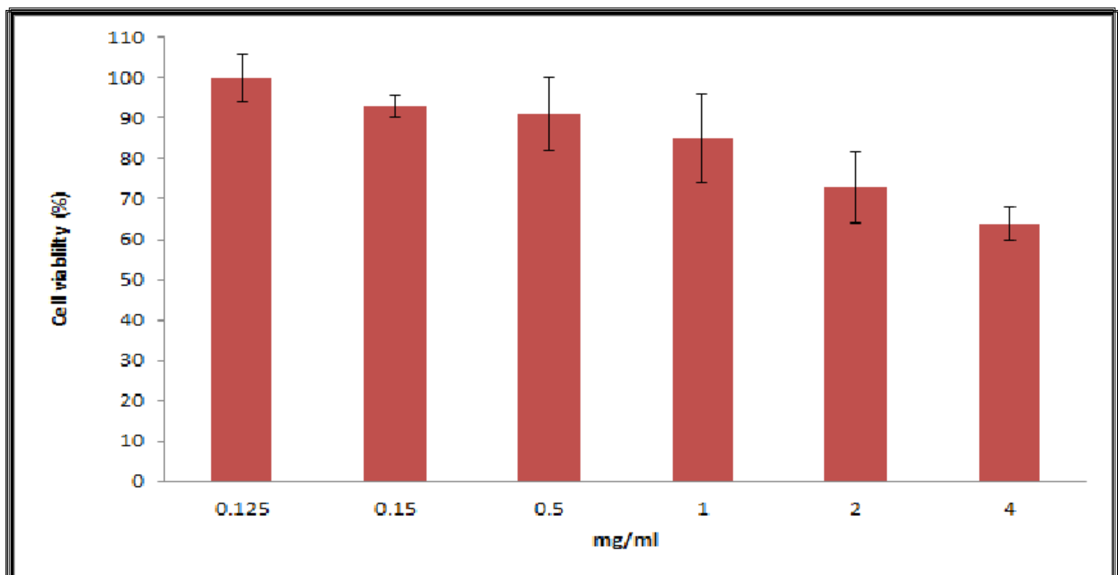


Figure 3.42: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *T. versicolor*. Each value is expressed as mean ± standard deviation (n=3).

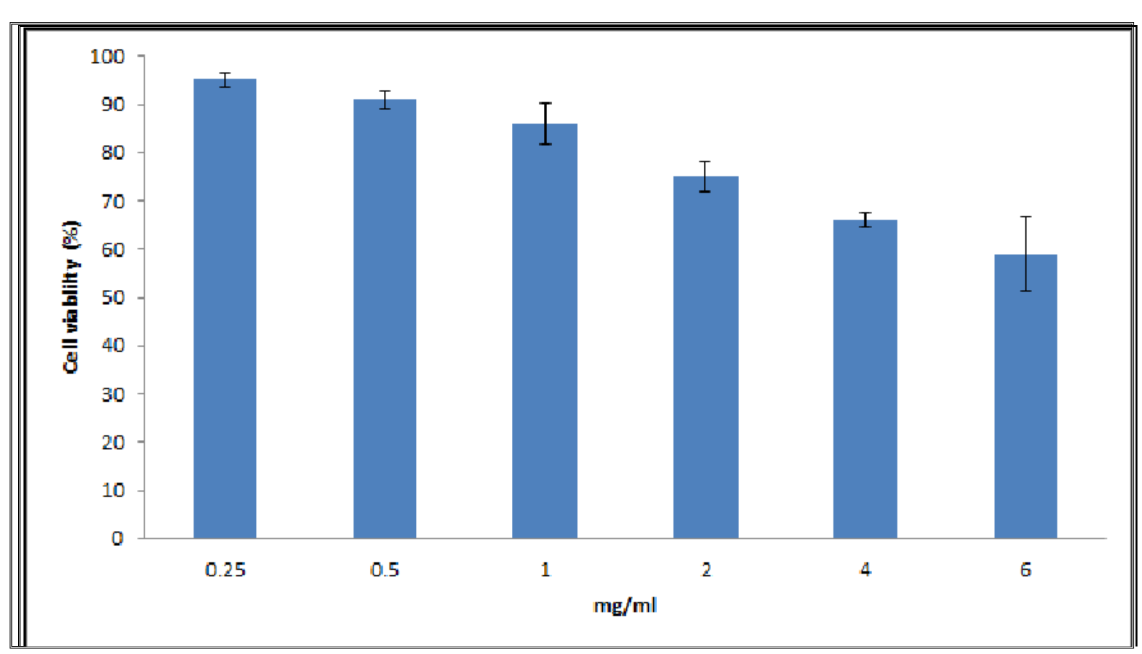


Figure 3.43: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *T. versicolor*. Each value is expressed as mean ± standard deviation (n=3).

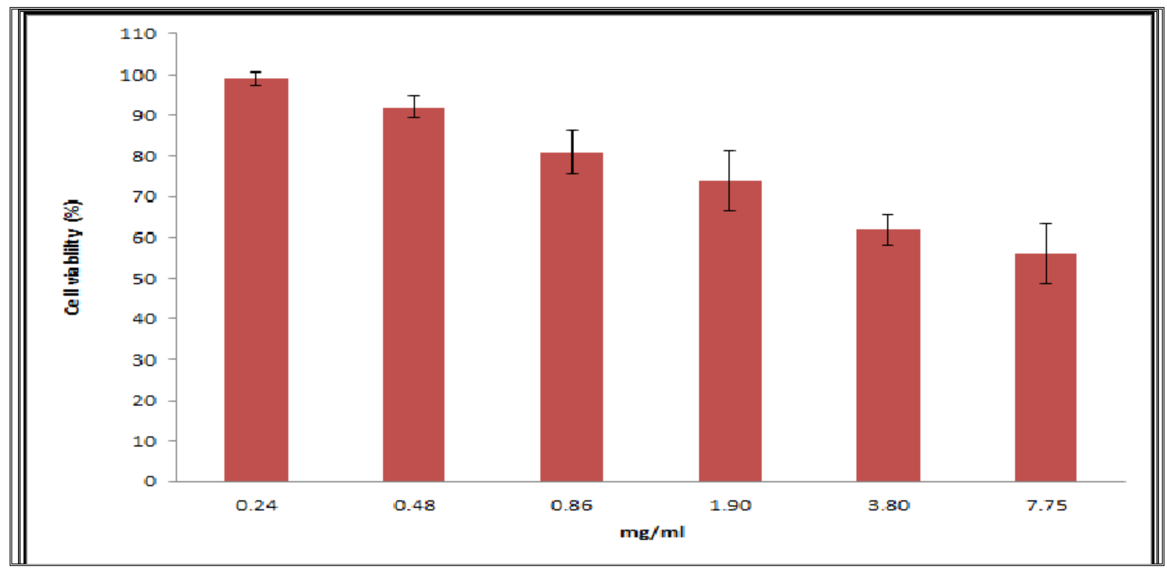


Figure 3.44: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *G. lucidum*. Each value is expressed as mean ± standard deviation (n=3).

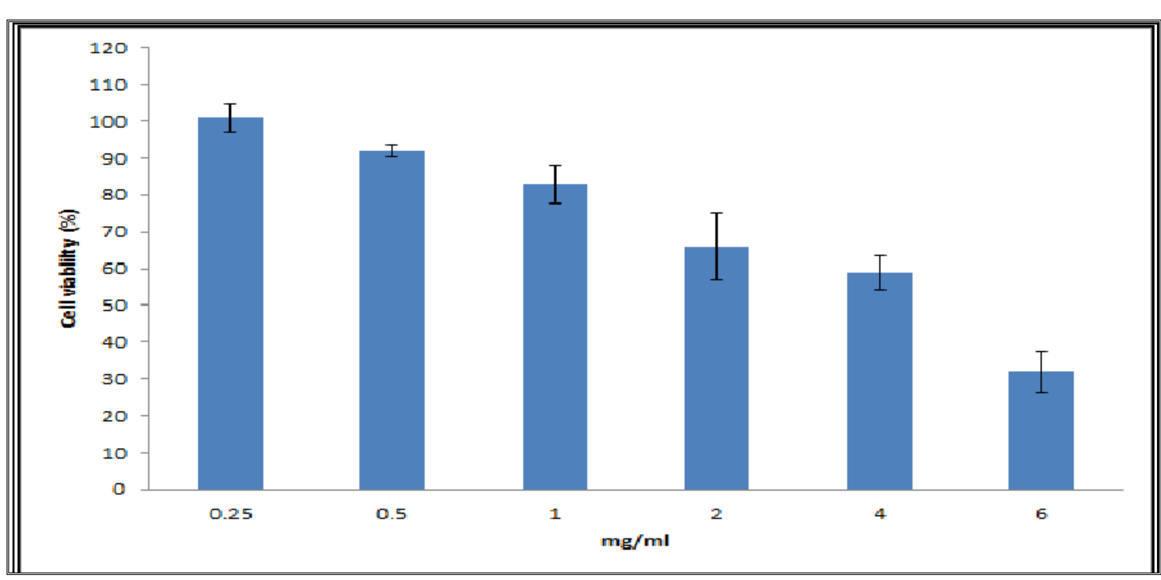


Figure 3.45: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *G. lucidum*. Each value is expressed as mean ± standard deviation (n=3).

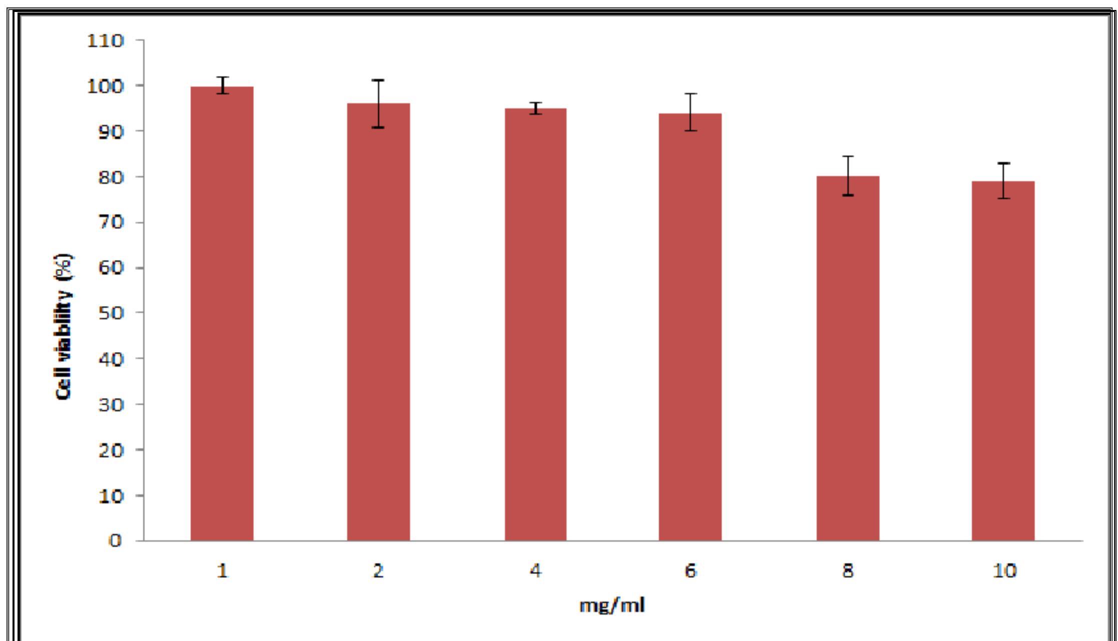


Figure 3.46: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *H. erinaceus*. Each value is expressed as mean ± standard deviation (n=3).

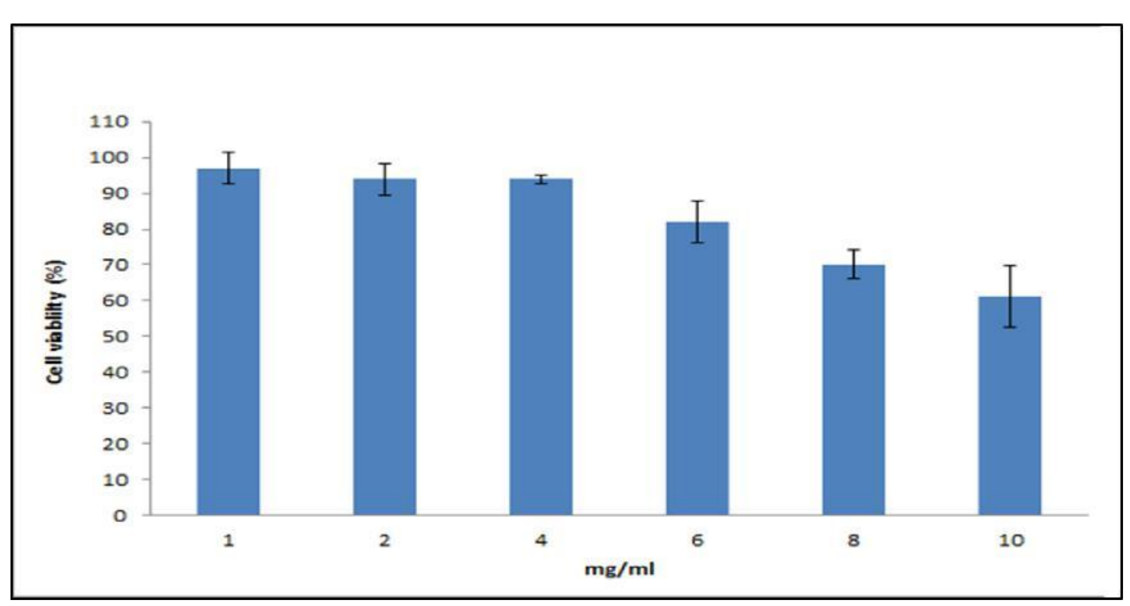


Figure 3.47: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *H. erinaceus*. Each value is expressed as mean ± standard deviation (n=3).

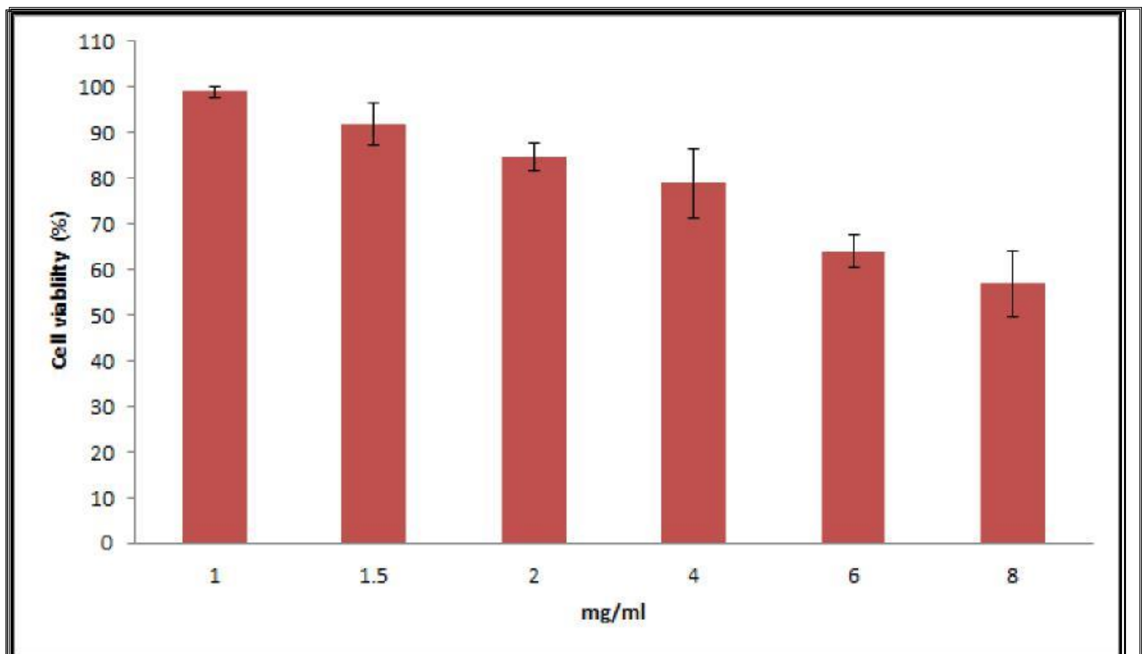


Figure 3.48: Cell survivals of Huh7 cells treated with various concentrations of hot-water extract of *P. citrinopileatus*. Each value is expressed as mean ± standard deviation (n=3).

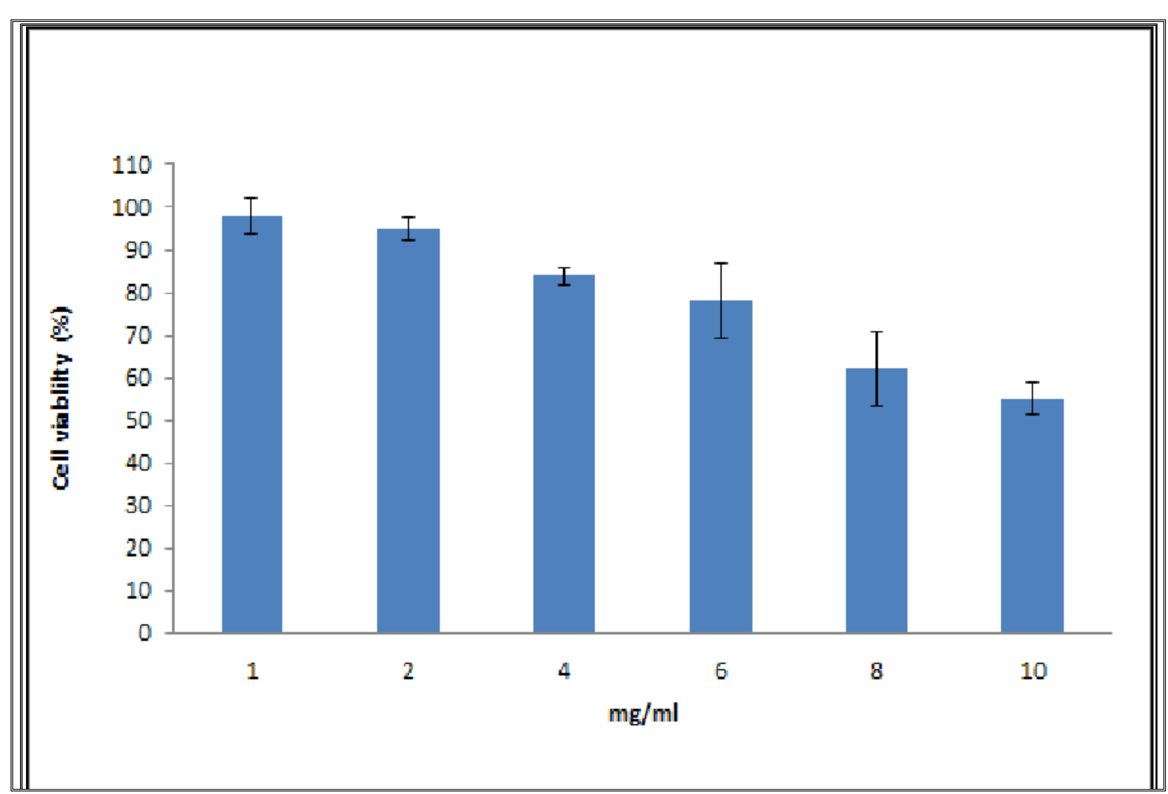


Figure 3.49: Cell survivals of Huh7 cells treated with various concentrations of cold-water extract of *P. citrinopileatus*. Each value is expressed as mean ± standard deviation (n=3).

3.7 Cellular antioxidant activity

The cellular antioxidant activity for the mushrooms ranged from 3557μmol QE/g of d.w. to 68μmol QE/g d.w. The cellular antioxidant activity values are shown in Table 3.5.

Table 3.5: Antioxidant capacity (μmol QE/g dried weight) of selected mushrooms using the cellular antioxidant activity assay. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

Mushroom	Cellular antioxidant activity (μmol QE/g)
Auricularia auricula-judae (Hot water)	126±4
Auricularia auricula-judae (Cold water)	236±26
Cordyceps militaris (Hot water)	2638±247
Cordyceps militaris (Cold water)	3557±305
Trametes versicolor (Hot water)	735±80
Trametes versicolor (Cold water)	550±41
Ganoderma lucidum (Hot water)	278±21
Ganoderma lucidum (Cold water)	107±12
Hericium erinaceus (Hot water)	68±6
Hericium erinaceus (Cold water)	106±10
Pleurotus citrinopileatus (Hot water)	192±20
Pleurotus citrinopileatus (Cold water)	340±20

In this study, 2´, 7´-dichlorofluorescin diacetate was taken up by Huh7 cells by passive diffusion and deactylated by cellular esterases to form polar 2´, 7´-dichlorofluorescin (DCFH), which is trapped within the cells. All of six selected mushrooms showed varying degrees of quenching ability of peroxyl radicals. C. militaris, a traditional medicinal mushroom, had a significantly higher antioxidant activity value than the other mushrooms (p<0.001) (Figure 3.50 to Figure 3.61).

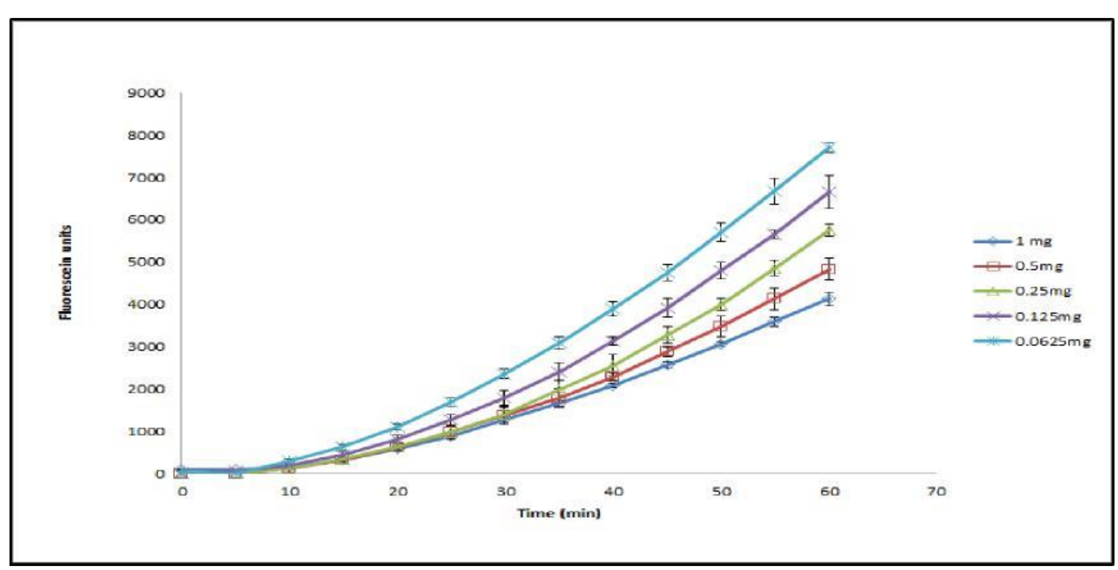


Figure 3.50: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *A. acuriculla-judae* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

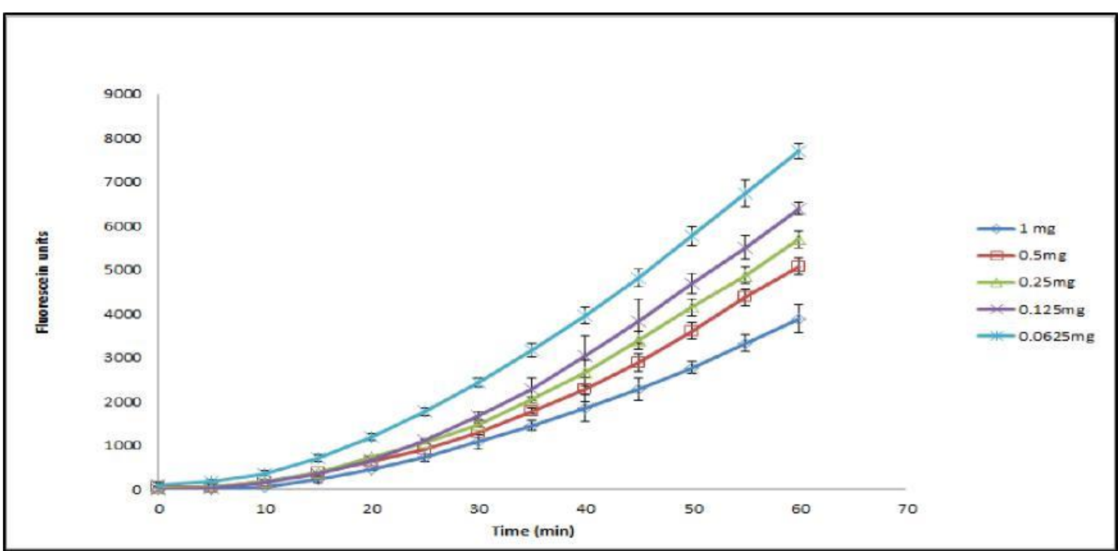


Figure 3.51: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *A. acuriculla-judae* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

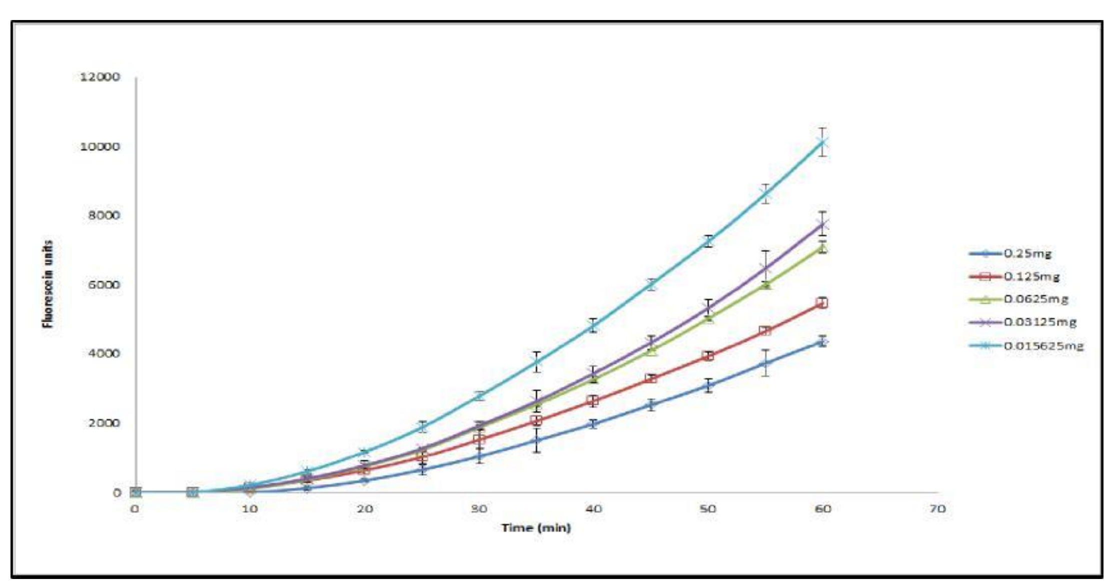


Figure 3.52: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *C. militari* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

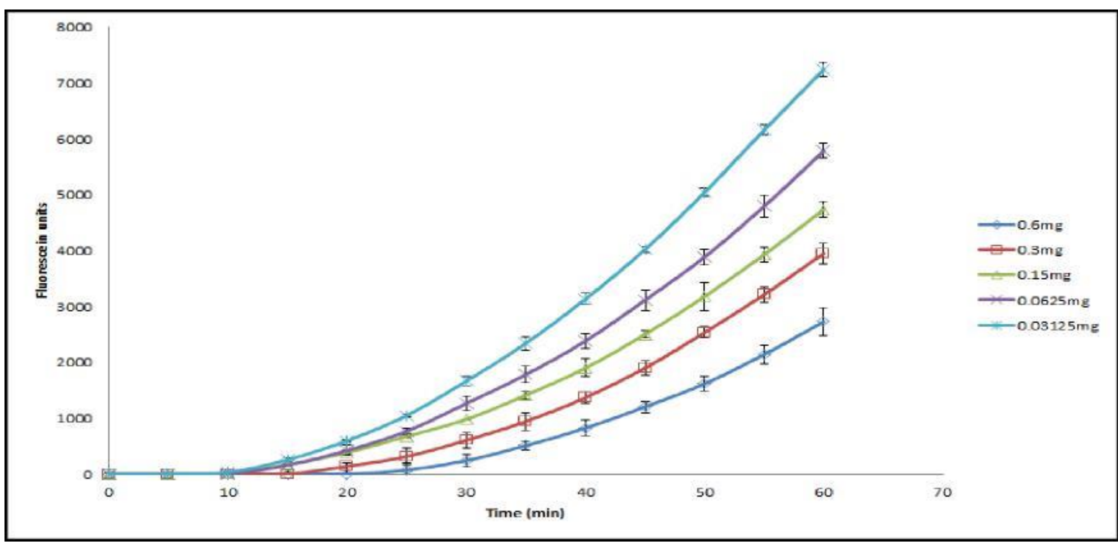


Figure 3.53: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *C. militari* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

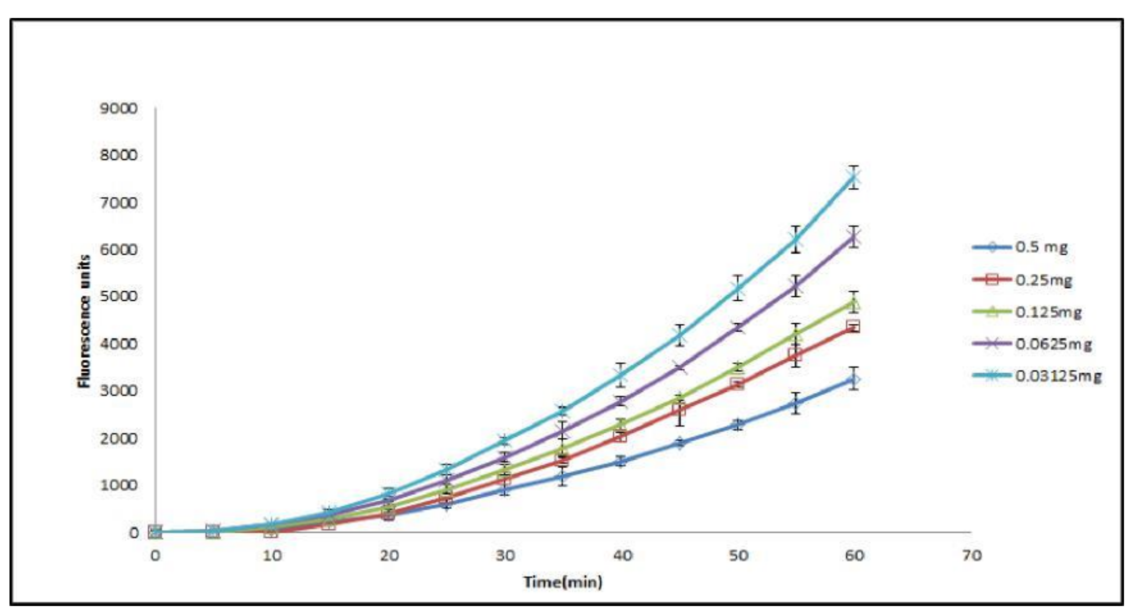


Figure 3.54: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *1. versicolor* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

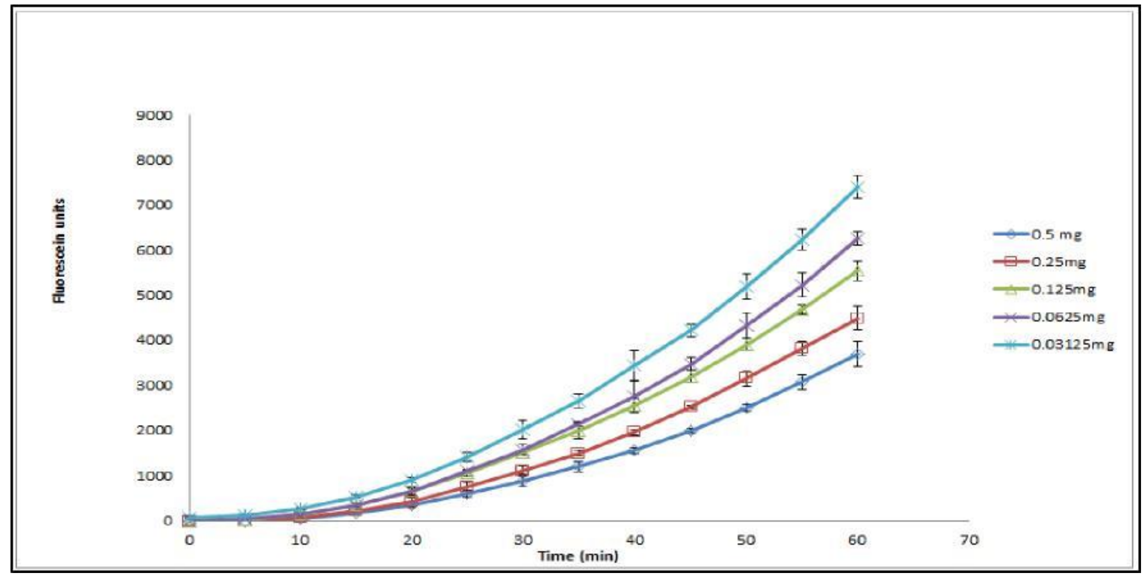


Figure 3.55: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *1. versicolor* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

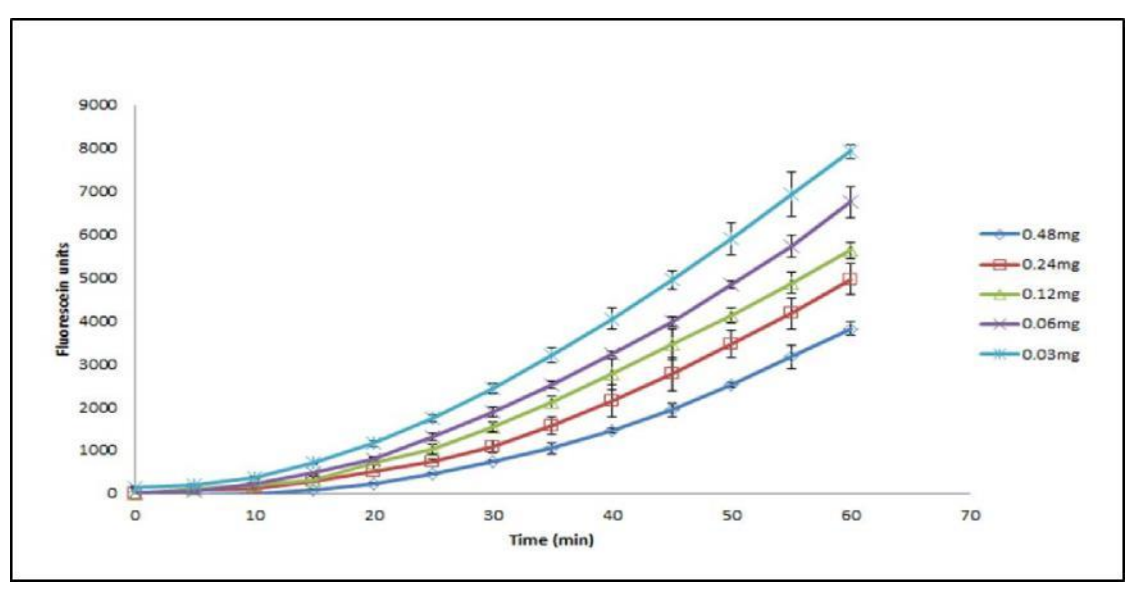


Figure 3.56: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *G. lucidum* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

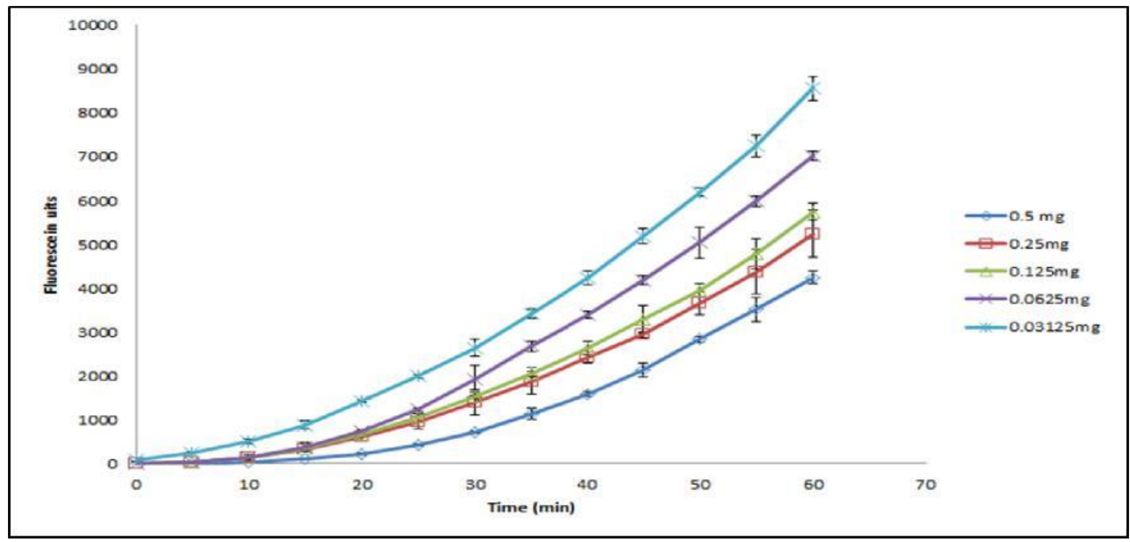


Figure 3.57: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *G. lucidum* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

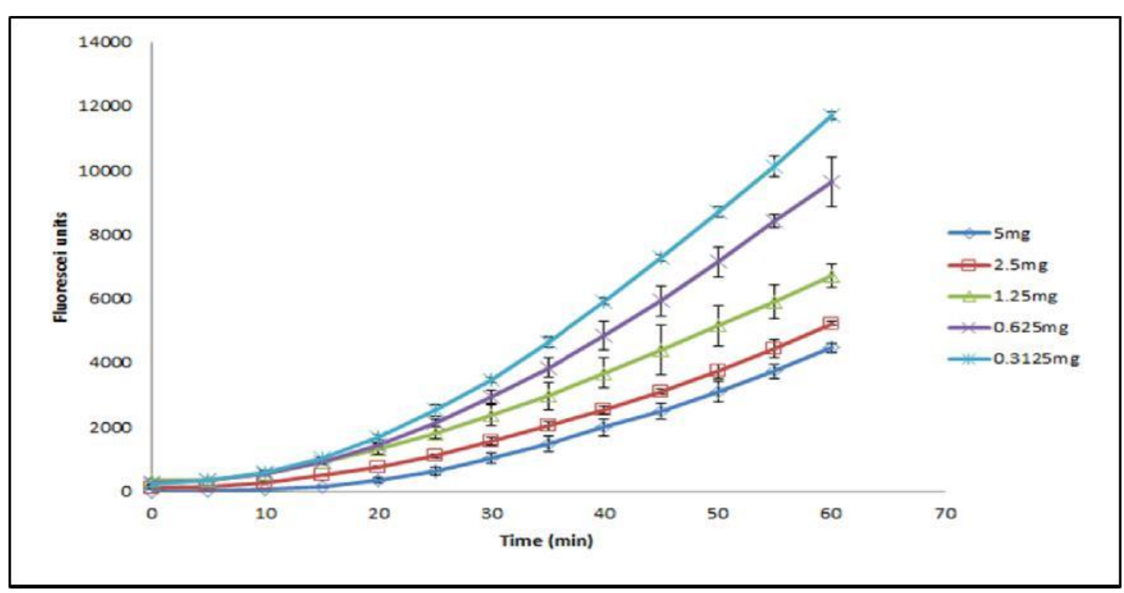


Figure 3.58: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *H. erinaceus* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

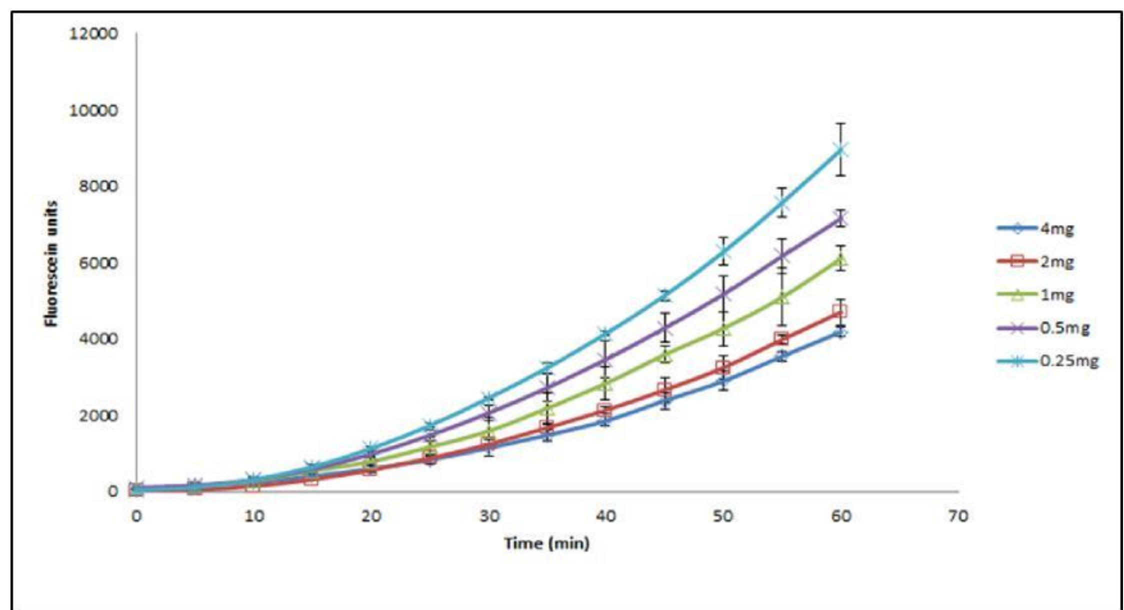


Figure 3.59: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *H. erinaceus* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

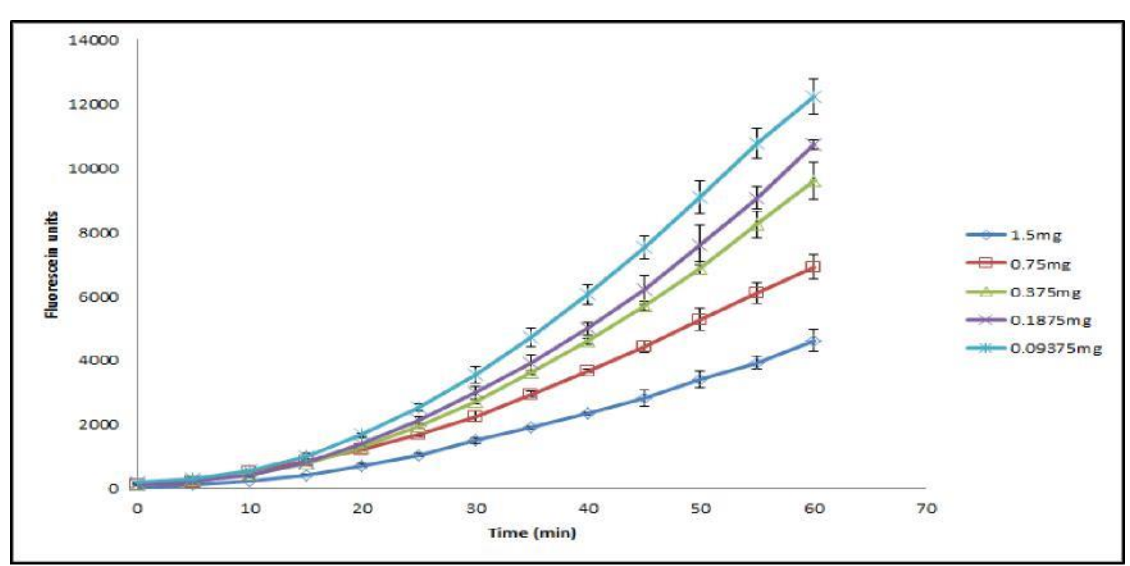


Figure 3.60: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *P. citrinopileatus* hot water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

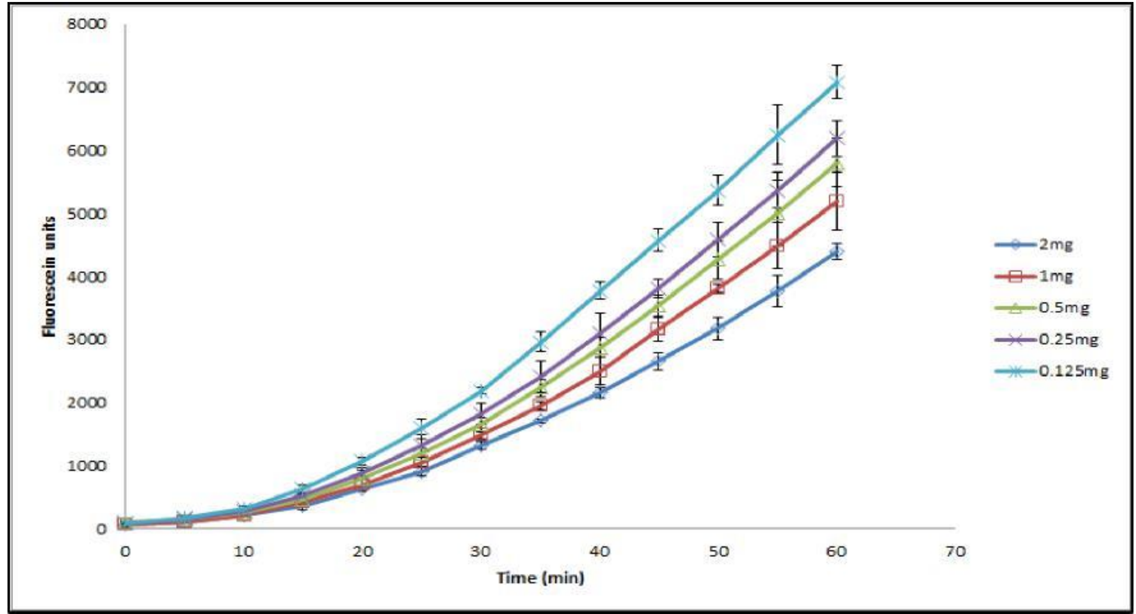


Figure 3.61: Peroxyl radical-induced oxidation of DCFH to DCF in Huh7 cells and inhibition of oxidation by *P. citrinopileatus* cold water extracts. Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

After sixty minutes of incubation, the highest cellular antioxidant activity values was *C. militaris* in both the hot- and cold-water extracts, followed by *T. versicolor* (in both extracts, the cold-water extract of *P. citrinopileatus*, the hot-water extract of *G. lucidum*, the cold-water extract of *A. auricula-judae*, the hot-water extract of *P. citrinopileatus*, the hot-water extract of *A. auricula-judae*, the cold-water extract of *G. lucidum*, and the cold-water extract of *H. erinaceus*, and the hot-water extract of *H. erinaceus* (p<0.05) (*see* Figure 3.62).

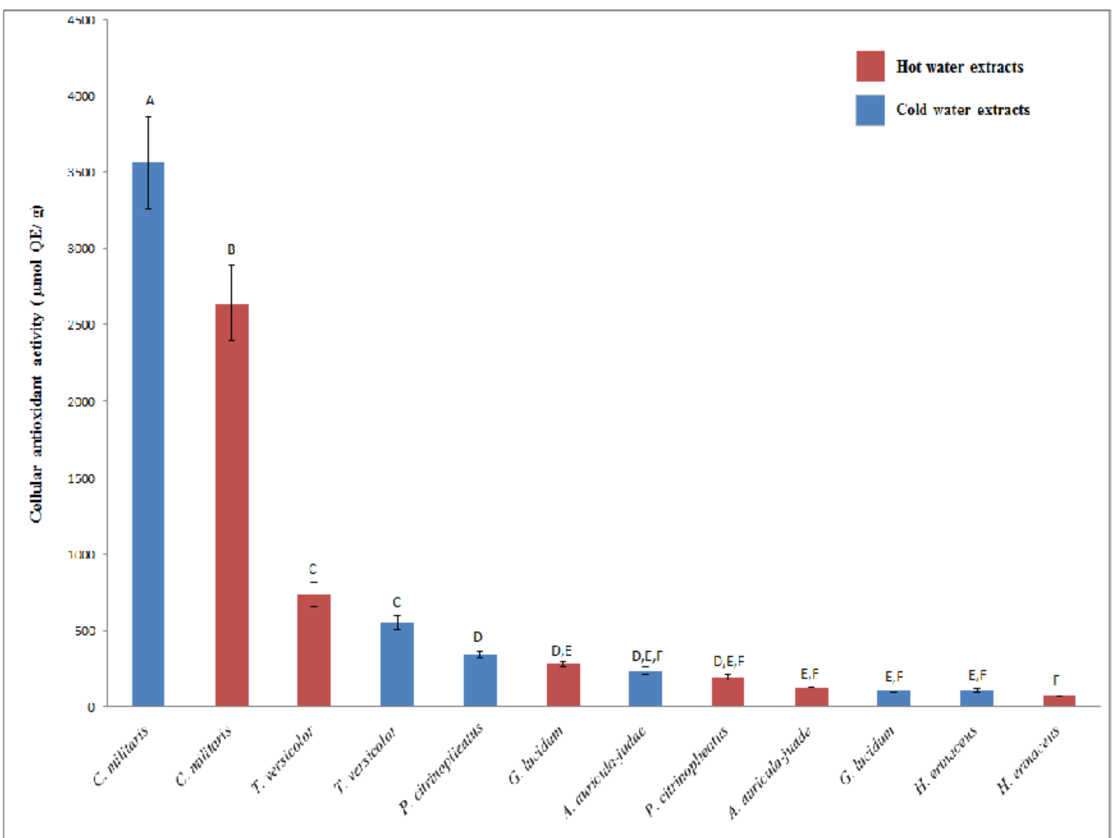


Figure 3.62: Cellular antioxidant activity of selected mushroom extracts after sixty-minute measurement. Bars with different letters are significantly different (p < 0.05). Each value is expressed as mean ± standard deviation (mean ± SD, n=3).

3.7 Correlation Analysis

The relationships in cellular antioxidant activity value between total phenolic contents, TEAC, FRAP, and DPPH were determined. The correlation coefficient for cellular antioxidant activity and total phenolic content of the hot-water extracts of selected mushrooms was significant at 0.94 (p<0.01). In contrast, the cellular antioxidant activity of the cold-water extracts of mushrooms was not significantly correlated to total phenolic content; the correlation coefficient was 0.56 (p=0.25) (Figure 3.63 and 3.64).

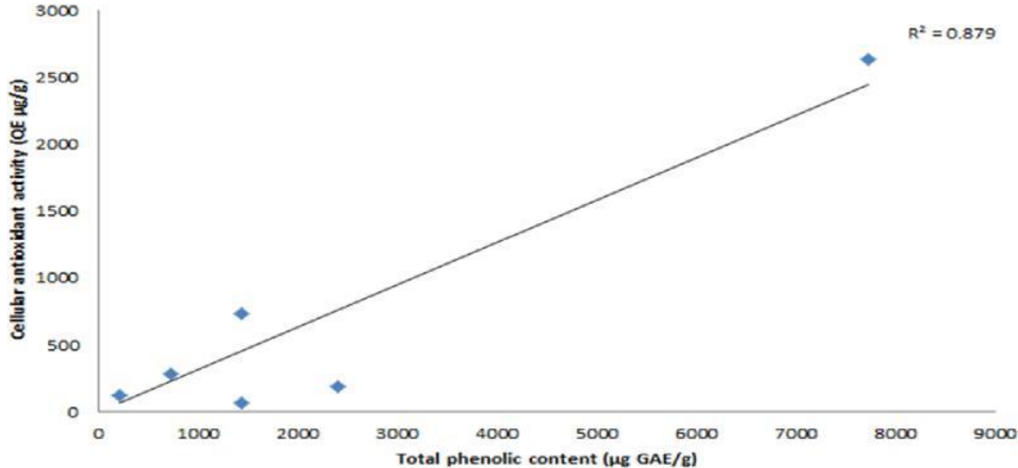


Figure 3.63: Correlation between total phenolic content and cellular antioxidant activity of hot-water extracts. Each value is expressed as mean (n=3).

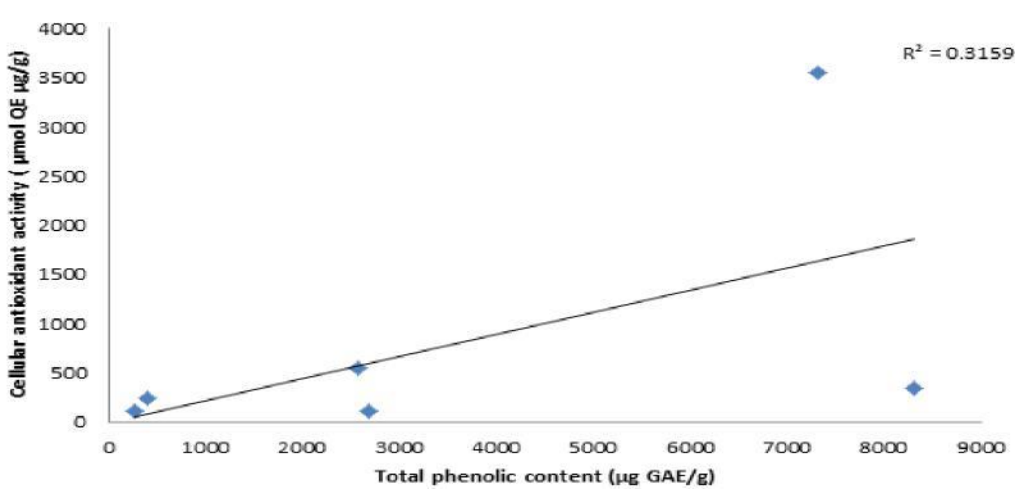
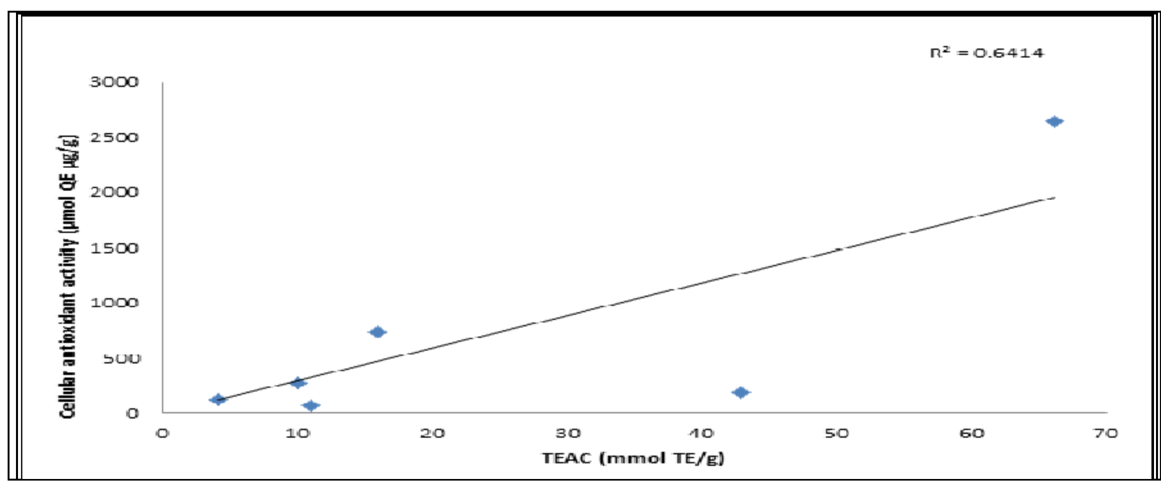


Figure 3.64: Correlation between total phenolic content and cellular antioxidant activity of cold-water extracts. Each value is expressed as mean (n=3).

The cellular antioxidant activity of the hot-water mushroom extracts was positively correlated with FRAP, and of the cold-water extracts was positively correlated with TEAC, whereas the antioxidant values of other chemical reactions for the mushrooms in both hot- and cold-water extracts were not significantly positively related to cellular antioxidant activity. Compared with the chemical-based antioxidant assays, total phenolic contents, TEAC, FRAP and DPPH, the correlation coefficients for the cellular antioxidant activity values of hot- and cold-water extracts and TEAC



were 0.8 (p=0.054) and 0.86 (p<0.05) respectively (Figure 3.65 and 3.66).

Figure 3.65: Correlation between TEAC and cellular antioxidant activity of hot-water extracts. Each value is expressed as mean (n=3).

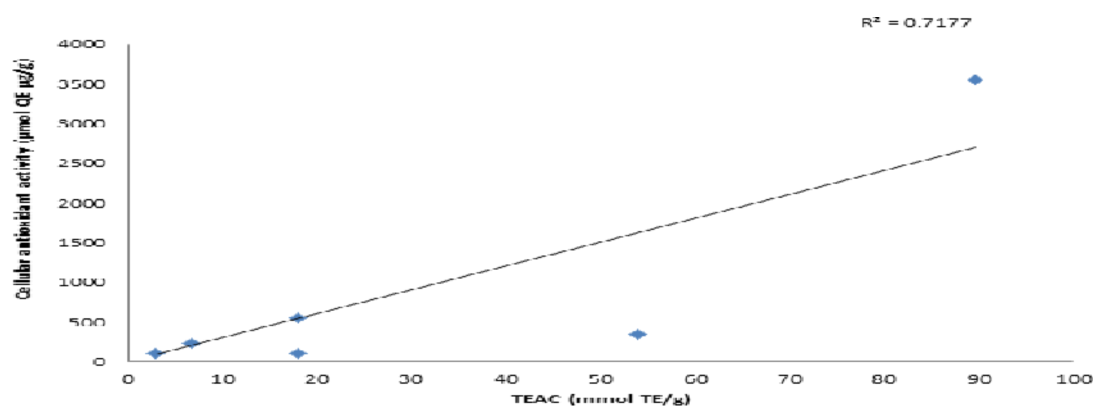


Figure 3.66: Correlation between TEAC and cellular antioxidant activity of cold-water extracts. Each value is expressed as mean (n=3)

The correlation coefficients for the cellular antioxidant activity values of hot- and cold-water extracts and FRAP were 0.93 (p<0.05) and 0.76 (p=0.079) respectively (Figure 3.67 and 3.68).

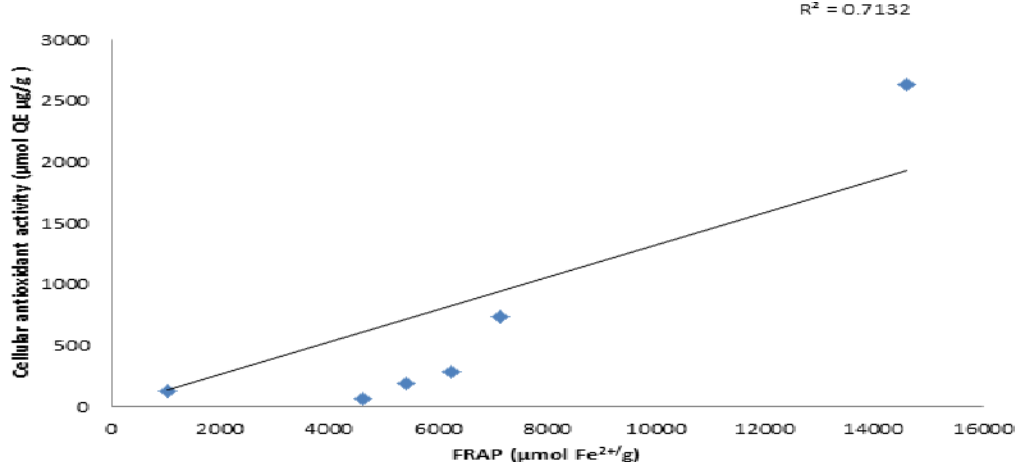


Figure 3.67: Correlation between FRAP and cellular antioxidant activity of hot -water extracts. Each value is expressed as mean (n=3).

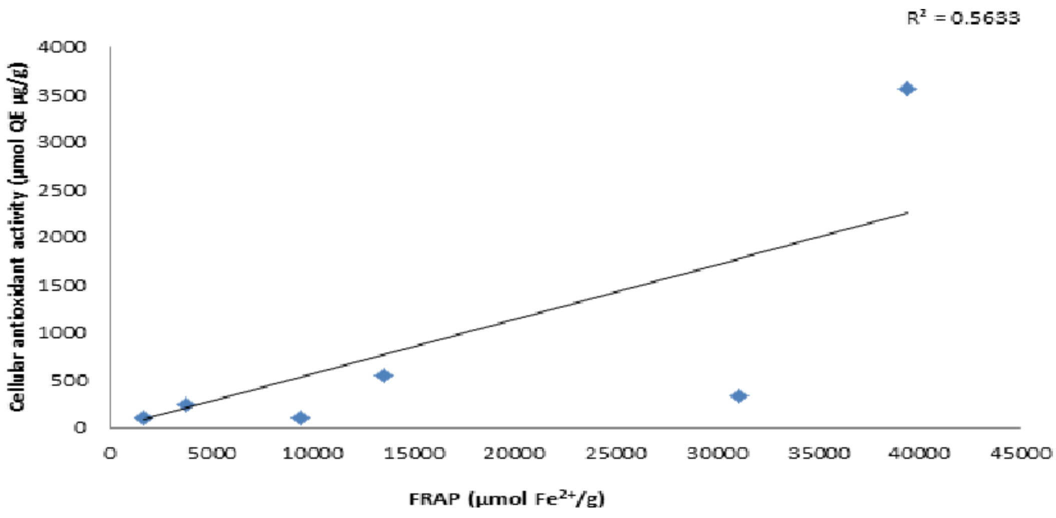


Figure 3.68: Correlation between FRAP and cellular antioxidant activity of cold-water extracts. Each value is expressed as mean (n=3).

The correlation coefficients for the cellular antioxidant activity values of hot- and cold-water extracts and DPPH were 0.56 (p=0.25) and 0.71(p=0.11) respectively (Figure 3.69 and 3.70).

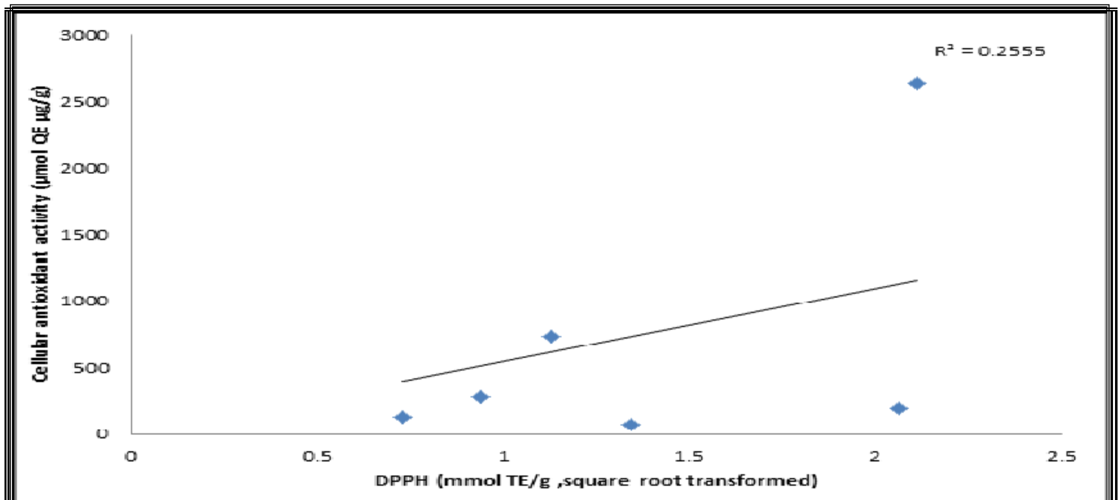


Figure 3.69: Correlation between DPPH and cellular antioxidant activity of hot-water extracts (DPPH value have been square root transformed). Each value is expressed as mean (n=3).

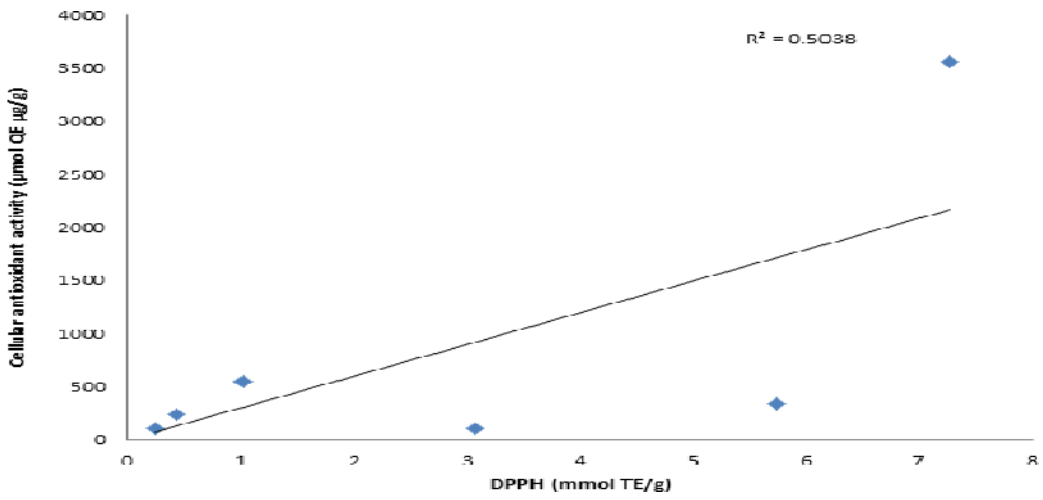


Figure 3.70: Correlation between DPPH and cellular antioxidant activity of cold-water extracts. Each value is expressed as mean (n=3).

Chapter 4 Discussion

4.1 Total phenolic contents

Mushrooms develop an analogous defence mechanism to those in plants against insects and micro-organisms by mechanisms such as the production of phenolic compounds (Vaz et al., 2011). For instance, melanins are phenolic compounds present in many mushrooms and it has been suggested that they are involved in the defence against pathogens (Islas-Flores et al., 2006; Berendsen et al., 2010). Alcalinaphenol A is a fungicide isolated from *Mycena alcalina* and is able to prevent other species from growing in the surrounding area (Spiteller, 2008). Moreover, in wound-activated chemical defence, mushrooms have developed different unique wound-activated chemical defence reactions involving the oxidation of phenols, such as 2-chloro-4nitro-phenol in *Stephanospora caroticolor* (Spiteller, 2008).

In this study, both hot- and cold-water mushroom extracts showed antioxidant activity, however, the magnitude of antioxidant potency varied with the type of extract. This could be due to the differences in concentrations and types of antioxidant compound present in the extracts. Overall, the amounts of phenolic contents extracted from the culinary mushrooms were higher than those from the medicinal mushrooms. The total average concentration of phenolic compounds in hot-water extraction was 2184μg of GAE/g and 1758μg of GAE/g and the total average in cold-water extracts was 4344μg of GAE/g and 1653μg of GAE/g in culinary and medicinal mushrooms respectively. A study of 39 medicinal and culinary herbs by Zheng and Wang (2001) also reported that culinary herbs had much higher total phenolic contents than medicinal herbs (Zheng and Wang, 2001; Kim et al., 2008). However, comparing edible mushrooms with medicinal mushrooms, which were cultivated mushrooms from Korea, Kim *et al.* (2008) reported that the total phenolic content of the medicinal mushrooms was higher than that of the edible mushrooms.

Generally, in this current study, the cold-water extracts showed more polyphenolic compounds than the hot-water extracts, whereas the hot-water extracts of *C. militartis*, *G. sinense, P. linteus* and *T. fuciformis* had more phenolic compounds than the cold-water extracts. Lee (2007b) reported similar findings that cold-water extracts of *H. marmoreus* had more polyphenolic compounds than hot-water extracts (Lee et al., 2007b). Yeh et al. (2011) also reported that cold-water extracts of *G.*

frondosa had more phenolic compounds than hot-water extracts (Yeh et al., 2011). A similar result was found in this current study. A similar result was also found in tea pollen extracts: a study of *Camellia sinensis* indicated that the cold-water extracts had higher flavonoid contents than hot-water extracts (Kao et al., 2011). In this current study, the cold-water extracts of *Pleurotus spp.* had higher phenolic contents than the hot-water extracts. Similar results were found in *Pleurotus sajor-caju* and *Pleurotus florida* by Shinde and Deshmukh (2012). In a study of *P. citrinopileatus*, Lee et al. (2007a) reported that hot-water extracts had higher phenolic contents than cold-water extracts. However, in this current study the cold-water extracts of *P. citrinopileatus* had higher phenolic contents than the hot-water extracts. A study of hot-water mushroom extracts reported that the level of phenolic contents was *G. lucidum* > *Agrocybe spp*. > *P. eryngii* > *V. volvaceae* > *L. edodes* > *H. erinaceus* > *A. auricular-judae* (Abdullah et al., 2011). In this current study the phenolic contents of hot-water extracts was *V. volvaceae* > *A. cylindracea* (Hong Kong and Taiwan) > *L. edodes* > *H. erinaceus* (China and Taiwan) > *P. eryngii* (Hong Kong and Taiwan) > *G. lucidum* > *A. auricular-judae* (China and Taiwan). The phenolic contents of cold-water extracts was *A. cylindracea* (Hong Kong) > *V. volvaceae* > *A. cylindracea* (Taiwan) > *L. edodes* > *P. eryngii* (Hong Kong and Taiwan) > *H. erinaceus* (China and Taiwan) > *G. lucidum* > *A. auricular-judae* (China and Taiwan). Tsai et al. (2007) reported that the phenolic contents of hot-water extracts of *A. subrufescens*, *A. cylindracea* and *B. edulis* had no significant difference (Tsai et al., 2007). In this current study, the phenolic contents of hot-water extracts were that *A. subrufescens* (Nantou, Taiwan) had higher total phenolic contents and there was no significant difference between *A. subrufescens* (Hong Kong) and *A. cylindracea* (Hong Kong and Taiwan), whereas *B. edulis* had the lowest phenolic contents compared with *A. subrufescens* and *A. cylindracea.* In cold-water extracts, the phenolic contents of *A. subrufescens* (Taiwan) and *A. cylindracea* (Taiwan) had no significant differences, and also had higher phenolic contents than *A. subrufescens* (Hong Kong), *A. cylindracea* (Hong Kong) and *B. edulis*. There was no statistical difference between *A. subrufescens* (Hong Kong), *A. cylindracea* (Hong Kong) and *B. edulis*, which is similar to the results which Tsai et al. (2007) reported. In this current study, the phenolic content of hot-water extracts of *Coprinus comatus* was 2457μg GAE/g, however, Tsai et al. (2009) reported a finding of 5600μg GAE/g (Tsai et al., 2009). A study of metholic extracts reported that *T. versicolor* had 2328μg GAE/g (Mau et al.,

2002). In this current study, the water extracts of *T. versicolor* were 1441 and 2575μg GAE/g in hot- and cold-water extracts respectively. Barros et al. (2007c) reported that the methanolic extract of *Scardon imbricatus* was 3760μg GAE/g (Barros et al., 2007c). In this current study, the hot- and cold-water extracts of *S. imbricatus* were 2330 and 3870μg GAE/g respectively. Mau et al. (2002) demonstrated that the methanolic extract of *H. erinaceus* was 120μg GAE/g (Mau et al., 2002). As results presented in Chapter 2 show, the amounts of the hot- and cold-water extracts of *H. erinaceus* (China and Taiwan) were 1442 and 1697μg GAE/g and 2676 and 2783μg GAE/g respectively. Lee et al. (2007a) demonstrated that the amount of phenolic compounds in hot-water extracts of *P. citrinopileatus* was higher than that of cold-water extracts. In this current study, the amount of phenolic compounds of the cold-water extract of *P. citrinopileatus* was higher than that of hot-water extracts. Samchai et al. (2009) reported that the cold-water extract of *P. linteus* was 596μg GAE/g (Samchai et al., 2009). In this current study, the hot- and cold-water extracts of *P. linteus* were 1154 and 244μg GAE/g respectively. Cheung et al. (2003) reported that the phenolic contents of water extracts of *L. edodes* and *V. volvacea* were 1330 and 1340μg GAE/g respectively (Cheung et al., 2003). In this current study, the phenolic contents of *L. edodes* were 1731 and 4604μg GAE/g in hot- and cold-water extracts respectively, and of *V. volvacea* were 5082 and 5226μg GAE/g in hot- and cold-water extracts respectively. Mau et al. (2001) reported that the amount of phenolic content of methanolic extracts of *T. fuciformis* was 1040 μg GAE/g; however, in this current study, the phenolic contents of hot- and cold-water extracts were 321 and 250μg GAE/g respectively.

FRAP

C. militaris, *G. amboinese* and *G. sinense* showed higher ferric reducing values than the other medicinal mushrooms used in this study. The hot-water extracts of *Ganoderma spp*., *P. linteus* and *W. extensa* had higher FRAP values than the cold-water extracts. In the culinary mushrooms, the hot-water extracts of *A. bisporus* and *V. volvacea* had higher FRAP values than the cold-water extracts. In a study of the leaf of *Melissa officinalis* L., Katalinic *et al.* (2006) pointed out that *M. folium* leaf infusions with hot (98°C) and cold (20°C) water could both release antioxidants and that preparations at higher temperature released more than twice the antioxidant capacity determined by FRAP (Katalinic et al., 2006). From a study of the seeds of

Lablab purpureus (L.) Sweet, Vijayakumari *et al*. (1995) suggested that a certain amount of polyphenols may react with other components to form an insoluble complex or may be oxidized during a long period of time of soaking at a higher temperature (Vijayakumari et al., 1995).

However, the opposite observation was found in *C. militaris*, *T. versicolor* and other selected mushrooms whose cold-water extractions had higher ferric reducing power than hot-water extractions. Similarly, a study of the fungus *Monascus spp.* found that cold-water extracts had higher reducing power than hot-water extracts (Lee et al., 2008). Lee et al. (2008) suggested that heating might cause thermal damage to certain reducing components (Lee et al., 2008). Lee et al. (2007a); Lee et al. (2007b) reported that cold-water extracts of *H. marmoreus* and *P. citrinopileatus* exhibited higher reducing power than their hot-water extracts (Lee et al., 2007a; Lee et al., 2007b). In this current study, the cold-water extracts of *P. citrinopileatus* showed higher FRAP value than the hot-water extracts.

In studies of hot-water extracts, *B. edulis* had higher reducing power than *P. citrinopileatus* > *A. cylindracea* > *A. subrufescens* > *C. comatus* (Tsai et al., 2007; Tsai et al., 2009). In this current study, the hot-water extracts of *A. subrufescens* (Hong Kong and Taiwan) had higher reducing power than those of *A. cylindracea* (Hong Kong and Taiwan) > *A. cylindracea* (Hong Kong and Taiwan) >*P. citrinopileatus* (Hong Kong and Taiwan) > *B. edulis* > *C. comatus*. In the cold-water extracts, *A. cylindracea* (Hong Kong and Taiwan) had higher reducing power than *A. subrufescens* (Hong Kong and Taiwan) > *P. citrinopileatus* (Hong Kong) > *B. edulis* > *P. citrinopileatus* (Taiwan) > *C. comatus*. In a study of Turkish wild mushrooms, the order of FRAP values was *B. badius* > *A. bisporus* > *P. ostreatus* (Elmastas et al., 2007). In this current study, the hot-water extract of *A. bisporus* showed the highest FRAP value, followed by *B. edulis* > *P. ostreatus*, and the cold-water extracts showed a similar order (*B. edulis* > *A. bisporus* > *P. ostreatus*).

DPPH

Compared with published reports of *P. citrinopileatus* in which cold-water extracts had higher DPPH scavenging ability than hot-water extracts, in this current study, the cold-water extract of *P. citrinopileatus* had higher DPPH scavenging value than the hot-water extract (Lee et al., 2007a). Another study of hot- and cold-water

extracts of *Pleurotus spp.* also reported that cold-water extracts exhibited higher antioxidant activity than hot-water extracts (Shinde and Deshmukh, 2012).

The same finding was made in the cold-water extracts of *Hypsizigus marmoreus* which had been reported to have had higher DPPH value than hot-water extracts (Lee et al., 2007b). Also, in a study of *V. volvacea* and *L. edodes*, the aqueous extract of *V. volvacea* showed higher DPPH scavenging ability than that of *L. edodes*, and in this current study similar results were observed (Cheung et al., 2003; Abdullah et al., 2011). Different authors have reported different DPPH values. In a study of methanolic extracts of *A. bisporus*, Savoie et al. (2008) reported that the DPPH value of strain X25, Bs0633 and Bs0118 were 4.97, 3.47 and 3.33mg/ml respectively. However, (Barros et al., 2009) reported that the DPPH value of methanolic extracts of *A. bisporus* was 9.61mg/ml. (Barros et al., 2007a; Barros et al., 2009). According to published data, *S. imbricatus* (methanolic extracts) (Barros et al., 2007c) had the most efficient DPPH radical scavenging ability, followed by *A. bisporus* (brown) (Barros et al., 2008) > *A. bisporus* (Reis et al., 2012) > *A. bisporus* (young) (Soares et al., 2009) > *A. bisporus* (mature) (Soares et al., 2009) > *A. bisporus* (Bs0118H)(Savoie et al., 2008) > *A. bisporus* (Bs0633) (Savoie et al., 2008) > *G. frondosa* (Mau et al., 2004) > *A. bisporus* (x25) (Savoie et al., 2008) > *G. lucidum*(Abdullah et al., 2011) > *S. imbricatus* (Barros et al., 2009) > *L. edodes* (Reis et al., 2012) > *P. ostreatus* (Reis et al., 2012) > *P. eryngii* (Reis et al., 2012) > *A. bisporus* (Barros et al., 2009) > *A. subrufescens* (Tsai et al., 2007) > *P. citrinopileatus* (Lee et al., 2007a) > *P. citrinopileatus* (Abdullah et al., 2011) > *B. edulis* (Tsai et al., 2007) > *P. citrinopileatus* (Lee et al., 2007a) > *V. volvacea* (Abdullah et al., 2011) > *A. auricula-judae* (Abdullah et al., 2011) > *A. cylindracea* (Tsai et al., 2007). In the results of the current study, the hot-water extracts of *V. volvacea* had the highest DPPH value > *A. bisporus* > *A. subrufescens* (Hong Kong) > *L. edodes* > *A. cylindracea* (Taiwan)> *P. citrinopileatus* (Taiwan) > *P. citrinopileatus* (Hong Kong) > *S. imbricatus* > *B. edulis* > *A. subrufescens* (Taiwan) > *A. cylindracea* (Hong Kong) > *H. erinaceus* (Taiwan) > *H. erinaceus* (China) > *P. eryngii* (Taiwan) > *P. eryngii* (Hong Kong) > *P. ostreatus* > *A. auricula-judae* (Taiwan) > *A. auricula-judae* (China) > *G. lucidum*. In the cold-water extracts, *V. volvacea* also had highest the DPPH value > *A. bisporus* > *A. subrufescens* (Hong Kong) > *A. cylindracea* (Taiwan)> *P.*

citrinopileatus (Taiwan) > *P. citrinopileatus* (Hong Kong) > *L. edodes* > *S. imbricatus* > *A. subrufescens* (Taiwan) > *H. erinaceus* (Taiwan) > *H. erinaceus* (China) > *G. frondosa* > > *P. eryngii* (Hong Kong) > *P. eryngii* (Taiwan) > *P. ostreatus* > *A. auricula-judae* (Taiwan) > *A. auricula-judae* (China).

TEAC

There are only a few published TEAC data on mushroom extracts. According to the published data, *A. bisporus* Bs0118H strain had the highest ABTS free radical scavenging ability followed by Bs036 strain > X25 strain > *V. volvacea* > *L. edodes* (Cheung et al., 2003; Savoie et al., 2008). In this current study, the same order was found in both hot- and cold-water extracts. Also, in this current study, hot-water extracts of the medicinal mushroom *P. linteus* showed higher antioxidant activity than *G. lucidum* and in the TEAC, FRAP and DPPH assays the results were similar to those of Lakshmi (Lakshmi et al., 2004). Instead of using hydrogen peroxide to generate $ABTS^{\bullet+}$ free radicals, in this current study the $ABTS^{\bullet+}$ was generated by using potassium persulfate, which gives higher sensitivity (Re et al., 1999). The hot-water extracts of *A. subrufescens* (Taiwan), *G. lucidum*, *G. sinense*, *G. frondosa*, *L. edodes*, *P. ostreatus*, *W. extensa* and *T. fuciformis* had higher TEAC values than the cold-water extracts, whereas the cold-water extracts of other mushrooms had higher values than the hot-water extracts. In their study of *Pleurotus spp*. Shinde and Deshmukh (2012) reported that the cold-water extracts had higher TEAC values than the hot-water extracts. Lee et al. (2007b) suggested that hot water might cause the thermal destruction of some antioxidant components. Due to the fat-soluble nature of β-carotene and tocopherols, Lee et al. (2007b) postulated that the naturally occurring antioxidants found in mushroom water extracts were ascorbic acid and phenols, and the ascorbic acid and phenol contents of *H. marmoreus* cold-water extracts were higher than those of hot-water extracts (Lee et al., 2007b).

The influence of heat processing on the antioxidant ability of edible mushrooms has already been reported. A study of *L. edodes* has shown that the concentration of phenolic content and antioxidant properties could be increased by cooking (Choi et al., 2006). On the other hand, some authors have reported that thermally-processed mushroom showed lower phenolic contents than a raw sample (Manzi et al., 2004; Barros et al., 2007a).

Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. Many studies have expected that herbal extracts would possess antioxidant properties because they are rich sources of phenolic compounds (Zheng and Wang, 2001; Wojdylo et al., 2007). It has been suggested that strains, period of mushroom harvest and shelf life cause the variations in the antioxidant properties of mushrooms. Ribeiro *et al.* (2007) suggested that the stability of phenol contents may be affected by the time since collection, which is suggested to be due to enzymatic and oxidative decomposition together with the different stress conditions to which mushrooms are subjected, as well as the different extraction methodologies, in addition to possible genetic variabilities. Savoie et al. (2008) demonstrated that there was different antioxidant activity between different *A. bisporus* strains. Savoie et al. (2008) reported that the $_{EC50}$ of reducing power of methanolic extracts of *A. bisporus* strains (X25, Bs036 and Bs0118H) were 3.37, 3.11 and 1.76mg/mL with phenolic contents 31, 49 and 40mg of GAE/g respectively. Another study of methanolic extracts carried out by Barros et al. (2008) reported that the $_{EC50}$ of reducing power of *A. bisporus* was 3.63 mg/ml with phenolic contents 4.5mg of GAE/g (Barros et al., 2008).

Moreover, different authors reported different results. In studies of *L. edodes*, Cheoung (2003), Yang (2002) and Choi (2006) reported phenolic contents of 1330µg of GAE/g, 3800µg of GAE/g and 6300µg of GAE/g respectively, whereas in this current study, the total phenolic contents of *L. edodes* were 1700 and 4600µg of GAE/g in the hot- and cold-water extracts respectively (Yang et al., 2002; Cheung et al., 2003; Choi et al., 2006).

In addition, studies of *P. ostreatus* have also reported varying phenolic content values: Fu (2002) reported 390µg of GAE/g and Elmastas (2007) reported 12100µg of GAE/g, whereas the phenolic value of *P. ostreatus* reported by Jayakumar (2009) was 5490µg of GAE/g. Similar values were found in this current study: the value of total phenolic content found in the cold-water extract of *P. ostreatus* was 5497µg of GAE/g, whereas that in the hot-water extract contained 2300µg of GAE/ g (Fu et al., 2002; Elmastas et al., 2007; Jayakumar et al., 2009).

A report on *Russula delica*, a mushroom collected from Portugal and Turkey, showed different phenolic profiles and in a study of *Fistulina hepatica*, different authors produced different results of phenolic content even with samples collected from the same region (Ribeiro et al., 2007; Yaltirak et al., 2009; Vaz et al., 2011).

In addition, in a study of the phenolic content and antioxidant activity of

vegetables, the measurement values of red beetroot were conflicting in a number of

reports. The measurement values of Stratil *et al*. (2006) were five times higher than those reported by Kähkönen (1999), but 20% lower than those of Vinson *et al* (2001) (Kähkönen et al., 1999; Vinson et al., 2001; Imeh and Khokhar, 2002; Stratil et al., 2006). Moreover, conflicting results have also been found in strawberries. In various studies of strawberries, different authors have found different results for the phenolic contents. The amount of phenolic content found by Proteggente (2002) was 3300μg of GAE/g whereas Heinonen (1998) found that the phenolic contents of strawberries was 16100μg of GAE/g (Heinonen et al., 1998; Proteggente et al., 2002).

Therefore, the amount of polyphenols and antioxidant capacity also depended on the method of extraction, and the deviations among the results from hot- and cold-water extraction can be explained by the fact that the content of phenolic compounds in some species of mushroom can be influenced. Lee et al. (2003) demonstrated that simple heat treatment on rice hull extracts could not cleave covalently bound phenolic compounds from rice hull. Jeong et al. (2004) suggested that the phenolic compounds of plants should be present in different bound statuses depending on species. Thus, effective processing steps for liberating phenolic compounds from various samples may be different (Jeong et al., 2004). There are various internal and external factors that might possibly influence the total phenolic contents in the presented results of observed antioxidant effects, for instance, agrotechnical processes, cultivars, soil composition, climate, geographic origin, and/or exposure to disease prevailing during growth (Manach et al., 2004; Schaffer et al., 2005; Stratil et al., 2006). It had been reported that the ability of an individual mushroom species to grow and fruit using different substrates is largely dependent on the ability of the fungus to use the major components of the substrates as a nutritional source (Buswell *et al.*, 1993). Shashirekha *et al.* (2005) suggested that the biomass produced as mushroom fruiting bodies shows clear chemical differences from different substrates.

Published data have reported the free radical scavenging ability as the efficient concentration (EC_{50}) (Fu et al., 2002; Yang et al., 2002; Cheung et al., 2003; Lakshmi et al., 2004; Lo and Cheung, 2005; Puttaraju et al., 2006; Yu et al., 2006; Dubost et al., 2007; Lee et al., 2007b; Ribeiro et al., 2007; Barros et al., 2008; Savoie et al., 2008; Tsai et al., 2008; RongYao et al., 2009; Abdullah et al., 2011; Yeh et al., 2011). The percentage of scavenging radicals is dependent on the initial DPPH radical concentration; however, EC_{50} depends on initial concentrations of free radicals, and the kinetic reaction between DPPH and antioxidants is not linear to DPPH

concentration. Trolox was used as a calibrating standard expressed as trolox equivalents which correlated very well with the biological redox properties of phenolics (Sánchez-Moreno, 2002; Stratil et al., 2006). Since phenolic compounds are processed in mushrooms, it could be considered that the antioxidant activity of mushrooms is related to the proportion of phenolic compounds present (Wada et al., 1996; Cheung et al., 2003). In this current study, the antioxidant ability of aqueous mushroom extracts were reported in TE/g instead of $_{EC50}$ since it is more accurate to use the absorbance variation (Karadag et al., 2009).

Phenols are known to be effective antioxidants; therefore, the high contents of total phenolic compounds in extracts were responsible for their effective antioxidant properties. In this current study, the TEAC, DPPH and FRAP of culinary mushrooms (hot- and cold-water extraction) were positively correlated with phenolic contents. On the other hand, for the medicinal mushrooms, the correlation analysis of FRAP and total phenolic content showed no significant correlation between hot-water extraction and FRAP value. There have been several investigations of antioxidant activity in medicinal herbs and plants showing that phenolic compounds play an important role in protecting oxidation damage (Bocco et al., 1998; Gazzani et al., 1998; Triantaphyllou, 2001; Zheng and Wang, 2001; Katalinic et al., 2006; Wong et al., 2006a; Wojdylo et al., 2007; Sulaiman et al., 2010). It also has been reported that plant and mushroom antioxidant abilities were well correlated with the proportion of phenolic compounds present (Visioli et al., 1998; Zheng and Wang, 2001; Puttaraju et al., 2006; Wong et al., 2006a; Turkoglu et al., 2007; Wojdylo et al., 2007; Kim et al., 2008). However, some authors have reported that there was not such a direct correlation between antioxidant activates and total phenol contents (Bocco et al., 1998; Kähkönen et al., 1999; Capecka et al., 2005; Hinneburg et al., 2006). A study of *A. bisporus* reported that strain Bs0633 had the highest phenolic concentrations in the extracts; however, it had the lowest antioxidant activity (Savoie et al., 2008).

The relationship between total polyphenols and total antioxidant activities is contradictory. Many studies have reported conflicting results between the potential natural antioxidant compounds and total phenolic contents because of the antioxidant assay methods that are used to evaluate antioxidant activity.

Antioxidant properties might be enhanced or decreased depending on their cultivation or environmental conditions, developmental stage, species, variety, storage

condition and so on (Kang and Saltveit, 2002). The loss of potential antioxidant

compounds during the extraction process may reflect the different antioxidant activities between hot- and cold-water extracts (Gazzani et al., 1998; Sun and Ho, 2005). Therefore, it is difficult to compare all these results unless they are based on the same assay and extraction process.

However, even when the same test is applied, the measurement of antioxidant values may vary from one study to another (Dávalos et al., 2004). This makes it difficult to make comparisons with previous reported data; also, the results are expressed in various ways, so the antioxidant activities of pure phenolic compounds are scattered in the literature and difficult to compare (Villano et al., 2005). The different extraction procedures, the quantities of extracts used in the reaction mixtures and the data expression are diverse in different published articles; therefore, it is difficult to compare the present results with published data on antioxidant activities in mushroom extracts.

It is obvious that it is not a simple matter to predict a full picture of the quality or quantity of the phenolic constituents in extracts by using the Folin-Ciocalteu procedure. Some compounds such as sugars and ascorbic acid may affect the results of a Folin-Ciocalteu assay. Therefore, it is difficult to predict the antioxidant activity based on the amount of total phenolic compounds present (Singleton and Rossi Jr, 1965; Kähkönen et al., 1999). Estimation of antioxidant capacity is dependent on the assays used. There is no single method that can lead to a complete evaluation of antioxidant activity in the extracts. The relationship between the antioxidant or scavenging activity and phenolic content is very difficult to establish using statistical tools because: 1. the antioxidant properties of single compounds within a group can vary remarkably so that the same levels of phenolics do not necessarily correspond to the same antioxidant responses; 2. the different methods used to determine the antioxidant activity are based on different mechanisms of reaction so that they often give different results; and 3. extracts are very complex mixtures of many different compounds with distinct polarity as well as antioxidant and pro-oxidant properties, sometimes showing synergistic actions by comparison with individual compounds (Parejo et al., 2002).

Table 4.1: Extraction solvent and antioxidant methods of published articles.

Mushroom species	Extraction methods	Antioxidant methods	Reference
Agaricus bisporus	1. 80% ethanol and 60°C water bath 2. 100°C hot water for 30 minutes 3. 95% Ethanol 4. Methanol 25°C for 1 hour 5. Methanol 25°C for 24 hours 6. Methanol for 24 hours	1. F-C 2. F-C and DPPH 3. F-C and DPPH 4. F-C and DPPH 5. F-C and DPPH 6. F-C, TEAC and DPPH	1. (Dubost et al., 2007) 2. (Abdullah et al., 2011) 3. (Fu et al., 2002) 4. (Reis et al., 2012) 5. (Barros et al., 2008) 6. (Savoie et al., 2008)
Agaricus subrufescens	Ethanol for 24 hours / heat with water at reflux for 1 hour	F-C and DPPH	(Tsai et al., 2007)
Agrocybe cylindracea	1. Ethanol for 24 hours / heat with 100ml water at reflux for 1 hour 2. Methanol at soxhlet extractor for 24 hours	1. F-C and DPPH 2. TEAC, DPPH and F-C	1. (Tsai et al., 2007) 2. (Lo and Cheung, 2005)
Auricularia auricula-judae	100°C hot water for 30 minutes	F-C and DPPH	(Abdullah et al., 2011)
Boletus edulis	1. Boiled in water 2. Ethanol for 24 hours / heat with distilled water at reflux for 1 hour 3. Boiled in water for 15 minutes	1. HPLC and DPPH 2. F-C and DPPH 3. HPLC and DPPH	1. (Ribeiro et al., 2007) 2. (Tsai et al., 2007) 3. (Puttaraju et al., 2006)
Coprinus comatus	Ethanol for 24 hour s and heat with distilled water at reflux for 1 hour	F-C and DPPH	(Tsai et al., 2009)
Cordyceps militaris	60°C distilled water for 30 minutes	F-C and TEAC	(Yu et al., 2006)
Trametes versicolor	Methanol for 24 hours	F-C and DPPH	(Mau et al., 2002a)
Ganoderma lucidum	1. 100°C hot water for 30 minutes 2. Methanol 24 hours 3. Petroleum ether / ethyl acteate, and 70% methanol in soxhlet extractor	1. F-C and DPPH 2. F-C and DPPH 3. FRAP, DPPH, TEAC	1. (Abdullah et al., 2011) 2. (Mau et al., 2002a) 3. (Lakshmi et al., 2004)
Ganoderma sinense	Methanol for 24 hours / distilled water for 3 hours	F-C and DPPH	(Rong Yao et al., 2009)
Grifola frondosa	1. 80% ethanol / heated to 60°C for one hour in water bath 2. Methanol for 24 hours 3. Ethanol for 24 hours / distilled water at 4°C for 24 hours / heat with distilled water at reflux for 3 hours	1. F-C 2. F-C and DPPH 3. HPLC and DPPH	1. (Dubost et al., 2007) 2. (Mau et al., 2002b) 3. (Yeh et al., 2011)

Hericium erinaceus	1. 100°C with distilled water for 30 minutes 2. Methanol for 24 hours	1. F-C and DPPH 2. F-C and DPPH	1. (Abdullah et al., 2011) 2. (Mau et al., 2002b)
Lentinula edodes	1. Petroleum ether, ethyl acetate, methanol and water using soxhlet extractor for 3 hours 2. 80% ethanol / heated to 60°C for 1 hour in a water bath 3. Methanol 25°C for 24 hours 4. Petroleum ether, ethyl acetate, methanol using soxhlet extractor for 24 hours 5. 100°C with distilled water for 30 minutes	1. F-C and DPPH 2. F-C 3. HPLC,DPPH 4. F-C 5. F-C and DPPH 6. F-C and DPPH 7. F-C and DPPH	1. (Cheung et al., 2003) 2. (Dubost et al., 2007) 3. (Yang et al., 2002) 4. (Cheung and Cheung, 2005) 5. (Abdullah et al., 2011) 6. (Fu et al., 2002) 7. (Reis et al., 2012)
Pleurotus citrinopuleatus	Cold water / heat with water at reflux for 1 hour	F-C and DPPH	(Lee et al., 2007a)
Pleurotus eryngii	1. 80% ethanol heated to 60°C for 1 hour in a water bath 2. 100°C distilled water for 30 minutes 3. Ethanol for 1 hour 4. Methanol for 1 hour	1. F-C 2. F-C and DPPH 3. F-C and DPPH 4. F-C and DPPH	1. (Dubost et al., 2007) 2. F-C and DPPH 3. (Fu et al., 2002) 4. (Reis et al., 2012)
Pleurotus ostreatus	1. 80% ethanol heated to 60°C for 1 hour in a water bath 2. Methanol for 24 hours 3. Ethanol using soxhlet extractor 4. Ethanol 5. Ethanol / cold water for 24 hours 6. Methanol 25° C for 1 hour	1. F-C 2. HPLC and DPPH 3. HPLC and F-C 4. F-C and DPPH 5. FRAP, TEAC, DPPH and F-C 6. F-C and DPPH	1. (Dubost et al., 2007) 2. (Yang et al., 2002) 3. (Jayakumar et al., 2009) 4. (Fu et al., 2002) 5. (Chirinang and Intarapichet, 2009)
Sarcodon imbricatus	Methanol for 24 hours in soxhlet extractor	F-C and DPPH	(Barros et al., 2007c)
Tremella fuciformis	Methanol	F-C and DPPH	(Mau et al., 2001)
Volvariella volvacea	1. Petroleum ether, ethyl acetate, methanol and water for 3 hours using soxhlet extractor 2. Petroleum ether, ethyl acetate, methanol for 24 hours using soxhlet extractor 3. 100°C distilled water 30 minutes 4. Ethanol	1. F-C and DPPH 2. F-C 3. F-C and DPPH 4. F-C and DPPH	1. (Cheung et al., 2003) 2. (Cheung and Cheung, 2005) 3. (Abdullah et al., 2011) 4. (Fu et al., 2002)

F-C: Folin-ciocalteu phenol reagent.

4.2 *In vitro* enzymatic digestion

Metabolism and excretion

In order to access the bloodstream for systemic distribution, phenolic compounds must pass through the enterocytes (Coulston and Boushey, 2008). Aglycones and free simple phenolic compounds, flavonoids (quercetin, genistein) and phenolic acid can be directly absorbed through the small intestinal mucosa (Bravo, 2009). However, the intestinal mechanisms of GI absorption of phenolic compounds tract remain unknown. It has been suggested that probably the phenolic compounds are too hydrophilic to penetrate the gut wall (Manach et al., 2004), and it is believed that the absorption of phenolic compounds into the enterocytes occurs through the action of monocarboxylic acid transporters, as well as by passive diffusion (Manach et al., 2004; Coulston and Boushey, 2008).

In the food matrix, expect for flavones, glycosylated forms are found in all flavonoids, and the absorption is influenced by glycosylation. Even so, the fate of glycoside in the stomach is not clear (Manach et al., 2004). Phenolic compounds are the substrates for the body's detoxification system. The detoxification system seeks to reduce the potential toxicity of foreign compounds, such as xenobiotic compounds, by metabolizing them to make them more readily excreted or to reduce their bioactivity. This detoxification system is composed of three primary activities: Phase I, II and III metabolism.

Phase I typically involves hydroxylation which is performed by cytochrome p450, a membrane-bound enzyme. The hydroxylation renders a hydroxyl group to substrates, which is a step of functionalization resulting in the substrates being more reactive for subsequent metabolism, such as phase II. Phase II involves a conjunction reaction in which a hydroxyl group on the foreign compound is modified by adding a sulphate, glucuronic acid or methyl group, and decreases the potential toxicity of xenobiotic compounds and facilitates their exretion into bile and/or urine by the liver and kidney. These reactions can occur at any phenol group on the molecule. Phase III, finally, involves the efflux of both native xenobiotics or phase I/II metabolites from the enterocyte as a means of lowering their intracellular concentration.

Polyphenols absorbed from the intestinal lumen by enterocytes occur in the phase II detoxification reaction in the cell interior. Polyphenols may be the circulated

into the liver by the portal vein prior to systemic circulation and extracted from the bloodstream by the kidneys and subsequently excreted in the urine (Coulston and Boushey, 2008; Bravo, 2009).

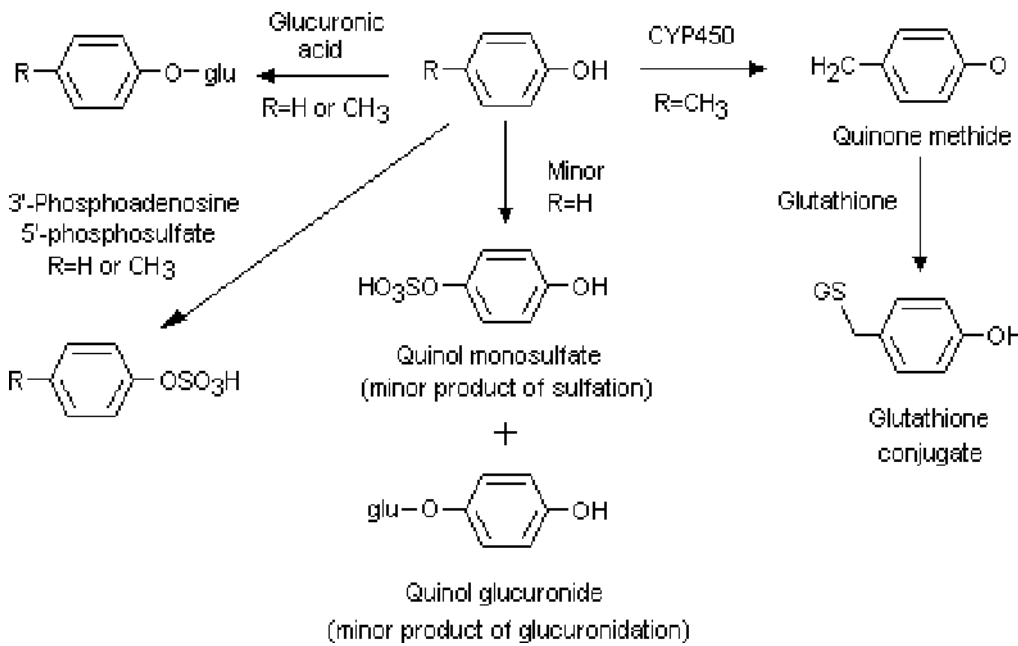


Figure 4.2 Metabolism of substituted and unsubtituted phenols (Sipes).

***In vitro* antioxidant assay**

Bio-availability refers to the fraction of an ingested nutrient that is available to the body for use in normal physiological functions or storage (Duthie et al., 2000). To exert a protective effect against free radical related diseases, potential antioxidant compounds have to be absorbed from the gut. To facilitate the evaluation of antioxidant compounds, *in vitro* enzymatic digestion models have been designed to simulate the human digestion system. Occurrence of oxidation and the action of antioxidants have different chemical and physical mechanisms. Three different *in vitro* antioxidant assays (TEAC, DPPH and FRAP) have been employed after a simulated GI digestion. Various foods, meals and supplements have been subjected to *in vitro* enzymatic digestion procedures to analyse the stability of antioxidants (Pérez-Vicente et al., 2002; Vallejo et al., 2004; Nagah and Seal, 2005;

Bermúdez-Soto et al., 2007). Since there are no studies to date that have provided information regarding the availability of antioxidants in selected mushroom extracts after digestion, this current study has provided information about the stability of potential antioxidants in mushroom extracts following digestion. By measuring the antioxidant activity of mushroom extracts after the *in vitro* digestion, it is possible to provide more biologically relevant data detailing the antioxidant properties of mushroom extracts.

In conclusion, according to the results presented by the TEAC, DPPH and FRAP assays, the potential antioxidant compounds of all of the mushrooms extracts studied could be released after enzymatic digestion, except for *C. militaris*. This suggests that potential bio-available antioxidant compounds may have been bound or associated with the soluble fraction of mushroom material, and most of the potential antioxidant compounds from the mushroom extracts would be released and their antioxidant activity increased under different pH conditions and enzymatic hydrolysis. In addition, there was no significant increase in the current study between the initial TEAC values of the *A. auricula* (the hot-water extract) and *H. erinaceus* (the cold-water extract) extracts and the TEAC values after *in vitro* digestion. A study of *B. edulis* showed similar result. The scavenging ABTS free radical ability of *B. edulis* did not significantly increase after digestion (Soler-Rivas et al., 2009). Although not measured using the TEAC assay, a study of fresh orange and apple juice also showed that there were no significant differences between the initial and the post-digestion FRAP values (Ryan and Prescott, 2010).

This observation suggests that potential antioxidant compounds in these mushroom extracts are resistant to GI treatment, and the *in vitro* enzymatic digestion process gives an estimated value of the antioxidant capacity of the potential antioxidant compounds that would be released and made available in an accessible form to be absorbed during digestion, and provide protection from oxidative damage within the gut lumen and intestinal mucosa (Nagah and Seal, 2005; Toor et al., 2008).

An increase in the antioxidant activity of mushroom extracts after enzymatic digestion has been reported by other researchers. In a study of *A. bisporus* and *L. edodes*, the ability to scavenge ATBS free radicals was found to increase significantly after *in vitro* digestion. The scavenging ability of DPPH free radicals by *A. bisporus*,

B. edulis and *L. edodes* was increased after *in vitro* digestion (Soler-Rivas et al., 2009).

Generally, in this current study, the aqueous mushroom extracts showed potential antioxidant ability while those of *C. militaris* showed decreased activity after *in vitro* enzymatic digestion in all antioxidant assays. This suggests that the degrading of potential antioxidant compounds in *C. militaris* extracts occurred during *in vitro* enzymatic digestion. This loss of antioxidant compounds after digestion steps has also been reported by Bermúdez-Soto et al. (2007). Polyphenols have been found to be highly sensitive to alkaline conditions, therefore, the degraded antioxidants in the extracts may potentially undergo slight structural transformation in different pH environments (Bermúdez-Soto et al., 2007; Ryan and Prescott, 2010). A numbers of studies on polyphenols, β-carotene and vitamin E have shown that the bio-availability of antioxidants in the small intestine could be very low (Burton and Traber, 1990; Clifford, 2004; Novotny et al., 2005).

A study of commercially available fruit juices showed that the FRAP value of fresh grapefruit and pineapple juice were significantly lower post digestion (Ryan and Prescott, 2010). Another study of pomegranate juice and broccoli discovered that the concentration of phenolic compound (anthocyanins and caffeolyquinic acid) were also significantly decreased after pancreatin-bile salt digestion (Pérez-Vicente et al., 2002; Vallejo et al., 2004). In studies of standard phenolic compounds, phenols (gallic acid, vanillic acid and caffeic acid) were rather stable compared with other phenolic compounds (catechin, rutin and quercetin) after gastric treatment. However, the concentration of gallic acid diminished by nearly 50% due to the pH value under intestinal conditions and the absorptivity of gallic acid was highly influenced by pH. Moreover, in an intestinal medium, the concentration of catechin, epicatechin, rutin, quercetin and kaempferol showed clear decreases (Troncoso, 2001). In addition, the decrease of antioxidant compounds was found in chokeberry, raspberry and red wine (McDougall et al., 2005a; McDougall et al., 2005b; Bermúdez-Soto et al., 2007). The processing of mushroom extracts can influence antioxidant activity results. Taking into consideration the different pH values in the medium may partially explain the losses of antioxidant ability.

It had been reported that after *in vitro* gastric digestion, total and individual simple phenolic acid, hydroxycinnamic acid derivatives and total sinapic and ferulic derivatives levels of broccoli extracts showed decreases (Vallejo et al., 2004). The

decrease of the level of potential antioxidant compounds after *in vitro* GI digestion has also been reported (Gil-Izquierdo et al., 2002; Pérez-Vicente et al., 2002; Vallejo et al., 2004). Vallejo et al. (2004) suggested that it is possible that pancreatin digestion liberates compounds able to associate with potential antioxidant compounds. In a study of mushroom phenolic compound concentration, Kim et al. (2008) reported that the phenolic acid level of mushrooms was higher than that of flavonoids (Kim et al., 2008). Polysaccharide extracted from mushrooms also showed antioxidant ability (Liu et al., 1997; Yu et al., 2006; Tseng et al., 2008). Therefore, polysaccharide degradation might also liberate antioxidants bounded to their complex structure exposing them to unfavourable media. Frontela et al. (2011) also suggested that it cannot be easily explained why there is a decrease in total phenolic content value after *in vitro* GI digestion between samples (Frontela et al., 2011).

Despite the decrease of antioxidant ability in *C. militaris* after *in vitro* enzymatic digestion, the increased antioxidant activity in all the other mushroom extracts after pancreatin digestion could perhaps be explained by the ability of mushroom extracts to produce more antioxidant compounds by hydrogen bonding and the subsequent release of these antioxidant compounds during enzymatic hydrolysis (McDougall et al., 2005b). Similar results showing an increase of antioxidant activity after digestion steps have also been found in various vegetables, fruit juices and whole grain food (Nagah and Seal, 2005; Ryan and Prescott, 2010; Wootton-Beard et al., 2011). This could be explained by the availability of antioxidant compounds during the first stage of the digestion process (Serrano et al., 2007). This may suggest that the potential antioxidant ability of antioxidant compounds in mushroom extracts would be more effective after digestion.

Soler-Rivas et al. (2009) and Ganguli et al. (2007) suggested that the longer a heat treatment was applied, the lower the antioxidant properties appeared on mushroom fruiting bodies (Ganguli et al., 2007; Soler-Rivas et al., 2009). It has been reported that the presence of protein and iron interacted with green tea polyphenols during *in vitro* enzymatic digestion resulting in a decrease of antioxidant ability (Alexandropoulou et al., 2006).Troncoso (2001) reported that the concentration of gallic acid and kaempferol were not affected after gastric treatment (HCl-pepsin treatment). However, when the phenolic compounds were submitted to intestinal

treatment (NaOH-pancreatin), the concentrations were clearly decreased (Table 4.3) (Troncoso, 2001). It is therefore likely that the potential antioxidant compounds within mushroom extracts may have undergone slight structural transformation, and therefore went undetected by the antioxidant method of analysis resulting in the various antioxidant values in each of the *in vitro* enzymatic digestion steps.

In general, the results obtained from the TEAC, DPPH and FRAP assays used in this study show that the mushroom extracts showed different antioxidant release patterns during *in vitro* enzymatic digestion. The variation in results may be because of the characteristic chemistry within each mushroom, and the fact that the chemical mechanisms behind each antioxidant assay are different (Nagah and Seal, 2005).

In brief, according to the results of the TEAC, FRAP and DPPH assays used in this study, potential antioxidant compounds of mushrooms extracts are resistant to *in vitro* enzymatic digestion (GI treatment), and release more potential antioxidant compounds that contribute to their total antioxidant activity. It may also suggest that the significantly lower post-digestion activity of *C. militaris* may suggest that the potential antioxidant compounds of *C. militaris* are unstable and easily degraded in the human GI tract.

Table 4.3: Phenolic compounds after gastric and intestinal treatment, modified from Troncoso (2001).

Phenolic compounds	Gastric treatment	Intestinal treatment
Gallic acid	Not affected	Decreased
p-Hydroxybenzoic acid	Not affected	Not affected
Tyrosol	Not affected	Not affected
Vanillic acid	Not affected	Not affected
Syringaldehyde	Not affected	Not affected
Caffeic acid	Not affected	Not affected
p-Coumaric acid	Decreased	Not affected
Ferulic acid	Decreased	Not affected
trans-Resveratrol	Not affected	Not affected
Catechin	Decreased	Decreased
Epicatechin	Decreased	Decreased
Rutin	Decreased	Decreased
Quercetin	Decreased	Decreased
Kaempferol	Not affected	Decreased

4.3 Cellular antioxidant activity assay

The cellular antioxidant quality of potential antioxidant compounds was determined for the mushrooms from their cellular antioxidant activity values. The effect of mushroom extracts on the prevention of oxidative damage to cells has not previously been reported. Compared with the chemical reagent assays, hot-water extracts of mushrooms had statistically positive relationships between the cellular antioxidant activity and the total phenolic content for mushrooms ($p<0.01$). A similar positive correlation has been found in fruits (Wolfe and Liu, 2007). In FRAP (the hot-water extracts) and TEAC assays (the cold-water extracts), mushroom extracts were found to be positively correlated with cellular antioxidant activities.

The absence of correlation between the total phenolic content (cold-water extracts), TEAC (hot-water extracts), FRAP (cold-water extracts), DPPH (both hot- and cold-water extracts) and cellular antioxidant activity was also found in the ORAC assay. Studies on common vegetables and broccoli extracts have demonstrated no agreement between ORAC and cellular antioxidant activities (Eberhardt et al., 2005; Song et al., 2010). This lack of correlation is likely to be due to the biological components of cellular antioxidant assays (Faulkner and Fridovich, 1993; Wolfe and Liu, 2007). It has also been reported that there was no significant correlation between ORAC value and cellular antioxidant activity in a study of standard flavonoids. Rutin and naringenin had high ORAC values but no activity on cellular antioxidant assay (Wolfe and Liu, 2008). On the other hand, galangin and epigallocatechin gallate had low ORAC values but they exhibited high activity in cellular antioxidant activity (Wolfe and Liu, 2008). The hot-water extract of mushroom *T. versicolor* in this study had significantly higher cellular antioxidant activity compared with the both hot- and cold-water extract of *P. citrinopileatus* ($p<0.001$), while in the chemical assays (total phenolic content, TEAC and DPPH), *P. citrinopileatus* exhibited higher antioxidant activity than *C. versicolor* ($p<0.001$). A similar result was also found in *H. erinaceus*. *H. erinaceus* had higher antioxidant activity in the chemical assays than *A. auricula-judae*, and *G. lucidum*, however; *H. erinaceus* had the lowest cellular antioxidant activity in both hot-and cold-water extracts. The permeability of membrane of potential antioxidant compounds is structure-depend. For example, polyphenolic compounds are thought to be the majority of exogenous antioxidants

which provide a protective effect against lipid oxidation. According to their chemical structures, flavanols are more hydrophobic than flavanones but in a liposomal membrane affinity test by fluorescence-quenching measurement, the membrane affinity of flavonols was greater than that of flavonoids (figure 4.1) (Oteiza et al., 2005). Therefore, the present data suggest that the chemical assays' estimates of potential antioxidants in the mushroom extract do not accurately reflect the antioxidant activity within cultured human cells.

Flavanone Flavanonol

Figure 4.1 Chemical structures of the flavanol and flavanone.

The cellular model of antioxidant activity assay has been used in the study of cell uptake, metabolism and distribution of flavonoids (Spencer et al., 2004). Although cellular antioxidant assays are more time consuming and require more equipment compared with chemical assays, they may more closely reflect the potential antioxidant components of mushroom extract activity within a biological environment (Eberhardt et al., 2005). Since the cellular antioxidant assay mimics the cellular processes of the potential antioxidant compounds by penetrating into the cells and reacting with free radicals that occur *in vivo*, the cellular antioxidant assay has more relevance to biological systems compared with chemical assays. The ability of potential antioxidant compounds to prevent lipid oxidation could be influenced by many interacting factors. For example, in biological membranes, ascorbic acid regenerates oxidized α-tocopherol to reactivate α-tocopherol. The antioxidant polar paradox hypothesis is not able to consistently predict the ability of antioxidant compounds to inhibit lipid oxidation in an oil/water emulsion. For instance, BHT and α-tocopherol are essentially not water soluble, however they do not inhibit lipid

oxidation in the corn oil in water emulsions. In contrast, gallic acid has been shown to be an effective antioxidant in corn oil in water emulsions (Alamed et al., 2009).

Cellular antioxidant activity values demonstrate some aspects of cell uptake, metabolism and the distribution of bioactive compounds, which are important modulators of bioactivity. This study provides more biologically relevant data on potential antioxidant compounds in mushroom extracts. Cellular measurements reflect antioxidant activities measured in whole animals and are shown to relate to health benefits (Crespy and Williamson, 2004). In this study, the potential antioxidant compounds of mushroom extracts moved beyond the measurement of simple chemical reactions towards a more complex biological system. Using cell-based testing systems *in vitro* would perhaps eliminate many of the limitations of chemical interaction-based assays.

Chapter 5 Conclusion and further work

With the remarkable enhancement of people's living standards coupled with the large growing ageing population, people in Taiwan are becoming more interested in diet and health and the demand for health-preserving foods is growing rapidly. In China and Taiwan, crude drugs have been used for centuries to enhance health and to prevent disease. Most consumption of mushrooms is based on traditional ways of preparation such as a tea, soup or beverage for the maintenance of health. A complex mixture of antioxidants is contained in food and beverages. For this reason, it is important to understand the composition and nutritional value of these products (Garcia-Salas et al., 2010). Infusion and decoction in water are the traditional methods for the preparation of mushrooms for culinary and medicinal use. This is a simply a way to mimic the ethnomedicinal methods used in folk therapies and to approximate the levels of antioxidants in mushrooms that might be taken and absorbed during normal dietary consumption (Triantaphyllou, 2001; Zheng and Wang, 2001).

In this study, the mushroom extracts were prepared in ways which mimicked the methods used in traditional folk medicine (Triantaphyllou, 2001), and were based on the traditional way of ingestion and preparation of crude material in China and Taiwan. For use in foods, extracts made with water are appropriate, less toxic, nutritionally more relevant and would have obvious advantages in relation to certification, practicality and safety (Wong et al., 2006b).

The Trolox equivalent antioxidant capacity (TEAC) parameter in ABTS free radical assay as well as DPPH assaying methods used in this investigation offers the benefit of allowing comparisons to be made with other food and plant materials examined by other authors. In genral, different antioxidant compounds have different chemical structures and features, therefore, it is clear that the variation in antioxidant activity detected by different methods depended on the nature of the oxidants and antioxidants and the chemistry involved in the reaction, reducing ability (in FRAP), and scavenging ability (in TEAC and DPPH).

The hydrolysable phenolic is believed to be partially hydrolysed during the digestion processes. The release of polyphenols of whole-grain cereal foods may also increase during digestion and cause the increase of antioxidant activity after digestion (Nagah and Seal, 2005). These results are important because they suggest that

antioxidants exert their effect within the digestive tract and provide an environment which protects the intestinal epithelium from pro-oxidative compounds (Fardet et al., 2008). Antioxidants may act as anticarcinogenic agents because they stimulate detoxification systems, especially phase II of the conjugation reaction. For example, the formation of carcinogens can be prevented by caffeic acid and ferulic acid and this blocks the interaction between carcinogens and critical cell macromolecules (Fardet et al., 2008).

In general, it can be concluded that the potential antioxidant compounds of the mushroom extracts were clearly affected by the *in vitro* digestion process. The amount of accessible antioxidant compounds from the mushroom extracts was found to be `much higher after *in vitro* digestion. According to the results presented, consumption of mushroom extracts may provide some degree of antioxidant property and further provide potential protection against oxidative damage, which is considered to cause arteriosclerosis and neurodegeneration. *In vitro* enzymatic digestion mimics the conditions in the human GI tract showing that the amount of antioxidant released from mushroom extracts into the human intestine may be higher than expected from the values predicted by chemical measurements. It suggests that antioxidants encounter an acidic medium in the stomach and will be substantially exposed to and reach higher antioxidant activity in the small intestine. It is therefore clear that the digestion process is an important factor influencing the antioxidant activity of selected mushrooms.

It suggests that proteolytic and pancreatic enzyme activity, and perhaps changes in pH, might generate new or more antioxidant compounds which have higher antioxidant activity than the original extracts (Soler-Rivas et al., 2009). The results of increase of antioxidant activity of selected mushroom extracts after digestion are important because they suggest that antioxidants exert their effect within the digestive tract and provide an environment which protects the intestinal epithelium from pro-oxidative compounds. In this current study, the enzymatic digestion steps give an estimate of the amount of potential antioxidant compounds in the mushrooms extracts that are available in an accessible form to be absorbed into the human mucosa. Study of *in vitro* digestion can therefore be used as a useful screen to examine the accessibility of antioxidants (Toor et al., 2008).

The present data obtained from chemical and cellular assays suggest that

estimates of the potential antioxidant activity of mushroom extracts may not accurately reflect the antioxidant activity within cultured human cells. Whether chemical assays reveal the extent of the actual antioxidant activity value of *P. citrinopileatus* and *H. erinaceus* or the activity of the antioxidant complex of *A. auricula-judae*, *T. versicolor* and *G. lucidum* were not efficiently shown in the chemical assays. The results of cellular antioxidant assays relate to cellular estimates of antioxidant activity within cells. Hence, in this stage, the results suggest that the actual antioxidant activity of *T. versicolor* could be much more than chemical reaction. In addition, this might help to explain that the ancient medicinal belief regarding *C. militaris* and *T. versicolor* is perhaps partly due to their potent antioxidant activity.

C. militaris exhibited more promising antioxidant potential than the other five mushrooms studied in chemical assays (phenolic content, TEAC, FRAP and DPPH) and cellular antioxidant assay. The most interesting aspect of this study was the *in vitro* digestion step and cellular antioxidants, which demonstrated the possible antioxidant activity within the human GI tract and the cell uptake and distribution. *C. militaris* showed a significant decrease after *in vitro* digestion steps, however, in the cellular antioxidant assays, it showed much higher cellular antioxidant values than the other five mushrooms ($p<0.001$). To the author's knowledge, there have been no previous studies which have demonstrated these data.

The benefits of the consumption of an antioxidant rich diet have been in doubt recently (Halliwell and Gutteridge, 2007). An epidemiological study in the Netherlands found an inverse correlation between heart disease and stroke and the dietary intake of flavonoids, which originate from tea, fruits and vegetables (Hertog et al., 1993; Halliwell and Gutteridge, 2007). A number of intervention trials have found that antioxidants failed to demonstrate benefits in humans (Rimm et al., 1993; Stampfer et al., 1993; Knekt et al., 1994; Halliwell and Gutteridge, 2007). These findings could be explained first by the suggestion that ROS may not actually be involved in some diseases and that antioxidants cannot encompass all symptoms. Second, in intervention trials, the subjects already have extensive pathology. Finally, the applied dosage may be wrong.

The evidence has shown that a low dosage of antioxidants might have more benefit than higher dosages of single agents. The uptake of other nutrients such as α-tocopherol and β-carotene might be interfered with at high doses of single agents (Halliwell, 2009). The timing of antioxidant administration may be also critical. For

example, vitamin C showed beneficial effects on processes in the early stage of atherosclerosis; however, if atherosclerotic plaques are already formed, the anti-atherogenic benefits of vitamin C may be negligible (Aguirre and May, 2008). Moreover, some clinical studies have been carried out on patients who were already suffering from vascular disease (Hodis et al., 1995; Azen et al., 1996). Further, if administration of antioxidants does not decrease the oxidative damage when diseases occur, then antioxidants will not have an effect on the diseases. One animal study showed benefits in murine models of amyotrophic lateral sclerosis, but not in human patients (Halliwell, 2001; Schnabel, 2008).

Another study of chronic obstructive pulmonary disease using several pharmacological antioxidants such as Erdosteine and Fudosteine suggested that these compounds may affect important outcomes in chronic obstructive pulmonary disease, such as steroid resistance, mucus hypersecretion and inflammation (Rahman and MacNee, 2012). Therefore, antioxidants may actually work in humans and act in the correct sites, such as crossing the blood/brain barrier, and terminate oxidative damage at those sites (Halliwell, 2009). Even so, in some studies, the health benefits of antioxidants such as β-carotene and vitamin E are ambiguous and paradoxical (Pearson et al., 2006; Halliwell and Gutteridge, 2007). Before extensive breeding programmes are undertaken to increase the understanding of antioxidant activity *in vivo* for mushroom extracts, it is necessary to estimate the antioxidant activity which reflects clinical effects and to carry out feeding studies to determine the benefit of antioxidant levels of mushroom extracts used as alternative remedies.

Further studies

The focus of this study has been to evaluate the antioxidant activity of selected mushrooms. According to cellular antioxidant assay, the medical mushroom, *C. militaris* showed the most potent antioxidant activity in this thesis. The results of *in vitro* tests obtained here can be used in evaluating the likelihood of antioxidant effects of mushroom extracts *in vivo*. The results also clearly show that the mushroom extracts have a wide range of antioxidant activity in different antioxidant approaches and the potential antioxidant compounds of mushroom extracts might contribute a certain degree of therapeutic benefits as alternative therapies for conditions such as neurodegeneration. Many studies have suggested that neurodegeneration is related to oxidative stress (Sayre et al., 1997; Keller et al., 2005; Castellani et al., 2006; Halliwell, 2009). A report has indicated that a high dosage of α-tocopherol could delay the deterioration of patients with Alzheimer's disease (AD), however, in patients with a progression of mild-cognitive impairment from AD, the same dose had no effect (Blacker, 2005; Halliwell, 2006). Therefore one major future research area is developing a novel antioxidant for neurodegenerative diseases (Castellani et al., 2006). Further identification, fractionation and isolation of the biologically-active chemical constituents are also required. Studies using *in vivo* models will lead to the estimation and standardization of the dosage, and to the distribution and excretion profile of these mushroom extracts under physiological conditions, and if the spectrum of potential antioxidant compounds is broad enough and the dosage low enough, they might be used as prophylactic agents. Small animal models could be the first step of such an *in vivo* study. Therefore, an investigation of the potential neuroprotective properties of mushroom extracts could be done by using mice (AD model).

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