THE EFFECT OF PHELLINUS TORULOSUS MUSHROOM EXTRACT ON ANTIOXIDANT ENZYMES

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Approval of the Graduate School of Natural and Applied Sciences, Atilim University.

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I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Sciences.

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ABSTRACT

THE EFFECT OF PHELLINUS TORULOSUS MUSHROOM EXTRACT ON ANTIOXIDANT ENZYMES

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In order to prevent free radical damages there has been an increasing interest in finding natural sources which can play as antioxidant. Even though several studies were done on mushrooms, Phellinus torulosus was not examined in details. In this study, the free radical scavenging capacity of the total phenolic and flavonoid contents of Phellinus torulosus mushroom extract was evaluated by DPPH and the antioxidant property was tested by catalase (CAT), superoxide dismutase (SOD) and glutathione- S-transferase (GST).

In this study, four different mushroom extracts have been prepared using cold water, hot water, methanol, and ethanol. As ethanol extract resulted in the highest contents of total phenolic and flavonoid (as 625.125 µg/ml and 463.5 µg/ml respectively), the ethanol extract was used in the following steps for determining the radical scavenging capacity and antioxidant property of the mushroom.

The free radical scavenging capacity of Phellinus torulosus ethanol extract was examined by using DPPH free radical. In this experiment by using quercetin and gallic acid as standards, DPPH radical scavenging capacity of mushroom extract was observed and IC50 value was calculated as 0.04353 g/l. The antioxidant property of

Phellinus torulosus ethanol extract was determined by observing its effect on GST. The result showed that the mushroom extract inhibited the activities of GST about 60%. The effect of ethanol extract of Phellinus torulosus on GST was shown with IC50 calculated in the range of 0.1609 to 0.9076 g/l. Again in the same experiment Phellinus torulosus ethanol extract was found to have no effect on CAT and SOD enzymes with less than 15% inhibition for both enzymes.

Keywords: Phellinus torulosus mushroom, free radical scavenging, antioxidants, DPPH assay, Catalase, Superoxide Dismutase, Glutathione-s-trasferase

ÖZ

PHELLINUS TORULOSUS MANTAR ÖZÜTÜNÜN ANTİOKSIDAN ENZİMLER ÜZERİNDE ETKİSİ

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Serbest radikaların zararlarını engellemek amacı ile Antioksidan olarak önemli rol oynayan doğal kaynakları bulmak için son zamanlarda artan bir ilgi oluşmuştur. Her ne kadar literatürde mantarlarla ilgili çalışmalar yapılmışsa da, Phellinus torulous detaylı olarak incelenmemiştir. Bu çalışmada Phellinus torulous mantar özütünün, toplam fenol ve flavonoid içerikleri, serbest radikal süpürücü etkinliği DPPH serbest radikalı ile test edilirken, antioksidan özelliği, Katalaz (KAT), süperoksit dismutaz (SOD) ve Glutathione-s-transferaz (GST) enzimleri ile test edilmiştir.

Bu çalışma da, soğuk su, sıcak su, metanol ve etanol kullanarak dört farklı mantar özütü hazırlanmıştır. Etanol özütü en yüksek toplam fenol ve flavonoid içeriği sahip olmasından dolayı (sırasıyla 625.125 µg/ml, 463.5 µg/ml) mantara ait radikal süpürücü etki ve antioksidan özellik tespiti aşamalarında etanol özütü kullanılmıştır.

Phellinus torulosus mantarının etanol özütünün radikal süpürücü etkisi DPPH serbest radikali kullanarak test edilmiştir. Gallic acid ve quercetin standartları kullanarak yapılan bu çalışmada mantar özütünün radikal süpürücü etkinliği tespit edilmiş ve bunun için IC50 değeri 0,04352 g/l olarak hesaplanmıştır. Phellinus torulosus mantarının etanol özütünün antioksidan özelliğini, GST enzimi üzerine etkisinin çalışılması ile gerçekleştirilmiştir. Sonuçlar göstermiştir ki mantarın özütü enzim aktivitesini %60 oranında inhibe etmiştir. Phellinus torulosus mantarının etanol özütünün GST enzimi üzerine etkisi için IC50 değeri 0,1609 - 0,9076 g/l aralığında hesaplanmıştır. Yine bu çalışma Phellinus torulosus mantarı etanol özütünün KAT ve SOD enzimleri üzerinde etkinliğini olmadığı her iki enzim için %15 den az inhibasyon sonucunda bağlı olarak gösterilmiştir.

Anahtar Kelimeler: Phellinus torulous mantarı, serbest radikal süpürücü, antioksidanlar, DPPH deneyi, Katalaz, Superoksit dismutaz, Glutathione-S-Transferaz

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DPPH	-	2,2-Diphenyl-1-picrylhydrazyl
SOD	-	Superoxide oxide dismutase
CAT	-	Catalase
GSH	-	Reduced Glutathione
GST	-	Glutathione-S-Transferase
CDNB	-	1-Chloro-2,4-dinitrobenzene
NADPH	-	Nicotinamide adenine dinucleotide phosphate
HRP	-	Horse reddish peroxidase
DHBS	-	Dichlorohydorxy benzene sulfonic Acid
4-AP	-	4-Amino antipyrine
EDTA	-	Ethylenediaminetetraacetic acid
NBT	-	Nitro blue tetrazolium chloride
QE	-	Quercetin equivalent
GAE	-	Gallic acid equivalent
TFC	-	Total flavonoid content
TPC	-	Total phenolic content
DMSO	-	Dimethyl sulfoxide
XOD	-	Xanthine oxidase
IC_{50}	-	The half maximum inhibitory concentration

CHAPTER 1

INTRODUCTION

Free radicals are produced in normal and/or pathological cell metabolism. Oxidation is vital for many living organisms in order to produce the energy required for the biological processes. On the other hand, the uncontrolled production of oxygen-derived free radicals can lead to many diseases such as cancer, rheumatoid arthritis, cirrhosis, arteriosclerosis and degenerative processes related to aging (Sarikurkcu et al., 2008).

As a protection system against free radical damage, almost all organisms have oxidative enzymes such as superoxide dismutase (SOD) and Catalase (CAT) or chemical compounds such as α-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione (Niki et al., 1994). However, as the mechanism of antioxidant protection becomes unbalanced by factors due to aging and deterioration of physiological functions, accelerated aging and other diseases occur. In order to help the protection system to reduce oxidative damage, antioxidant supplements or antioxidant containing foods may be useful (Halliwell and Gutteridge, 2003; Mau et al., 2004; Gulcin et al., 2002a,b).

The antioxidant ability of plants tissue is directly related to their activity of “free radical scavenging enzymes” (superoxide dismutase, catalase, peroxide, etc.) as well as the contents of antioxidant substances primarily phenolic compounds, carotenoids, tocopherol and ascorbic acid (Bartosz, 1997).

Mushrooms have been eaten for centuries all over the world and they are well known for their soft texture and mild flavour. There are evidences showing that they are good source of nutrients and bioactive compounds that are beneficial to human body (Chang, 2011). In several studies, it has been observed that mushrooms have photo chemicals and other compounds which are strong antioxidants. These compounds seem to sweep oxygen derived free radicals which are normally generated in natural metabolism of aerobic cells. In circulation most of the free radicals are neutralized by enzymes of antioxidant defence system of the organism such as superoxide dismutase or catalase but the non-enzymatic compounds such as ascorbic acid and carotenoid present in mushrooms also act as antioxidants (Fang et al, 2002).

In this study, the antioxidant properties of a mushroom species called "Phellinus torulosus" will be evaluated.

1.1 Free radicals and Reactive oxygen species (ROS)

Free radicals can be defined as substances containing one or more unpaired electrons in atomic or molecular orbitals (Haliwell & Gutteridge, 1999). This unpaired electron (s) usually causes the free radicals to be highly reactive. Radicals derived from oxygen are the most important free radicals generated in living systems (Miller, Buettner & Aust, 1990). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($O_2.^-$) (Miller et al, 1990). Superoxide radicals are mostly generated within the mitochondria of a cell (Cadenas & Sies, 1998). The mitochondrial electron transport chain is vital for the mammalian cells as it is the primary source of ATP. During energy transduction, a small number of electrons "leak" to oxygen prematurely; forming the oxygen free radical superoxide which can cause implications and lead to a variety of diseases (Kovacic, Pozos, Somanathan, Shangari & O'Brien, 2005; Valko, Izakovic, Mazur, Rhodes &Telser, 2004). In vivo under stress conditions an excess of superoxide releases "free iron" from iron containing molecules. The released Fe^{+2} can participate in the Fenton reaction generating highly reactive hydroxyl radical ($Fe^{+2} + H_20_2 \rightarrow Fe^{+3} + .OH + OH^-$). Therefore under stress conditions, $O_2.-$ acts as an oxidant of 4Fe-4S cluster-containing enzymes leading to the production of .OH from H_20_2 by making Fe^{+2} available for Fenton reaction (Valko et al. 2006; Leonard, Harris & Shi, 2004). Other oxygen derived reactive radicals which can be formed in living systems are peroxyl radicals (ROO.). The simplest peroxyl radical is HOO, which is the protonated form of superoxide (O_2.-) and is usually termed either hydroxyl radical or perhydroxyl radical.

1.2 Oxidative damage to DNA, lipids and proteins

At high concentrations, Reactive Oxygen Species abbreviated as ROS can be important factors for damage to cell structures, nucleic acids, lipids and proteins (Valko et al., 2006). The hydroxyl radical is known to react with all components of the DNA molecule, damaging both the purine and pyrimidine bases and also the deoxyribose backbone (Haliwell & Gutteridge, 1999). One of DNA lesion studied is the formation of 8-OH-G. The oxidative damage causing permanent modification of genetic material results in the first step involved in mutagenesis, carcinogenesis and aging.

The metal-induced generated Reactive Oxygen Species do not only attack on DNA, but also they attack other cellular components such as polyunsaturated fatty acid residues of phospholipids, which can easily be oxidised (Siems, Grune & Esterbauer, 1995). The generated peroxy radical ($ROO^{\cdot}$) can undergo a cyclisation reaction to endoperoxides with the final product of the peroxidation process being malondialdehyde (MDA) Marnett, 1999, Wang et al., 1996).
Besides malondialdehyde, another important aldehyde product of lipid peroxidation is 4- hydroxyl-2-nonenal (HNE). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats. HNE is weakly mutagenic but appears to be the major toxic product of lipid peroxidation.

The oxidation mechanism of proteins by ROS has also been studied. Here, amino acids, simple peptides and proteins were exposed to ionising radiation causing the formation of hydroxyl radicals or a mixture of hydroxyl/superoxide (Stadman, 2004). The side chains of all amino acid residues of proteins are susceptible to oxidation. When the cysteine residues are oxidized, disulphides are formed between protein thiol groups (-SH) and low molecular weight thiols, particularly GSH (S-glutathiolation). The content of carbonyl group formed by various mechanisms is a good measure of ROS-mediated protein oxidation. All discussed above is illustrated in figure. 1. In this figure reaction 1 refers to the reduction of oxygen to form superoxide anion radical by NAD(P)H oxidase and xanthine oxidase or non-enzymatically by redox-reactive compounds such as the semi-ubiquinone compound of the mitochondrial electron transport chain. In reaction 2, superoxide radical is converted to hydrogen peroxide by superoxide dismutase (SOD). In reaction 3, Hydrogen peroxide is scavenged by the enzyme glutathione peroxide (GPx) using GSH as electron donor. In reaction 4, the oxidised glutathione (GSSG) is reduced back to GSH by the enzyme glutathione reductase using NADPH as the electron donor. Reaction 5 refers to Fenton reaction converting hydrogen peroxide to reactive hydroxyl radical using some transition metals. In reaction 6 the hydroxyl radical obtains an electron from polyunsaturated fatty acid (LH) to form a carbon centred lipid radical (L.). In reaction 7 lipid radical (L.) can react with molecular oxygen to give a lipid peroxy radical (LOO.). If lipid peroxy radical (LOO.) is not reduced by antioxidants, the lipid peroxidation occurs (reactions 18-23, and 15-17). Reaction 8 shows the reduction of lipid peroxy radical (LOO.) by the reduced form of vitamin E (T-OH) to form a lipid hyrdoperoxide and a radical of vitamin E (T-O.) In reaction 9, vitamin E is regenerated by vitamin C leaving behind the ascorbyl radical ($Asc.^{-}$). Reaction 10 refers to the regeneration of vitamin E by GSH. In reaction 11, the oxidised glutathione (GSSG) and the ascorbyl radical ($AS\text{-}sc.^{-}$) are reduced back to GSH and ascorbate monoanion, $AscH^{-}$ by the dihyrolipoic acid (DHLA) which is turned into α-lipoic acid (ALA). Reaction 12 refers to the generation of DHLA from ALA using NADPH. In reaction 13 lipid hyroperoxides are reduced to alcohols and dioxygen by GPx using GSH as the electron donor. Then lipid peroxidation starts. In reaction 14 if lipid hyroperoxides reacts with Fe +2 lipid alkoxyl radical (LO.) is formed but if it reacts with Fe +3 lipid peroxyl radical (LOO.) is formed and after certain chain reactions (reaction 15 to 17) 4-hydroxy-nonenal is formed which is then converted to glutathiyl adduct. Reaction 18 shows that a peroxy radical can form a cyclic peroxide next to a carbon-centred radical and in reaction 19 this radical can form a hyroperoxide by reduction (not shown) or it can undergo a second cyclisation to form a bicyclic peroxide which finally give rise to the production of malondialdeyde in reaction 20. In reactions 21 to 23, Malondialdehyde can react with DNA bases as shown in the figure 1.

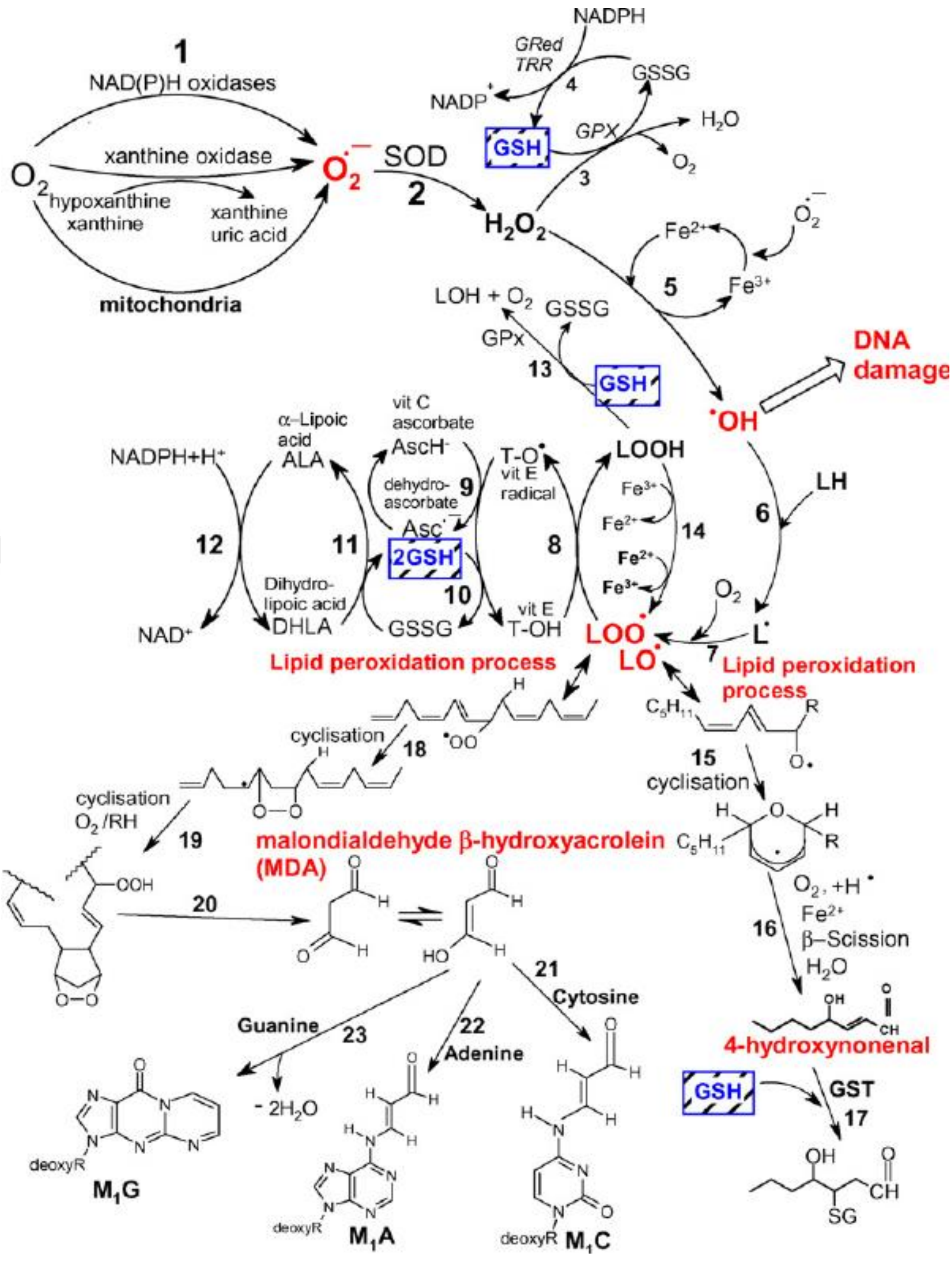

Figure 1. Pathways of ROS formation

1.3 Antioxidant Protection System

Humans have evolved an extensive antioxidant protection system in order to protect the cells and organs against reactive oxygen species (ROS). This system includes a variety of components both endogenous and exogenous in origin that function interactively and synergistically to neutralize free radicals (Table 1) (Mark Percival, 1998). These components include:

a. Endogenous Antioxidants
    - Bilirubin
    - Thiols, e.g. glutathione, lipoic acid, N-acetyl cysteine
    - NADPH and NADH
    - Ubiquinone (Coenzyme Q10)
    - Uric Acid
    - Enzymes:
        - Copper/zinc and manganese-dependant superoxide dismutase
        - Iron-dependant glutathione peroxidase
        - Selenium-dependant glutathione peroxidase

b. Dietary Antioxidants
    - Vitamin C
    - Vitamin E
    - Beta Carotene and other carotenoids and oxycarotenoidse, eg., lycopene and lutein.
    - Polyphenols, e.g., falvonoids, flavones, flavonol’s and proanthocyanidins

c. Metal Binding Proteins
    - Albumin (Copper)
    - Ceruloplasmin (Copper)
    - Metallothionein (Copper)
    - Ferritin(iron)
    - Myoglobin (iron)
    - Transferrin (iron)

Table 1. Various ROS and corresponding neutralizing antioxidants

ROS	Neutralizing Antioxidants
Hydroxyl radical	Vitamin C, Glutathione Flavonoids, Lipoic acid
Superoxide radical	Vitamin C, Glutathione, Flavonoids, Lipoic acid, SOD
Hydrogen peroxide	Vitamin C, Glutathione, beta carotene, vitamin E, Flavonoids, lipoic acid
Lipid Peroxide	Beta carotene, vitamin E, Ubiquinone, flavonoids, Glutathione peroxidase

Defence mechanisms against free radical-induced oxidative damage include removal of free radicals where enzymes such as catalase, superoxide dismutase, GPx and thiol-specific antioxidants play important roles, reducing free radicals by donating electrons which is done by Glutathione, Vitamin E and Vitamin C or by protection through binding metal ions or other protective proteins such as stress or heat shock proteins. (B. Halliwell, J. Gutteridge, 1999).

In other words, there are two main mechanisms played by antioxidants (Rice-Evans CA, Diplock AT.1993). In the first mechanism, antioxidant donates an electron to free radical and breaks the free radical generating chain from the beginning. In the second mechanism reactive nitrogen species initiators are removed by quenching chain-initiating catalyst. Therefore Antioxidants may have different protecting mechanisms namely electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation (Krinsky NI. 1992).

Under normal conditions, there is a balance between both the activities and the intracellular levels of antioxidants. This balance is essential for the survival of organisms and their health.

In case this balance is not managed then the created oxidative stress can lead to various implications and pathological conditions involving cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing (Sies H., 1985, Dalle Donne et al, 2006, Valko et al, 2006, Niki E., 1997). In general these diseases can be divided into two groups: The first group called as "mitochondrial oxidative stress" conditions which include the diseases with the thiol/disulphide redox state shifting and impairing glucose tolerance (Cancer and diabetes mellitus); The second group include diseases with "inflammatory oxidative conditions" where either activity of NAD(P)H oxidase is enhanced (such as in atherosclerosis and chronic inflammation) or ROS production is induced by xanthine oxidase (such as in ischemia and reperfusion injury). The aging process occurs by damaging causes of free radicals (such as lipid peroxidation, DNA damage, protein oxidation) (Harman 1992).

Biomarkers are considered good proof for demonstrating that various acute and chronic diseases are related to oxidative damage. In order to have a meaningful evaluation, such biomarkers must be measured on healthy and ill subjects for long periods. Table 2 gives a summary of well-known biomarkers of oxidative damage of for various human diseases. Recently Dalle-Donne and co-workers have published an excellent review on such biomarkers of oxidative stress in human diseases (Dalle-Donne et al., 2006).

In general, the antioxidant defense systems act at different levels such as preventive, radical scavenging, repair and de novo, and the fourth line of defense, i.e., the adaptation.

The first line of defense is the preventive antioxidants, which reduce the formation of free radicals. The most important free radical formation is Fenton reaction which is decompositions of hydrogen peroxide induced by metal ion. In order to lower the incidence of such reactions, some antioxidants reduce hydrogen peroxides to alcohols and water in advance. This way, the occurrence of next chain reactions is prevented.

Glutathione peroxidase, glutathione-S-transferase, phospholipid hydroperoxide glutathione peroxidase (PHGPX), and peroxidase are known to decompose lipid hydroperoxides to corresponding alcohols.

Table 2. Biomarkers of oxidative damage associated with some human disease

Diseases/biomarkers	
Cancer	Parkinson's disease
MDA	HNE
GSH/GSSG ratio	GSH/ GSSG ratio
NO_2- Tyr	Carbonylated proteins
8-OH-Dg	Iron level
Cardiovascular disease	Ischemia/reperfusion
HNE	F_2-isoprostanes
GSH/GSSG ratio	GSH/ GSSG ratio
Acrolein	Atherosclerosis
NO_2- Tyr	MDA
F2-isoprostanes	HNE
Acrolein	Acrolein
Rheumatoid arthritis	F_2-isoprostanes
F_2-isoprostanes	NO_2-Tyr
GSH / GSSG ratio	diabetes mellitus
Alzheimer's disease	MDA
MAD	GSH/ GSSG ratio
HNE	S-glutathionylated proteins
GSH / GSSG ratio	F_2- isoprostanes
F2-isoprostanes	NO_2 - Tyr
NO_2- Tyr	AGE
AGE	

Abbreviations: MAD, malondialdehyde; HNE, 4-hydroxy-2-nonenal;AGE, advanced glycation end products; 8-OH-dG, 8-hydroxy-20-deoxyguanosine; GSH, reduced glutathione; GSSG, oxidized glu-tathione; NO_2 –Tyr, 3-nitro- tyrosine

In particular PHGPX can reduce hydroperoxides of phospholipids which are part of membrane structure. Glutathione peroxidase and catalase reduce hydrogen peroxide to water.

The second line of defense includes antioxidants that scavenge the active radicals to lower chain initiation and/or break the chain propagation reactions. There are certain endogenous radical-scavenging antioxidants some hydrophilic (Vitamin C, uric acid, bilirubin, albumin, and thiols) and others are lipophilic (vitamin E and ubiquinol).

The third line of defense is the repair and *de novo* antioxidants including proteolytic enzymes, proteinases, proteases, and peptidases found in the cytosol and in the mitochondria of mammalian cells. They can detect and degrade or remove oxidized proteins and prevent their accumulation.

The DNA repair systems namely glycosylases and nucleases also play an important role in the total defense system against oxidative damage.

There is another important function called adaptation where appropriate antioxidant is formed and transported to the right site by certain signal for the generation and reactions of free radicals to the right site. (Niki E.1993).

1.3.1 Enzymatic Antioxidants

Cells are protected against oxidative stress by an interacting antioxidant enzyme (Sies H. 1985). Such enzymes donate their electrons to free radicals thereby causing them to become stable so the free radicals are not reactive any more. These enzymes can also end the free radical generating chain reaction thereby minimizing their damages. For instance, the superoxide formed by certain processes is first converted to hydrogen peroxide by superoxide dismutase and then further reduced to give water by catalase and various peroxidase. (Magnenat JL, Garganoam M, Cao J. 1998).

1.3.1.1 Superoxide Dismutase

Superoxide dismutases (SODs) found in extracellular fluids of aerobic cells (Johnson F et al. 2005) convert superoxide anion into oxygen and hydrogen peroxide (Zelko I, et al., 2002) (Banniste J, et al 1987). Based on the metal cofactor they bind, they fall in to three main classes as follows: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni type which binds nickel (Wuerges J. et al., 2004).

In higher plants, SOD isozymes can be found in different cell compartments. For instance, Mn-SOD is present in mitochondria and peroxisomes. Fe-SOD is found in chloroplasts mostly but has also been found in peroxisomes, and CuZn-SOD has been detected in cytosol, chloroplasts, peroxisomes, and apoplast (Wuerges J et al., 2004, 49. Corpas FJ, et al., 2001, Corpas FJ et al., 2006)

In humans and in other mammals and chordates, three forms of superoxide dismutase are present. SOD1 (as a dimer containing two units) is located in the cytoplasm, SOD2 is found in the mitochondria, and SOD3 is extracellular. Both SOD2 and SOD3 are tetramers with four subunits. SOD1 and SOD3 have copper and zinc, but SOD2 has manganese in its reactive center (Cao X, et a., 2008).

1.3.1.2 Catalase

Catalase is an enzyme present in all known animals mostly in liver (Eisner T et al., 1999). This enzyme catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani P et al., 2004). Hydrogen peroxide is a harmful by-product of many normal metabolic processes. In order to prevent its damage, it must be quickly converted into less dangerous substances such as oxygen and water (Gaetani G et.al., 1996),

1.3.1.3 Glutathione systems

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases. This system is found in animals, plants, and microorganisms (Meister A, et al., 1983). Glutathione peroxidase is an enzyme which catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. In animals four different glutathione peroxidase isozymes are found (Brigelius-Flohe R., 1999). Glutathione peroxidase 1 is the most abundant and is a very good scavenger of hydrogen peroxide, but glutathione peroxidase 4 is most active with lipid hydroperoxides.

The glutathione S-transferases enters reactions mostly with lipid peroxides. These enzymes are located mostly in liver and also play important role in detoxification during metabolism (Hayes J, et al., 2005). In general, GST enzymes act in phase II of drug metabolism. These enzymes catalyze the conjugation of glutathione with many compounds formed in phase I of drug metabolism. In such reactions, a bond is formed between sulfur atom of glutathione and the substrate and such product can easily be eliminated from the cell (Chasseaud, 1979 and Mannervik 1985). In chemotherapy, there is an overexpression of GST in tumors which cause resistance to the therapeutics in patients. Thus, GST inhibition may be a helpful tool in chemotherapy (Morrow et al. 1998).

1.3.2 Nonenzymatic Antioxidants

1. 3.2.1 Ascorbic acid

Ascorbic acid or “vitamin C” is a monosaccharide antioxidant found in both animals and plants. It is a vitamin which cannot be synthesized in humans and thus it must be obtained from the diet (Smirnoff N., 2001). Most other animals can produce this compound in their bodies and do not require it in their diets. In cells, it is found in its reduced form by reaction with glutathione, which can be catalyzed by protein disulfide isomerase and glutaredoxins (Meister A., 1994).

An important function of Ascorbic acid is that it can neutralize hydrogen peroxide (Padayatty S, et al., 2003). Additionally, it is a substrate for the antioxidant enzyme ascorbate peroxidase which is considered a protection mechanism against stress in plants (Shigeoka S. et al., 2002).

1.3.2.2 Glutathione

Glutathione is a cysteine-containing peptide found in most forms of aerobic life (Meister A, et al., 1983). It is not required in the diet as it is synthesized in cells. It has reducing function acting on certain metabolites and enzymes. Also, it has the ability to react with oxidants directly (Meister A., 1988). Due to its high concentration and central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants (Matill HA.,1947) In some organisms, glutathione is replaced by other thiols, such as by mycothiol in the actinomycetes, or by trypanothione in the kinetoplastids.(Fairlamb AH, et al., 1992.,).

1.3.2.3 Melatonin

Melatonin, also known chemically as N-acetyl-5-methoxytryptamine (Nassar E, et al., 2007) is a hormone naturally found in animals and in some other living organisms, including algae (Caniato R, et al., 2003) Melatonin is a powerful antioxidant that can easily cross cell membranes and the blood–brain barrier (Reiter RJ, et al. 1997). The mechanism of melatonin is different from other antioxidants in a way that it is not reduced or oxidized repeatedly. When it is oxidized by free radicals, it cannot be reduced back to its previous form. Instead, it forms several stable end-products. Because of this property, Melatonin has been called as a terminal (or suicidal) antioxidant (Tan DX, et al., 2000).

1.3.2.4 Tocopherols and tocotrienols (Vitamin E)

There are eight related tocopherols and tocotrienols with antioxidant properties collectively called as Vitamin E (Herrera E, et al., 2001). Among these, α-tocopherol has been most studied as it is mostly available in biological systems (Brigelius-Flohe R, et al., 1999). It has been claimed that the α-tocopherol form is the most important lipid-soluble antioxidant. It protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction (Traber MG, et al., 2007). This way, it can remove the free radical intermediates thereby preventing the chain reaction occurrence to continue. This reaction produces oxidized α-tocopheroxyl radicals that can be recycled back to the active reduced form by other antioxidants, such as ascorbate, retinol, or ubiquinol (Wang X, et al.1999).

1.3.2.5 Uric acid

It has been thought that Uric acid may have been evolved to function as ascorbate in human evolution (Jaeschke H, et al., 2002). Uric acid is responsible for about half of antioxidant ability of plasma.

1.4 Plants as source of antioxidant

Synthetic and natural food antioxidants are used routinely in foods and medicine especially those containing oils and fats to protect the food against oxidation. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are examples of synthetic phenolic antioxidants which have been used in food industry, cosmetics, and therapeutic industry. However, certain physical properties of such synthetic antioxidants such as their high volatility and instability at elevated temperature, strict regulations of their use as food additives, and their carcinogenic nature as well as the consumer preferences have drawn the attention of manufacturers to shift from synthetic to natural antioxidants (Papas AM. 1999). Due to increasing risk factors of human to various deadly diseases, it has been a worldwide tendency to use natural substance present in medicinal plants and dietary plants as therapeutic antioxidants. There are reports showing the inverse relationship between the dietary intake of antioxidant-rich food and medicinal plants and incidence of human diseases. The use of natural antioxidants instead of synthetic antioxidants in food, cosmetic, and therapeutic industry has been favored because of their low cost, being highly compatible with dietary intake and having no harmful effects for human. There are evidences that certain antioxidant compounds found in plant sources have ability to scavenge free radical or reactive oxygen species (Brown JE, et al., 1998). In some studies, many vegetables such as potato, spinach, tomatoes, and legumes (Furuta S, et al., 1997) as well as certain fruits such as berries, cherries, citrus, prunes, and olives showed strong antioxidant potentials (Wang H, et al., 1996). Additionally, it has been shown that green and black teas contain up to 30% of the dry weight as phenolic compounds with antioxidant properties (Lin JK, et al., 1998).

1.4.1 Mushrooms as exogenous antioxidants

The fact that several species of mushrooms contain certain free radicals or reactive oxygen species scavenger have made mushrooms attractive as beneficial nutrition and good source of drugs development (Guerra –Dore et al., 2007). Barros et al. (2009) reported that mushroom flavonoids can act as free radical scavengers terminating the radical chain reactions which occur during the oxidation of triglycerides in the food system.

In another study, phenolic compounds, alkaloids, saponins, flavonoids, tannins, sterols, titerpenes, coumarins and cyanogenic glycoside have been detected in wild mushrooms analysed in Sudan and in Nigreia (Adebayo et al., 2012, Egwim et al., 2011,Ehssan & Saadabi, 2012).

At least, three review articles have mainly described medicinal properties of mushrooms, including antioxidant (Ferreira ICFR et al., 2009) anticancer (Patel et al., 2012) and antibacterial activities (Alves MJ, et al., 2012). The antioxidants found in mushrooms are primarily phenolic compounds (phenolic aids and flavonoids), as well as tocopherols, ascorbic acid and carotenoids.

In addition, a number of other review articles have explained pharmacological and medicinal properties of edible mushrooms, including antimicrobial, anticancer, antioxidant, antiviral, immunomodulatory, immununosuppressive, anti-allergic, anti-inflammatory, and anti-cholestral activities (Rathe S., et al, 2012). Among other medicinal properties, the antioxidant activity of edible mushrooms can be used in daily life as it may associate to preventing oxidative stress naturally which is influenced by lifestyle habits (Sakano N., et al., 2009).

1.4.1.1 Phellinus torulosus

Phellinus torulosus or its binomial name Fuscoporia torulosa is a wood-dexay fungus, which causes a white rot of heartwood in dead and living trees in Europe and in Coniferous trees in North America (Ainsworth M., 1993). Until recently this species was known as Phellinus torulosus. However, a phylogenetic study in 2001 resulted in the Phellinus genus being split into five new genera, and P. torulosus being renamed to Fuscoporia torulosa.

The scientific classification is as follows:

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycotina

Order: Agaricomycetes,

Family: Hymenochaetales

Genus: Hymenochaetaceae

Species: Fuscoporia

Binomial name: Fuscoporia torulosa (Pers.) (T. Wagner & M. Fisch, 2001).

For this study, the mushroom samples were collected from Belgrad forest (Istanbul), on beech stump, N 41° 11' - E 28° 58', 85 m, 15.11.2014, Akata 6256.

Figure 2. Photograph of Phellinus torulosus

1.5 Scope of this study:

The aim of this study is to determine the total phenolic content and total flavonoid content of Phellinus torulosus and to evaluate the effect of the antioxidant and free radical scavenging properties of Phellinus torulosus by evaluating the activities of GST, CAT and SOD in reaction mixture of Phellinus torulosus extract with these enzymes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Phellinus torulosus mushroom samples were collected from Belgrade Forest in Istanbul by Assoc. Prof Dr. Ilgaz Akata at Department of Botany of Ankara University, Gallic Acid (Merk), Dimethyl Sulfoxide (DMSO) (Carloerba), Folin Ciocalteus Phenol Reagent (Sigma-Aldrich), Sodium Carbonate (Fisher Scientific), Quercetin (Sigma), Absolute Ethanol (Merk), Sodium Acetate (Aklar Kimya), Aluminum Chloride (Merk), Methanol, Mono Potassium Phosphate and Di Potassium Phospate (Riedel.dehaen), Reduced form of Glutathione (Sigma-Aldrich), 1-Chloro-2,4-Dinitrobenzene (CDNB) (Fluka), Bovine Liver Cytosol extracted in our laboratory from the bovine liver which was obtained from slaughter house in Kazan , Ankara, 2,2-Diphenyl-1-Picrylhyrazyl (DPPH) (Calbiochem), Hydrogen Peroxide (H_2O_2) (J.T. Baker), Dichlorohydorxy Benzene Sulfonic Acid (DHBS) (Sigma), 4-Amino Antipyrine (AP) (Acros), Horse ReddishPeroxidase (HRP), Sodium Azaide (Acros), Catalase (Sigma-Aldrich), Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate (EDTA) Sigma), Nitro Blue Tetrazolium Chloride (NBT) (Thermo), Xanthine Oxidase (Calbiochem), Xanthine (Sigma-Aldrich).

2.2 Methods

2.2.1 Extraction details of cold water, hot water, methanol and ethanol methods of Phellinus torulosus mushroom

Phelinus torulosus was obtained in dried form from Assoc. Prof. Dr. İlgaz Akata, Department of Botany, Ankara University, Ankara, Turkey.

Using liquid nitrogen, the dried mushroom was ground and turned into powder for extraction. In this study, four extraction methods were used in order to extract P. torulosus mushroom.

These were cold water, hot water, methanol and ethanol extraction method (Table 3). The concentration of dried extract was determined in each method as mg of dried extract per ml of 99.9% DMSO in which they were dissolved (Table 3). Then the dissolved extracts were kept in -20°C to use for further analysis. The detail of each of extraction procedure is given in Table 4. For the cold water and hot water extractions method of Yeh, J. Y., 2011 was followed and for methanol and ethanol extraction method of Coruh et al., 2007 was used.

Table 3. Concentration of prepared extracts

Extraction Method	Concentration of Mushroom as mg of dried extract/ml of DMSO
Cold water	0.176
Hot water	2.69
Methanol	14.09
Ethanol	5.5

Table 4. Procedures of extraction methods.

Extraction Methods	Addition to solvent	Centrifugation	Filtration	Times of extraction	Filtration	Evaporation
Cold water	2 g of dried powder was stirred with 20 ml of distilled water at room temperature for 24 hours	_	Under pressure	Two times	Under pressure	Evaporation to dryness using 80 rpm rotary evaporator at 60 °C
Hot water	2 g of dried powder was boiled with 70 ml of distilled water for one hour	3000 rpm for 45 minutes	Under pressure	Two times	Under pressure	Evaporation to dryness using 80 rpm rotary evaporator at 60 °C
Methanol	1 g of dried powder was dissolved in 100 ml methanol, stirred at room temperature for 30 minutes and left in 4° C for 24 hours	6000 rpm at 4°C for 10 minutes	_	_		Evaporation to dryness using 80 rpm rotary evaporator at 45 °C
Ethanol	1 g of dried powder was dissolved in 100 ml Ethanol, stirred at room temperature for 30 minutes and left in 4° C for 24 hours	6000 rpm at 4°C for 10 minutes	_	_		Evaporation to dryness using 80 rpm rotary evaporator at 45 °C

2.2.2 Determination of Total Phenolic Content (TPC) of Phellinus torulosus

The Phenolic content of P. torulosus was determined using Folin-Ciocalteu's assay (Slinkard and Singleton, 1977). First four different concentrations of Gallic Acid (from 0 to 200µg/ml) were prepared and dissolved in DMSO and the phenolic content was measured and the calibration curve was drawn and used as standard. The calculated concentrations of the mushroom extracts and Gallic Acid are given in Table 5. The reaction components of the phenolic content determination assay are given in Table 6. In this assay 1 ml of 1:10 diluted Folin-Ciocalteu Reagent was added to 100 µl of mushroom extracts and incubated in dark for 5 minutes. Then 1 ml of 2% w/v of Sodium Carbonate is mixed and again incubated for one hour in dark at room temperature. Then the absorbance was measured at 750 nm. In this assay; in the presence of phenolic compounds in an alkaline solution;

phosphomolybdic-phosphotungstic acid (Folin) reagent is reduced to a blue-colored complex. The absorbance at this particular wavelength shows the amount of potentially oxidizable phenolic groups. The blank contains DMSO instead of mushroom extract. The total phenolic contents of four extracts were measured by using gallic acid standard curve and were expressed as µg of gallic acid equivalent (GAE) per ml of extract volume.

Table 5. The concentrations of Phellinus torulosus extract prepared by four different methods which were used for total phenolic content determination using gallic acid as standard

Dilution	Concentrations (µg/ml)				
	Cold water extract	Hot water extract	Methanol extract	Ethanol extract	Gallic Acid Standard
1	176	2690	14090	5500	50
2	88	1345	70450	2750	100
3	-	-	-	-	200

Table 6. The reaction components of phenolic content determination assay.

Stock	Added Volume
Cold water extract	100 µl from each concentration given in table 5.
Hot water extract	
Methanol extract	
Ethanol extract	
Gallic acid	
10 fold diluted Folin-Ciocalteu's reagent with distilled water	1 ml
Incubation for 5 minutes in dark	
2% w/v Sodium Carbonate solution	1 ml
Incubation for one hour in dark at room temperature	
Read at 750 nm	

The reaction mixture containing DMSO instead of mushroom extract was used as blank.

2.2.3 Determination of Total Flavonoid Content (TFC) of Phelinus torulosus

The total flavonoid content of the extracts was determined by using the aluminum chloride colorimetric method (Chang et al., 2002). Initially four different concentrations of quercetin (from 0 to 200µg/ml) were prepared and the flavonoid content was measured and the calibration curve was drawn and used as standard. The calculated concentrations of the sample extracts and quercetin are given in table 7.

The reaction mixture is given in table 8. In this assay, 250 µl of mushroom extracts was mixed with 750 µl of absolute ethanol, 50 µl of 10% w/v aluminum chloride, 50 µl of 1M Sodium acetate and 1 ml of DMSO. Then the mixture was incubated for 30 minutes in dark and the absorbance was read at 415 nm. The principle of this assay is that aluminum chloride ($AlCl_3$) can form acid stable complexes with flavonoids which can be measured calorimetrically and thus the absorbance measured reflects the amount of flavonoid in the extract tested. The reaction mixture containing DMSO instead of mushroom extract was used as blank.

As it was found that the phenolic and flavonoid content of ethanol extract was the highest comparing to the extracts of other methods, all following experiments were done using ethanol extract. The enzymatic assays other than GST were carried out by using multi-mode plate reader (Figure 3). GST activity was measured using UV visible spectrophotometer.

Table 7. The concentrations of Phellinus torulosus extract prepared by four different methods which were used for total flavonoid content determination using quercetin as standard.

Dilution	Concentrations (µg/ml)				
	Cold water extract	Hot water extract	Methanol extract	Ethanol extract	Quercetin Control
1	176	2690	14090	5500	25.0
2	88	1345	70450	2750	50.0
3	-	-	-	-	100.0
4	-	-	-	-	200.0

Table 8. The reaction components of flavonoid content determination assay

Stock	Added Volume
Cold water extract	250 µl from each concentration given in table 7.
Hot water extract	
Methanol extract	
Ethanol extract	
Quercetin	
Absolute ethanol	750 µl
10% w/v Aluminum Chloride	50 µl
1 M Sodium Acetate	50 µl
DMSO	1 ml
Incubation for 30 minutes in dark	
Read absorbance at 415 nm	

The reaction mixture containing DMSO instead of mushroom extract was used as blank

Figure 3. Multi-mode plate reader with 96 well plate (Molecular Devices Spectramax M2)

2.2.4 Determination of free radical scavenging assay

The free radical scavenging capacity of the ethanol extract was determined using 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay (Sharma, Bahat, 2009). Different concentrations of Gallic acid and Quercetin were used as positive control and are mentioned in table 9. The different concentrations of ethanol extract used in this assay are shown in table 10.

Table 9. Concentrations of Gallic acid and Quercetin used in DPPH assay

Dilutions	Concentrations (µg/ml)			
	Stock Quercetin	Final Quercetin	Stock Gallic Acid	Stock Gallic Acid
1	180.00	5.40	540.00	16.20
2	60.00	1.80	180.00	5.40
3	20.00	0.60	60.00	1.80
4	6.67	0.20	20.00	0.60
5	2.22	0.07	6.67	0.20
6	0.74	0.02	2.22	0.07
7	0.25	0.01	0.74	0.02

Table 10. Concentrations of ethanol extract used in DPPH assay

Dilutions	Stock ethanol extract concentration mg/ml	Final ethanol extract concentration mg/ml
1	0.712	0.1068
2	0.237	0.0356
3	0.079	0.0119
4	0.0263	0.00396
5	0.00876	0.00132
6	0.00292	0.00044
7	0.000973	0.000146

The reaction mixture is given in table 11. In this assay, based on the procedure 6 µl of ethanol extract and the standards was added to 144 µl and mixed with 50 µl of 200 mM DPPH. Then the mixture was incubated for 25 minutes in dark at temperature 21-25°C. After incubation, the absorbance was read at 517nm. The reaction mixture without ethanol extract was used as blank keeping the total reaction volume the same. DPPH assay is based on the ability of 2,2-diphenyl-1-picrylhydrazyl, a stable free radical to decolorize from purple to yellow color in presence of antioxidants. The DPPH contains an odd electron, which is responsible for the absorbance at 517nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound of sample extract, the DPPH is decolorized. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517nm induced by antioxidants.

The percent inhibitions were calculated for each extract concentrations and were plotted against concentrations. The DPPH radical scavenging activity of ethanol extract was expressed as IC50 value calculated from dose-response curve.

Table 11. The reaction mixture and procedure in DPPH assay

Stocks	Volumes
Standards/ Ethanol extract	6 µl
Methanol	144µl
200 µM DPPH	50µl
Incubation for 25 minutes in dark at 21-25° C	
Read absorbance at 517 nm	

The reaction mixture without mushroom extract was used as blank keeping the total reaction volume the same.

2.2.5 Determination of Catalase Enzyme Activity

This assay is based on measuring the remaining hydrogen peroxide once the action of catalase enzyme is stopped by the addition of sodium azide. The remaining hydrogen peroxide was determined by measuring the formation of red quinoneimine dye by colorimetric method (Aebi, 1984; Bai et al., 1999; Fossati et al., 1980). However the assay was miniaturized for micro plate application (Işgor et al., 2008) and the absorbance was measured at 520 nm. For this assay, 4 µl of ethanol extract was added to 20 µl of 100 U/ml Catalase obtained from Sigma, 50 µl of 10 mM hydrogen peroxide (H_2O_2), 26 µl of 50 mM phosphate buffer. The mixture was incubated for 2 minutes in dark and then the reaction was stopped by the addition of 50 µl of 15 mM sodium azide (NaN_3) and then the mixture was incubated in dark for 5 minutes. Then 5 µl of this mixture was added to 255 µl of chromogen with horse reddish peroxidase (for each 5 ml chromogen, 5 µl of HRP was added) and then incubated for 40 minutes in dark. After the termination of incubation period, the absorbance was read at 520 nm. Chromogen was prepared by mixing 1 ml of 1.25 mM of 4 amino antipyrine (4-AP), 1 ml of 10 mM dichloro hydroxyl benzene sulfonic Acid (DHBS) and 3 ml of 150 mM phosphate buffer). The enzyme activity was determined by measuring the remaining H_2O_2 calculated from calibration curve for hydrogen peroxide concentration ranging from 0.00192 mM to 0.12308 mM. The concentration of ethanol extract used in this assay is given in Table 12 and the reaction mixture and the related procedure is illustrated in Table 13.

Table 12. Concentrations of ethanol extract used in catalase assay

Dilutions	Stock ethanol extract concentration mg/ml	Final ethanol extract concentration mg/ml
1	0.079	40×10^{-6}
2	0.026	13×10^{-6}
3	0.0086	4×10^{-7}
4	0.0029	1×10^{-7}
5	0.00097	33×10^{-8}
6	0.00032	11×10^{-8}

Table 13. The reaction mixture used in CAT Assay

Stock	Volume Added
Chromogen in total of 5 ml	
1.25 mM 4 Amino Antipyrine	1ml
10 mM Dichloro Hydroxyl Benzene Sulfonic Acid	1ml
150 mM Phosphate Buffer	3 ml
For each 5 ml chromogen, 5 µl HRP was added	
Reaction Mixture	
Ethanol Extract	4 µl
100 U/ml Catalase	20 µl
10 mM Hydrogen Peroxide	50 µl
50 mM Phosphate Buffer	26 µl
Incubation for 2 minutes	
15 mM Sodium Azide	50 µl
Incubation for 5 minutes	
Above mixture + Chromogen	5 µl + 255 µl
Incubation for 40 minutes and read absorbance at 520 nm	

Reaction mixture without enzyme was used as blank keeping the total reaction volume the same.

2.2.6 Determination of Superoxide Dismutase (SOD) Enzyme Activity

The principle for determining the SOD enzyme activity in this assay is based on the inhibition of nitroblue tetrazolium (NBT) reduction by using method of Işgor, B. S., 2013. The NBT is reduced by the activity of superoxide and forming blue colored Formazan which is measured at 560 nm. The concentration of ethanol extract used in this assay is shown in Table 14. In this assay, 213 µl of assay mixture was mixed with 10 µl of cytosol which was used as the source of SOD enzyme, and 57 µl of deionized water, 5 µl of Ethanol extract and 5 µl of xanthine oxidase was added. Then the mixture was incubated for 30 minutes in dark and then the absorbance was read at 560 nm. Assay mixture was composed of 3 µl of 25mM NBT (nitroblue Tetrazolium), 150 µl of 0.3 mM xanthine and 75 µl of 200 mM sodium carbonate buffer with 10 mM EDTA The reaction mixture and the procedure for this assay is illustrated in Table 15.

Table 14. Concentrations of ethanol extract used in SOD assay

Dilutions	Stock ethanol extract concentration mg/ml	Final ethanol extract concentration mg/ml
1	0.712	123×10^{-4}
2	0.237	41×10^{-4}
3	0.079	14×10^{-4}
4	0.026	47×10^{-5}
5	0.0086	16×10^{-5}
6	0.0029	54×10^{-6}

Table 15. The reaction mixture used in SOD Assay

Reagents	Volumes added (µl)	Contents
Assay Buffer	213	3 µl of 25mM NBT, 150 µl of 0.3 mM Xanthine, 75 µl of 200 mM Sodium Carbonate Buffer with 10 mM EDTA
Cytosol	10	Directly from stock
Deionized Water	57	180 ohm and 22 micro filtered
Ethanol extract	5	
Xanthine Oxidase	5	2 U/ml
Incubation for 30 minutes and read absorbance at 560 nm		

2.2.7 Determination of Glutathione-S-Transferase (GST) Enzyme Activity

This assay is based on formation of the conjugate product of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione according to Habig et al., (Habig et al, 1974) with small modifications. The cytosol extracted from bovine liver in our laboratory was used as the source of GST enzyme. The activity of GST enzyme was measured by using ultraviolet spectrophotometer. In the assay mixture 50 µl of 20 mM CDNB substrate was mixed with different volumes of ethanol extract. The reaction was started by adding 50 µl of bovine cytosol. The total volume of assay mixture was 1 ml and the absorbance was measured at 340 nm. The enzyme activity was determined as micromoles of conjugated product of GSH and given as micromole/min.µl. The reaction mixture containing 50 µl of 0.2 M K_2PO_4 instead of enzyme was used as blank. The control was assay mixture without ethanol extract keeping the total reaction volume the same.The concentration of ethanol extract used in this assay is shown in Table 16 and the reaction mixture and the procedure for this assay is illustrated in Table 17.

Table 16. Components of assay mixture used in GST enzyme assay

Components	Volumes added (ml)
50 mM Reduced Glutathione	0.8
0.2 M K_2PO_4, pH 6.5	20.0
Deionized water	13.2

Table 17. The concentrations of Ethanol extract and added volume in GST assay

Ethanol extract Volume added µl	Ethanol extract Concentration mg/ml
5	0.0275
20	0.11
50	0.275
75	0.4125
100	0.55
200	1.1

CHAPTER 3

RESULTS

3.1 Extraction methods for Phellinus torulosus mushroom

In order to extract P. torulosus mushroom samples, four different extraction procedures were carried out. Methanol extraction was found to be the method with the highest yield and the highest concentration of mushroom extract as 14.09 mg/ml DMSO. The method with the lowest percent yield and the lowest concentration was cold water extraction as 0.176 mg/ml DMSO. (Table 19).

3.2 Determination of Total Phenolic Content (TPC) of Phellinus torulosus

The total phenolic contents of four different extracts of P. torulosus were measured. It was found that ethanol extraction resulted in the highest concentration of phenolic content and therefore the ethanol extract was used for further assays such as DPPH and antioxidant enzymes. The phenolic content was expressed as 625.125 µg of gallic acid equivalent/ml of extract (Table 20).

3.3 Determination of Total Flavonoid Content (TFC) of Phellinus torulosus

The total Flavonoid contents of four different extracts of P. torulosus were measured. It was found that the ethanol extraction resulted in the highest concentration of flavonoid content as well and was expressed as 463.5 µg of quercetin equivalent/ml of extract (Table 20). Again since the ethanol extract had the highest flavonoid content, this extract was used for all following assays.

Table 18. Percent Yield and Concentrations of Phellinus torulosus mushroom extracts prepared in DMSO

Mushroom Extracts	Yield %	Concentrations of extracts mg/ml
Cold water extract	0.15	0.176
Hot water extract	6.45	2.69
Methanol extract	7.75	14.09
Ethanol extract	2.2	5.5

Table 19. TPC and TFC of Phellinus torulosus mushroom extract

Mushroom Extracts	Total Phenolic Content µg GAE*/ ml	Total Flavonoid Content µg QE**/ml
Cold water extract	106.38	37.145
Hot water extract	298.66	102.19
Methanol extract	575.58	454.375
Ethanol extract	625.125	463.5

* The total phenolic content was expressed as µg of gallic acid equivalent per ml of extract.

**The total flavonoid content was expressed as µg of quercetin equivalent per ml of extract.

3.4 Determination of DPPH activity

In this assay, different concentrations of quercetin and gallic acid standards were used as positive controls and different concentrations of ethanol extract were used as given under methods in Table 9 and Table 10. The capacity of ethanol extract for stabilizing free radical DPPH is shown as dose-response curve (Figure 4.) and it was concluded that the DPPH activity of ethanol extract was inhibited over 70% and IC50 value was 0.04353 g/l.

IC50 values of quercetin and gallic acid standards were 0.031 g/l and 0.0096 g/l respectively.

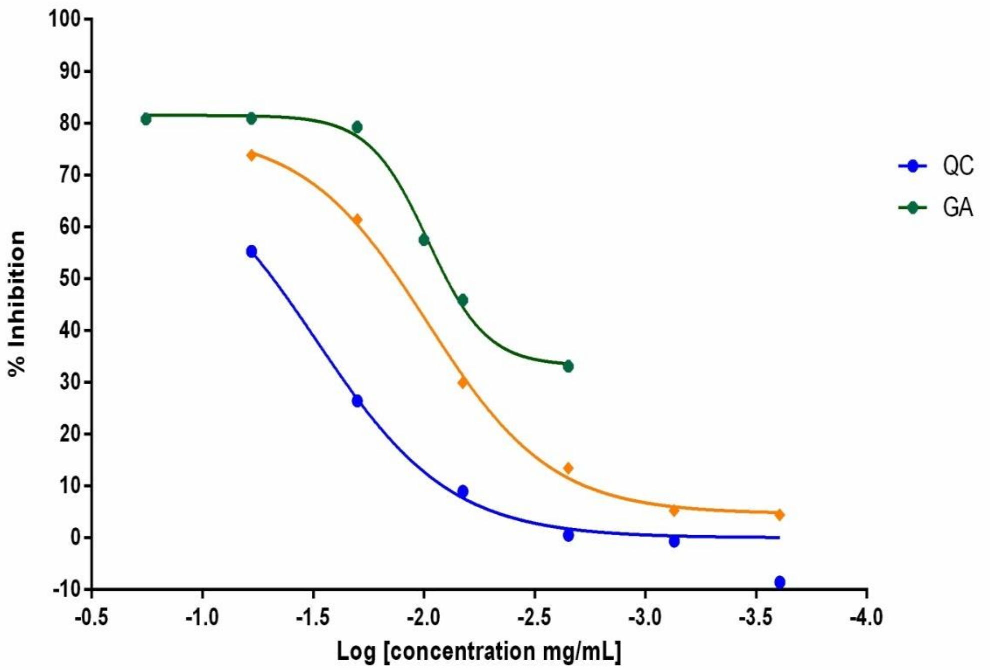

Figure 4. The effect of Phellinus torulosus extract on DPPH activity by using quercetin and gallic acid as standards

3.5 Determination of Catalase activity

In this assay, six different concentrations of ethanol extract were used as given in Table 12 under methods. It was found that ethanol extract of Phellinus torulosus inhibited catalase activity about 15% with respect to control and IC50 value was calculated as 3.641x 10^{-7}g/l (Figure 5).

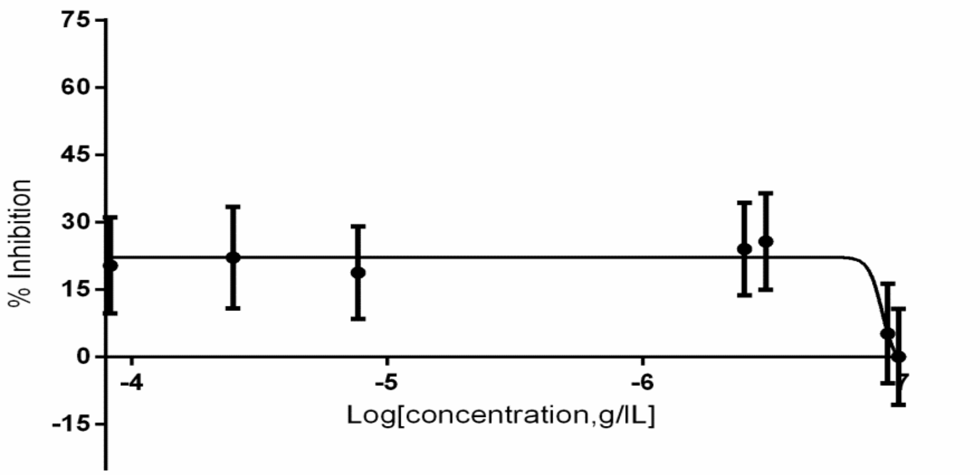

Figure 5. The effect of Phellinus torulosus extract on catalase enzyme with respect to control

3.6 Determination of SOD activity

The bovine liver cytosol with protein amount of 0.928 mg/ml was used as source of SOD. In this assay six different concentrations of ethanol extract were used as seen in Table 14 under methods.

The SOD activity with ethanol extract is shown in Figure 6. The inhibitory percent was found to be about 15 percent and IC50 value was 8.12 x 10^{-4} g/l.

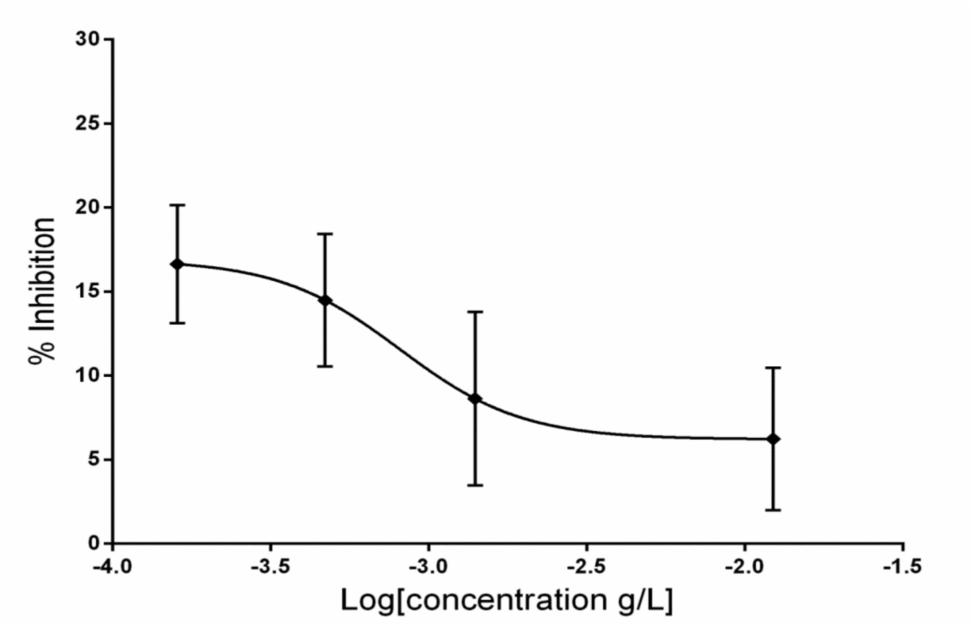

Figure 6. The effect of Phellinus torulosus extract on SOD enzyme with respect to control

3.7 Determination of GST activity

The bovine liver cytosol with protein amount of 0.928 mg/ml was used as source of GST enzyme. The concentrations of ethanol extract used in this assay were given in Table 17 under methods.

The GST activity with ethanol extract is shown in Figure 7. It was shown that the concentrations of ethanol extract used in this assay inhibited GST activity. The inhibitory percent with respect to control was found to be more than 60% and IC50 value was IC50=0.1609 to 0.9076 g/l.

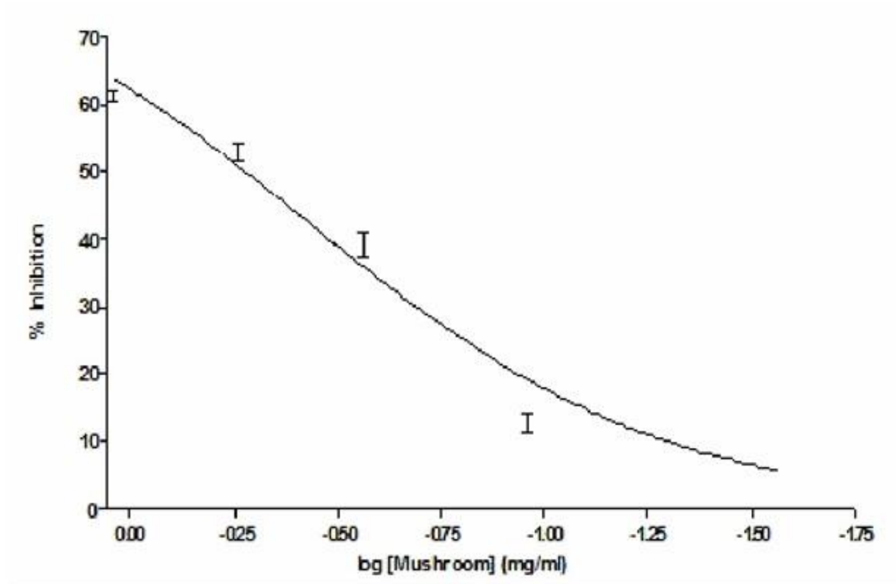

Figure 7. The effect of Phellinus torulosus extract on GST enzyme with respect to control

Discussion

It is a long time that edible mushrooms have been considered one of the most prominent functional foods. At least three review articles have exclusively focused on certain medicinal properties of mushrooms including antioxidants (Ferreira ICFR et al., 2009), anticancer (Patel S. and Goyal A. 2012) and antibacterial activities (Alves MJ et al., 2012).

In addition, a number of other review articles have shown pharmacological and medicinal properties of edible mushrooms, including antimicrobial, anticancer, antioxidant, antiviral, immunomodulatory, immunosuppressive, anti-allergic, anti-inflammatory and anticholestral activities (Rathee S et al., 2012).

Antioxidants donate an electron to stabilize a free radical. When a free radical is neutralized in this way, it is not able to cause damage. For this reason, today's health science is interested in antioxidants from natural sources as defense mechanisms against various diseases. Antioxidants protect our body against free radicals and mushrooms are rich sources of these antioxidants (Mau Jl, et al., 2004) (Puttaraju NG et al., 2006), (Oyetayo FL., 2007).

There are reports which have shown the antioxidant properties of cultivated edible mushroom species. One such study is a review done by Ferreira et al., 2009. In most of these studies antioxidant activity is measured through in vitro assays including DPPH inhibition, reducing power, ferric chelating and biochemical assays including lipid peroxidation inhibition. Additionally a number of compounds related to antioxidant activity have been identified, including polyphenols (phenolic acids, phenylpropanoids, lignin, melanins, tannins and flavonoids), ß-caroten and ascorbic acid (Barros L., et al., 2009).

In our study, among the four extraction methods used to extract Phellinus torulosus mushroom, the ethanol extract resulted in the highest amount of Phenolic and Flavonoid content. The phenolic content was expressed as 625.125 µg of gallic acid equivalent/ml of extract and flavonoid content was expressed as 463.5 µg of quercetin equivalent/ml of extract.

In one study (Hip Seng Yim et al., 2009) four species of edible wild mushrooms and one cultivated mushroom were used to investigate how extraction solvent (methanol, ethanol, acetone, water and hexane) affected the yield of phenolic substances which were comprised of total phenolic (TP), total flavonoids (TF) and condensed tannins (CT). Results showed that water extracts had the highest TP, TF and CT of all edible wild and cultivated mushrooms. Then keeping water as extraction solvent and taking TP as indicator, the extraction time and the temperature were varied. Content of TP with optimal extraction time and temperature were in descending order as follows: Pleurotus Sp. (1046.87 mg GAE/100g, 330 min at 40°C), Lentinus ciliates (801.08 mg GAE/100g, 150 min at 50°C), Pleurotus osteratus (cultivated) (798.55 mg GAE/100g, 330 min at 50°C), Hygrocybe Conica (442.37 mg GAE/100g, 240 min at 60°C), Schizophyllum commune (427.31 mg GAE/100g, 240 min at 25°C). Extraction time and temperature with water as extraction solvent were found to have a critical role in extracting total phenolic in edible wild mushrooms.

The TP and TF content of Phellinus torulosus mushroom obtained in our study may be too low comparing to above values, however this difference may be attributed to genetic factors of different species. Additionally, differences in particle size, type of phenolic complex mixtures, solvent concentration, extraction time and temperature, solvent to solid ratio, moisture content of mushrooms and pH contributed to the differences between the studies. Thus for a particular sample extract, all contributing factors in the extraction procedure should be optimized in order to get the highest yield of total phenolic and flavonoid content.

In various antioxidant assays using mushrooms, certain bioactive components have been detected, isolated and investigated. It has been observed that such bioactive compounds introduce hydroxyl groups and other hydrogen and electron donating groups to scavenge the free radical species (Imtiyaz A. S. 2014). DPPH assay is popular in natural product antioxidant studies. One of the reasons is that this method is simple and sensitive. The principle of this assay is that theoretically a hydrogen donor is an antioxidant. DPPH radical accepts hydrogen from an antioxidant to form stable DPPH compound. The antioxidant effect is proportional to the disappearance of DPPH radical in test samples which shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow when DPPH is formed by obtaining hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm. (Macdonald-Wicks, L. K. et al, 2006) (Moon , J.K., et al, 2009).

In the same study discussed above (Imtiyaz A. S. 2014), the superoxide radical scavenging abilities of two polypore mushrooms called Ganoderma lucidum and Trametes hirsuta were examined. In this study the superoxide was generated by autoxidation of hydroxylamine hydrochloride in presence of NBT (nitroblue tetrazolium salt) which gets reduced to nitrite and can be measured at 560 nm similar to the method of our study. Decreased in absorbance of the reaction mixture indicated the increase in superoxide anion scavenging activity and percentage of inhibition was calculated. The results showed that Ganoderma lucidum had higher scavenging activity (66.00 ± 0.98%) than Trametes hirsuta (57.14±0.54) at the level of 100 µg/ml in the reaction mixture. However they revealed significantly lower scavenging potential when compared with ascorbic acid.

In a recent study, the antioxidant activity and total phenolic content of extracts of ten species of Phellinus mushroom including Phellinus torulosus collected from northeast Thailand has been investigated (Seephonkai, P. et al., 2011). In this study the samples were tested for their radical scavenging activity by DPPH method and total phenolic content by Folin-Ciocalteu method. The results showed that some of the sample extracts had strong radical scavenging activity with the IC50 ranging from (7.30 ±0.34) to (19.80 ± 0.13) µg/ml. IC50 values were in the range of the standard antioxidant used such as quercetin, ascorbic acid and butylated hydroxytoluene (BHT). The strongest scavenging activity as comparable to quercetin was found to be 80% in crude ethanol extract of P. torulosus having IC50 value of

7.30±0.34 µg/ml. It was also shown that P. pint species contained the highest total phenolic content with a value of 87.76 ±1.00 equivalent gallic acid expressed as mg GA/ 100 mg of extract and the total phenolic content of P. torulosus was 78.34 ± 0.27 mg GA/ 100 mg of extract which was higher than the other phellinus species tested.

In general the results indicated that crude extracts from Phellinus mushrooms have a potential to be natural antioxidant source. To our knowledge there has not been any studies done for evaluation related to the effect of P. torulosus on Catalase, SOD or GST enzyme activity in literature in order to compare with our study. However in a study it was shown that the highest antimicrobial activity of this mushroom was measured in ethanol extract of the mushroom. Based on the result of this study, Phellinus torulosus had antimicrobial activity against some gram (+) and gram (-) bacteria, yeast, filamentous fungi and actinomycetes (B. Dulger et al., 2005).

As a more comparative study similar to our study, the antioxidant defense systems namely Glutathion-S-transferase, Glutathione peroxidase and catalase of some plants from Asteraceae were evaluated. The results showed that the acetone extract of C. solstitalis flowers can inhibit GPx and GST enzymes with IC50 value of 79 ng/ml and 232 ng/ml respectively and that the methanolic extract of C. avense leaves has high free radical scavenging potential and antioxidant activity with IC50 value of 366 ng/ml (Suheda Koc, et al. 2015).Over the past decade, advances have been made in research on antioxidant properties of Phellinus torulosus so called Iranian wild mushrooms. In vitro antioxidant activity of this mushroom has been reported. The total methanol extract of P. torulosus showed a significant antioxidant activity, but the butanol fraction has a stronger activity than the total methanolic extract (Hokmollahi F., et al, 2012).

CONCLUSION

In this study, the antioxidant property of Phellinus torulosus was examined. The results showed that the ability of this mushroom to donate hydrogen to DPPH radical is high with IC50 of 0.04353 g/l and therefore it can be considered as a good antioxidant for removal of free radical and prevent their possible damage. Additionally, upon the examination of the mushroom extract on Catalase, SOD and GST, it was found that ethanol extract of Phellinus torulosus inhibit GST more than 60% with IC50 value of 0.1609 to 0.9076 g/l which means that the compounds in this mushroom can lower the activities of GST enzyme. This may be useful in chemotherapies or other cases where the activities of these enzymes are much higher than normal. However Phellinus torulosus could not inhibit SOD and CAT significantly with inhibition of about 15% for both enzymes. The calculated with IC50 value for SOD was 8.12×10^{-4} g/l and IC50 value for CAT was calculated 3.641×10^{-7} g/l which means that these enzymes are not affected.

As a final point, it can be a good idea to compare various mushroom species to determine the ones with the highest phenolic and flavonoid contents and best free radical scavenging abilities and antioxidant properties. The selected species can then be cultivated and can be used in vitro for further discoveries and can even be tested on cancer patients who do not heal with chemotherapy. In future this by itself can be considered a breakthrough in treatment of cancer or other free radical related diseases.

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