THE EFFECT OF POLYPORUS SQUAMOSUS 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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ABSTRACT

THE EFFECT OF POLYPORUS SQUAMOSUS MUSHROOM EXTRACT ON ANTIOXIDANT ENZYMES

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Recently, an increasing interest in searching natural antioxidant sources has led to extensive studies on mushrooms. In this study, Polyporus squamosus was examined for determination of its total phenolic and flavonoid contents. The free radical scavenging property was then evaluated by DPPH assay and finally the effect of Polyporus squamousus was tested on 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. It was observed that cold water extract resulted in the highest contents of total phenolic and flavonoid (as 223.60 µg GAE /ml and 61.25 µg QE /ml respectively), Therefore, the cold water extract was used for further experiments.

Based on the results obtained, it was found that DPPH radical scavenging capacity of mushroom extract is relatively low with 35% and IC50 value was 20.55 g/l with respect to quercetin and gallic acid as standards. In order to evaluate the antioxidant property of Polyporus squamosus extract, its effect on catalase, superoxide dismutase and glutathione-S-transferase was observed. It was found that the cold water extract of Poluporus squamosus had about 60% activation on catalase with respect to control and IC50 value of 2.810 g/l and surprisingly it had an activation effect as high as 95% and IC50 value was 11.94 g/l on SOD but nearly no effect on inhibition of GST

with the inhibitory percent with respect to control found to be just around 7.2% and IC50 value was 1.089 g/l.

Keywords: Polyporus squamosus mushroom, free radical scavenger, antioxidants, DPPH assay, Catalase, Superoxide Dismutase, Glutathione-S-transferase

ÖZ

POLYPORUS SQUAMOSUS MANTAR ÖZÜTÜNÜN ANTİOKSIDAN ENZİMLER ÜZERİNDE ETKİSİ

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Doğal antioksidan kaynakları bulmak için son zamanlarda artarak oluşan bir ilgi mantarlar üzerinde geniş kapsamda çalışmaların yapılmasına yol açmıştır. Bu çalışmada Polyporus squamosus mantar özütünün toplam fenol ve flavonoid içerikleri incelenmiştir. Sonra radikal süpürücü etkisi DPPH serbest radikali kullanarak test edilmiştir ve son olarak Polyporus squamosus mantarın etkisi Katalaz (KAT), süperoksit dismutaz (SOD) ve Glutathione-S-transferaz (GST) enzimleri üzerinde test edilmiştir.

Bu çalışma da, soğuk su, sıcak su, metanol ve etanol kullanarak dört farklı mantar özütü hazırlanmıştır. Soğuk su özütünün en yüksek toplam fenol ve flavonoid içeriğine sahip olduğu saptanmıştır (sırasıyla 223,60 µg GAE/ml ve 61,25 µg KE/ml). Bu nedenle bir sonraki deneyler için soğuk su özütü kullanılmıştır.

Elde edilen sonuçlara göre, mantar özütünün radikal süpürücü etkisi gallik asit ve quersetin standartları kullanarak %35 ve IC50 değeri 20,55 g/l olarak nispeten düşüktür. Polyporus squamosus özütün antioksidan özellikleri değerlendirmek için, KAT, SOD, GST enzimleri üzerindeki etkinliği incelenmiştir.

Sonuçlar göstermiştir ki kontrole göre Polyporus squamosus mantarının soğuk su özütü KAT üzerinde % 60 aktivasyon göstermiş ve IC50 değeri ise 2,810 g/l olarak hesaplanmıştır. SOD enzimi üzerinde % 95 oranda aktivasyon gösteren mantarın IC50 değeri de 11,94 g/l olarak hesaplanmıştır. Bu çalışmada Polyporus squamosus

mantarının GST üzerine hemen hemen hiç etkisi olamadığı ve kontrole göre ancak % 7.2 ve IC50 değerinde 1.089 g/l olarak bir inhibasyon yaptığı bulunmuştur.

Anahtar Kelimeler: Polyporus squamosus 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

It is a well-known fact that oxygen is an element vital for life (Mohammed AA, 2004), however it can have harmful effects in some cases (Bagchi, 1998). The main cause of such harmful effects of oxygen is the generation and reactivity of certain compounds called Reactive Oxygen Species abbreviated as ROS. Not until Gershman published the free radical theory in 1954, oxygen toxicity could be clearly described. This theory explains that the reduced forms of oxygen can partially be the main cause of toxicity of oxygen (Gerschman, R, et al., 1954). Such Oxygen free radicals are generated during normal metabolism and as they are not stable and tend to be very reactive, they can act as oxidants or reductants giving or accepting electron from other molecules (Cheeseman, K. H., 1993).

In order to prevent free radicals to cause damages, organisms have developed powerful defense mechanisms with enzymatic and non-enzymatic antioxidants (Cadenas, E, 1997). There are also external antioxidants which are included in foods, vitamins and herbs. These so called natural antioxidants exist in all parts of plants. There are also many documents that mushrooms are also good sources of strong antioxidants which will be discussed in details in this thesis again.

In this thesis, the antioxidant properties of a mushroom called Polyporus squamousus will be evaluated.

1.1 Reactive oxygen species

Reactive oxygen species abbreviated as ROS is a general term used for a group of oxidants that are either free radicals themselves or are able to generate free radicals. A free radical is a molecule with one or more unpaired electron in its outer shell (Bahorun T. et al., 2006). The oxygen radicals are the most important class of radicals produced in living systems (Valko M.,et al, 2006 ; Miller DM, et al., 1990) There are different kinds of free radicals and some are called reactive nitrogen species (RNS). Among different free radicals we can name hydroxyl ($OH^{\cdot}$), Superoxide ($O_2^{\cdot -}$), nitric oxide ($NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxyl ($ROO^{\cdot}$) and lipid peroxyl ($LOO^{\cdot}$). There are also some other oxidants which can generate free radicals. We can name hydrogen peroxide (H_2O_2), Ozone (O_3), singlet Oxygen, hypochlorous acid, nitrous acid (HNO_2), peroxynitrite, dinitrogen trioxide and lipid peroxide as examples of such molecules. Radical species are generally more reactive than non-radical species and this makes the radicals to be less stable (Pham-huy LA, et al., 2008). Free radicals are formed by hemolytic cleavage of a chemical bond and redox reactions and when they are formed they can start chain reactions (Bahron T, et al., 2006).

1.2 Generation of ROS

As one source we can discuss about Fenton's & Haber's reactions described by Fridovick, Holliwell and Gutteridge (Fridovich I, 1984 ; Halliwell B. Gutteridge JMC, 1989) These reactions explain the production of superoxide radical ($O2^{\cdot -}$) resulting from the reduction of molecular oxygen. Such reaction occurs during electron transport chain in mitochondria.

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

The superoxide can undergo a dismutation reaction to form hydrogen peroxide.

$$O_2^{\cdot -} + O_2^{\cdot -} \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is neutralized by catalase or glutathione peroxidase or can undergo Fenton's reaction reacting with iron or copper ion to form a more reactive radical called hydroxyl radical:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + HO^{-}$$

ROS can also be produced in endoplasmic reticulum by cytochrome P450 reductase which forms superoxide anions in order to detoxify toxic chemicals where NADPH and NADH provide electrons for reduction of cytochrome b5 and cytochrome P450.

Such detoxification reactions as well as the electron transport chain in mitochondria are considered endogenous sources of ROS generation. There are also exogenous sources of ROS production such as cigarette smoking, industrial wastes, radiation, viral and bacterial infection. Among the pathological sources we can name metabolism of pollutants and drugs, immune cell activation, inflammation, ischemia, infection and cancer (Noori S., 2012).

1.3 Beneficial effects of ROS

ROS and RNS are produced normally during the metabolism. As a matter of fact at low or moderate amount, these free radicals can have beneficial effects. These include physiological roles in cellular responses to anoxia or defending against infection as well as the induction of mutagenic response (Valko et. al., 2006). Another advantageous aspect is to maintain "redox homeostasis" thereby preventing oxidative stress. As defense mechanism, the white blood cells like neutrophils, macrophages and monocytes invade pathogenic microbes preventing various diseases. (Droge, w, 2002).

Other beneficial effects of ROS and RNS is related to the regulation of cellular signaling systems For instance, nitric oxide (NO) is an intercellular messenger for modulating blood flow, thrombosis, and neural activity. NO has also an important defense role for killing intracellular pathogens and tumors. Additionally, free radicals can enhance mitogenic responses (Pacher P, et al., 2007) (Genestra M., 2007) (Halliwell B., 2007). In other words, low or moderate levels of ROS/RNS are vital to human health.

1.4 Harmful effects of ROS

The harmful effect of free radical, ROS/RNS in particular is the biological damage they cause which is generally called oxidative stress. In biological system when the production of free radicals exceed the power of defense mechanisms it leads to accumulation of these reactive radicals which can disturb the balance between pro-oxidant /antioxidant reactions (Kovacic, P., 2001).

There are evidences showing that at the high concentrations, ROS can harm various cellular molecules such as nucleic acids, lipids and proteins (Valko M. et al, 2006). The hydroxyl radical can react with DNA molecule and damage purine and pyrimidine bases as well as deoxyribose backbone (Halliwell B., & Gutteridge, J. M. C, 1999). An example of permanent modification of DNA we can name the formation of 8-OH-G which is the most studied DNA lesion. DNA damages in general are considered major causes of mutagenesis, carcinogenesis and ageing.

Other compounds inside the cell such as phospholipids found in fatty acids which can easily be oxidized can also be damaged by metal induced generated ROS (Siems, W. G., et al, 1995). The peroxy radicals (ROO.) formed in this way can be cyclized forming endoperoxide, the precursors of malondialdehyde (MDA) which is the final and the most toxic product of lipid peroxidation (Fedtke, N., et al, 1990, Fink, S.P et al., 1997, Mao, H., et al., 1999).

Oxidation of proteins has also been studied. In these studies the amino acid, peptides or proteins were treated with radiations causing the production of hydroxyl and superoxide radicals (Standtman, E. R., 2004). Cysteine, methionine, arginine and histidine are shown to be the most sensitive amino acids which can easily be oxidized by reactive oxygen species. The oxidation of proteins can alter the mechanism of signal transduction, enzyme activity, heat stability and proteolysis susceptibility leading to ageing process (Freeman, B. A., Carpo, J. D. , 2004).

1.5 The diseases caused by oxidative stress

There are evidences that many human diseases are caused by oxygen derived free radical reactions (Pham-Huy LA, 2008), (Valko M. et al., 2007).

Neurodegenerative disorder like alzheimer's disease, Parkinson's disease, multiple sclerosis, memory loss and depression. Diseases associated with premature infants as well as cardiovascular diseases like atheroscelerosis, ischemic heart disease and hypertension. Some other diseases related to pulmonary, renal, liver, eye and gastrointestinal disorders. Auto immune diseases like rheumatoid arthritis. Diabetes, AIDS, ageing process, tumors and cancers. Figure 1 shows the diseases in humans caused by oxidative stress. (Lien Ai Pham-Huy et al., 2008)

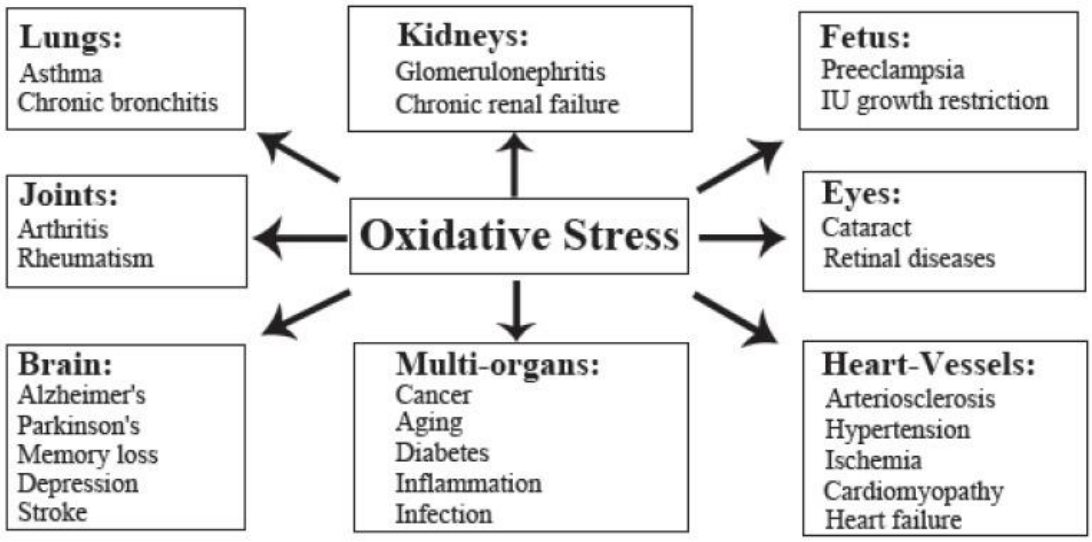

Figure 1. Diseases in humans caused by oxidative stress

1.6 Antioxidants and the mechanism of their action

An antioxidant is a stable molecule which can neutralize free radicals by donating an electron to them and thereby reducing their potential to cause damage. Antioxidants can undergo certain reactions with free radicals and stop their production before they can give any harm to cellular components (Halliwell B., 1995).

Antioxidants can act as radical scavenger, hydrogen or electron donor, enzyme inhibitor or metal chelator. The endogenous antioxidants are classified as enzymatic or non-enzymatic and are found in the intracellular and extracellular environment (Frie B.,1988). There are two main mechanism of action suggested for antioxidants. The first is to break the chain production of free radicals by giving them an electron and the second is to remove ROS/RNS initiators by quenching the related catalysts (Rice-Evans CA, 1993). Also, in some cases, an antioxidant can play as a pro-oxidant by generating toxic ROS/RNS (Young et al., 2001).

Some antioxidants are formed in the body during normal metabolism and some other are found in diet (Shi HL., et al., 1999). The antioxidants which are formed naturally in the body are called endogenous antioxidants and the ones which are or externally supplied through foods are called exogenous antioxidants.

1.6. 1 Endogenous antioxidants

Endogenous compounds in cells can be grouped in to two classes as enzymatic antioxidants and non-enzymatic antioxidants.

The most well-known antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRx) (Willcox JK, et. Al., 2004), (Valko M, et. Al., 2005). As the first enzyme of defense system SOD converts the superoxide radical($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by reduction. Hydrogen peroxide is then converted to water and oxygen by catalase (CAT) or glutathione peroxidase (GPx). GPx also reduces lipid or non-lipid hydroperoxides by oxidizing glutathione (GSH) (Bahorun T, et al., 2006), (Droge W. 2002), (Young et al., 2001).

In fact a system called glutathione system includes glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferases. This system plays role as antioxidants and also serve as general detoxification metabolism (Hayes, J. 2005).

1.6.1.1 Glutathione S-transferase

Glutathione S- transferase (GST) isoenzymes are involved in many activities and participate in many different kinds of reactions. The first GST family known was soluble or cystolic enzyme (Mannervik, 1985). All soluble GSTs are divided into eight classes depending on their sequences called alpha (α) kappa (κ), mu (μ), pi (π), omega(ω), sigma(σ), theta(Θ) and zeta (ξ) (Board et al., 1997). The second GST family is microsomal transferases which are membrane associated proteins (Jakobsson et al., 2000).

In general, GST enzymes play role in detoxification of damaging chemicals such as environmental pollutants, carcinogens and medicines used in chemotherapy. For this reason in chemotherapy, GST activity is enhanced and such overexpression in tumors leads to the resistance to therapeutics and this causes problem for patient rather than benefit (Morrow et al., 1998). GST enzymes have a crucial role in phase II durg-metabolism converting compounds formed in phase 1 drug metabolism to more water soluble and less toxic compounds which can be easily excreted from the body (Chasseaud, 1979 and Mannervik, 1985).

1.6.1.2 Superoxide dismutase

Superoxide dismutase (SOD) enzymes are found in all aerobic organisms and their function is to breakdown superoxide anion into oxygen and hydrogen peroxide (Zelko I., et al., 2002 and Banniste et al., 1987). Depending on the metal cofactor SOD enzymes fall into three main families: Cu/Zn types bind to both copper and zinc, Fe and Mn types bind to either iron or manganese and finally Ni type which binds to nickel (Wuerges J., et al., 2004).

In higher plants, SOD isozymes can be found in different areas inside the cell. 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, there are three forms of superoxide dismutase. SOD1 (as a dimer containing two units) is found in the cytoplasm, SOD2 is found in the mitochondria, and SOD3 is extracellular. Both SOD2 and SOD3 are tetramers with four subunits. In SOD1 and SOD3 copper and zinc are found and in SOD2 manganese is found in its reactive center (Cao X, et al., 2008).

1.6.1.3 Catalase

Catalase (CAT) is an enzyme found in all aerobic organisms. The function of this enzyme is to facilitate the breakdown of hydrogen peroxide to water and oxygen (Chelikani P et al., 2004). Hydrogen peroxide is considered a toxic product normally produced in the body during metabolism. In order to prevent it to cause damage, it should be decomposed into less dangerous substances such as oxygen and water as soon as possible (Gaetani G et.al., 1996). Catalase is generally used in all parts but it occurs in higher concentration mostly in liver (Eisner T et al., 1999).

1.6. 2 Non- enzymatic antioxidants

The non-enzymatic antioxidants can be classified into metabolic antioxidants and nutrient antioxidants. The metabolic antioxidants are classified as endogenous antioxidants as they are formed inside the body during metabolism. Examples of such antioxidants are lipoid acid, glutathione, L-ariginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc (Droge W. 2002), (Willcox JK et al., 2004) The nutrient antioxidants are classified as exogenous antioxidants as they can not be formed in the body and should be obtained as food or supplements in diet. Examples of such antioxidants we can name vitamin E, vitamin C, carotenoids, trace metals, flavonoids, omega-3 and omega-6 fatty acids.

1.7 Antioxidant supplements

Antioxidant supplements can be obtained either by extracting natural foods or by chemical synthesis. Even though there has been an increasing interest in such supplements, as they are not similar to natural antioxidants in structure, their use may be limited by some important factors such as lack of prospective studies, side effects of long term use and the required dosage. Also in case the antioxidant supplements

are taken at levels much higher than the recommended dietary intakes (RDI), they can act as pro-oxidants. Considering all above, it is preferred to use the antioxidants from a diet rich in fruits and vegetables and not from supplements. There are evidences that certain synthetic antioxidant compounds are even toxic or mutagenic and because of this there has been an increasing interest in natural antioxidants (Nagulendran K., et al., 2007).

1.8 Antioxidants from natural sources

Plants have developed a powerful defense system against oxidative stress. The complexes found in plants can act as radical scavenger and covert the radicals to less reactive compounds. It has been shown that certain class of compounds known as phytochemical compounds such as phenols, flavonoids and carotenoids are able to scavenge free radicals such as $O_2^{\cdot -}$, OH^- or lipid peroxy radical LOO in plasma (Manach, C., et al., 1998).

Natural oxidants present in foods have attracted high interest due to their safety as well as their nutritional and therapeutic effects. The interest in search for natural replacements for synthetic antioxidants has led to evaluating the antioxidant properties in plant sources. Several studies were done to show the antioxidant properties of various vegetables such as potato, spinach and legumes (Furuta S., et al., 1997). Strong antioxidant activities have been found also in fruits like berries, cherries, citrus, prunes and olives (Wang H., et al., 1996). Green and black teas are shown to have antioxidant potential as they contain phenolic compounds up to 30% of their dry weight (Lin JK, et al., 1998).

There have been studies about antioxidant features of wild mushrooms and it was shown that certain phenolic compounds, tocopherols, ascorbic acid and caretoinds extracted from mushrooms showed to be strong antioxidants.

1.9 Medicinal mushrooms

According to Chang and Miles (Chang ST, Miles PG. 1992) mushroom is a micro fungus with a fruit body able to grow either on the earth or underground. This group of higher microfungi belongs to to basidiomycetes and ascomycetes that can be edible or non-edible. Mushrooms have nutritional value composing of 90% water and 10% dry matter (Sanchez C. 2010 and Oei P, 2003). Mushrooms contain various vitamins such as thiamine, riboflavin, ascorbic acid, ergosterol and niacin and essential amino acids. They also have proteins, fats, ash, and glycosides, volatile oils, tocopherols, phenolic compounds, flavonoids, carotenoids, folates and organic acids (Sanchez C. 2004 and Patel S, 2012).

Mushroom extracts contain certain compounds each unique to specific mushroom. The phenolic compounds found in mushrooms include phenolic acids, flavonoids, hydroxybenzoic acids, hydroxycinnamic acids, lignans, tannins, stilbenes and oxidized polyphenols. These are aromatic hydroxylated compounds with one or more aromatic rings and one or more hydroxyl groups giving them their antioxidant properties. Like other organisms mushrooms have developed such defense system to protect themselves against oxidative stress. There are reports showing that such phenolic compounds show antioxidant activities acting as free radical inhibitors, peroxide decomposers, metal inactivators or oxygen scavengers (Dziezak JD, 1986 and Yagi K., 1970). Besides, they can induce the synthesis of endogenous antioxidant molecules in the cell (Cote J, et al., 2010 and D'Archivio M. et al., 2010).

According to what has been discussed above, mushrooms may prove to be useful candidates for search for bioactive compounds particularly that such compounds can be formed in their fruiting part in relatively short time and also through culturing optimum quantities of such active products can be produced. The mushrooms themselves can be added to diet or the antioxidant compounds they contain may be extracted and used as functional ingredients in order to reduce oxidative damages.

1.10 Polyporus squamosus

This is a fan shaped mushroom with central stalk, in white-yellow color with brown scales and white pore surface. It gives fruits in spring-fall and exists on hardwood trees. It is a basidiomycete bracket fungus, known as dryad's saddle and pheasant's back mushroom. It is edible being found in North America, Australia, Asia, and Europe creating a white rot in living and dead hardwood trees. (Spahr DL. (2009). Edible and Medicinal Mushrooms of New England and Eastern Canada , Richmond, Calif: North Atlantic Books. pp. 131–35. ISBN 1-55643-795-1. Retrieved 2010-05-28). A photograph of this mushroom is shown in Figure 2. Polyporus squamosus smells like watermelon rind and has a mild nutty flavor.

Figure 2. Polyporus squamosus

Classification of Poluporus squamosus is as follows (Kirk P.M. 2017, Species Fungorum (version Jan 2016).:

Kingdom: Fungi
Phylum: Basidiomycota
Class: Agaricomycetes
Order: Polyporales
Family: Polyporaceae
Genus: Polyporus

1.11 Scope of this study

The aim of this study is to determine the total phenolic content and total flavonoid content of Polyporus squamosus and to evaluate free radical scavenging potential as well as determining the effect of Polyporus squamosus extract on the activities of GST, CAT and SOD.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Polyporus squamous 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 Reddish Peroxidase (HRP), Sodium Azide (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),SOD(Sigma).

2.2 Methods

2.2.1 Preparation of Polyporus squamousus mushroom extract

The extract was prepared by using cold water, hot water, methanol and ethanol. Polyporus squamousus was obtained in dried form from Assoc. Prof. Dr. İlgaz Akata, Department of Botany, Ankara University, Ankara, Turkey.

Prior to the extraction procedure, the dried mushroom was ground to make power using liquid nitrogen. Then four extraction methods using cold water, hot water, methanol and ethanol was used. The detail of each of extraction procedure is given in Table 3. (Table 1). 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.

The concentration of dried extract in each method was determined as µg of dried extract per ml of 99.9% DMSO (Table 2). Then the dissolved extracts were kept in -20°C to use for further analysis.

Based on the results obtained with the cold water extraction with the highest % yield and concentration, the cold water extract was preferred to continue with determination of phenolic and flavonoid assays as well as the following enzyme activity assays. The new cold water extract was prepared and shown to be 0.0144 g of dried extract/ml of DMSO which was used for further analysis.

Table 1. 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	Three times	Under pressure	Evaporation to dryness using 80 rpm rotary evaporator at 60 °C
Hot water	2 g of dried powder was boiled with 25 ml of distilled water for one hour	3000 rpm for 45 minutes	Under pressure	Three times	Under pressure	Evaporation to dryness using 80 rpm rotary evaporator at 60 °C
Methanol	1 g of dried powder was dissolved in 2 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 2.5 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

Table 2. Concentration of extracts prepared

Extraction method	Dry weight of mushroom (g)	Dry weight of extract (g)	Concentration of mushroom extract in DMSO (g/L)
Cold water	2	0.590	29.5
Hot water	2	0.317	12.67
Methanol	1	0.0826	41.3
Ethanol	1	0.029	11.6

2.2.2 Determination of total phenolic content (TPC) of polyporus squamousus

The Phenolic content of Polyporus squamousus was determined using Folin-Ciocalteu's assay (Slinkard and Singleton, 1977). This assay is based on measuring blue colored complex of the reduced Folin reagent. First four different concentrations of Gallic Acid (from 50 to 400µg/ml) were prepared and dissolved in DMSO. After measuring the phenolic content, the calibration curve was drawn and used as standard. The calculated concentrations of the cold water extract and Gallic Acid are given in Table 3. The reaction components of the phenolic content determination assay are given in Table 4. 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 added and incubated for one hour in dark at room temperature. Then the absorbance was measured at 750 nm. The blank contains DMSO instead of mushroom extract. The total phenolic content of cold water extract was measured by using gallic acid standard curve and was expressed as µg of gallic acid equivalent (GAE) per ml of extract volume.

Table 3. The concentrations of Polyporus squamousus cold water extract used for total phenolic content determination using gallic acid as standard

Dilution	Cold water extract µg/ml	Gallic Acid Standard µg/ml
1	14400	400
2	7200	200
3	3600	100
4	1800	50

Table 4. The reaction components of phenolic content determination assay.

Stock	Added Volume
Cold water extract	100 µl from each concentration given in table 2.
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 polyporus squamousus

The total flavonoid content of the extracts was determined by using the aluminum chloride colorimetric method (Chang et al., 2002). First four different concentrations of quercetin (from 25 to 200µg/ml) were prepared. The flavonoid content of each concentration was measured and the calibration curve was drawn and used as standard. The calculated concentrations of the cold water extract and quercetin are given in Table 5.

The reaction mixture is given in Table 6. 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. At this wavelength, the absorbance shows the amount of stable complexes of aluminum chloride ($AlCl_3$) and

flavonoid calorimetrically. Thus the absorbance 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 cold water extract was the highest comparing to the extracts of other methods, all following experiments were done using ethanol extract. The enzymatic assays were carried out by using multi-mode plate reader (Figure 3).

Table 5. The concentrations of Polyporus squamousus cold water extract used for total flavonoid content determination using quercetin as standard.

Dilution	**Cold water extract µg/ml**	**Quercetin Standard µg/ml**
1	14400	200
2	7200	100
3	3600	50
4	1800	25

Table 6. The reaction components of flavonoid content determination assay

Stock	Added Volume
Cold water extract	250 µl from each concentration given in table 5.
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 cold water extract was determined using 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay (Sharma, Bahat, 2009). Table 7 shows various concentrations of Gallic acid and Quercetin which were used as positive control and Table 8 gives the different concentrations of cold water extract used in this assay.

Table 7. Concentrations of Gallic acid and Quercetin used in DPPH assay

Dilutions	Concentrations (µg/ml)			
	Stock Quercetin	Final Quercetin	Stock Gallic Acid	Final 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 8. Concentrations of cold water extract used in DPPH assay

Dilutions	Stock Cold water extract concentration mg/ml	Final Cold water extract concentration mg/ml
1	4.8	0.144
2	1.6	0.048
3	0.53	0.0159
4	0.18	0.0054
5	0.06	0.0018
6	0.02	0.0006
7	0.0066	0.000198

The reaction mixture is given in table 9. In this assay, based on the procedure 6 µl of cold water 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. At this wave length, in the presence of antioxidant, the stable DPPH radical having an odd

electron is measured which is represented by change of color from purple to yellow. In other words, the reduction capability of DPPH radicals was determined by decrease in its absorbance at 517nm induced by antioxidants. The reaction mixture without cold water extract was used as blank keeping the total reaction volume the same. The percent inhibitions were calculated for cold water extract and standard concentrations and were plotted against concentrations. The DPPH radical scavenging activity of cold water extract was expressed as IC50 value calculated from dose-response curve.

Table 9. The reaction mixture and procedure in DPPH assay

Stocks	Volumes
Standards/ cold water 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

In this assay when the activity of catalase is inhibited by the addition of sodium azide, the remaining hydrogen peroxide is measured calorimetrically which is represented by the formation of red quinoneimine dye. (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 cold water 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 calibration curve for hydrogen peroxide concentration ranging from 0.00192 mM to 0.12308 mM was plotted and the catalase enzyme activity was determined by measuring remaining H_2O_2 calculated based on the calibration curve. The concentration of cold water extract used in this assay is given in Table 10 and the reaction mixture and the related procedure is illustrated in Table 11.

Table 10. Concentrations of cold water extract used in catalase assay

Dilutions	**Stock cold water extract concentration mg/ml**	**Final cold water extract concentration mg/ml**
1	4.8	0.128
2	1.6	0.043
3	0.53	0.0141
4	0.18	0.0048
5	0.06	0.0016
6	0.02	0.00053

Table 11. 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	
Cold water 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

For this assay method of Işgor, B. S., 2013 was used which is based on the inhibition of nitroblue tetrazolium (NBT). In this method NBT is reduced by superoxide to form blue colored Formazan which is measured at 560 nm. The concentration of cold water extract used in this assay is shown in Table 12 and the procedure for this assay is illustrated in Table 13.

The reaction mixture contained total of 235 µl of assay buffer as described in table 13. To this assay buffer, 5 µl of SOD enzyme 3.64 U/ml , 5 µl of cold water extract and 5 µl of xanthine oxidase were added. Then this mixture was incubated for 30 minutes in dark and then the absorbance was read at 560 nm.

Table 12. Concentrations of Cold water extract used in SOD assay

Dilutions	Stock Cold water extract concentration mg/ml	Final Cold water extract concentration mg/ml
1	4.8	0.096
2	1.6	0.032
3	0.53	0.0106
4	0.18	0.0036
5	0.06	0.0012
6	0.02	0.0004

Table 13. The reaction mixture used in SOD Assay

Reagents	Volumes added (µl)	Contents
Assay Buffer	235	10 µl of 25mM NBT, 75 µl of 0.3 mM Xanthine, 150 µl of 200 mM Sodium Carbonate Buffer with 10 mM EDTA
SOD	5	3.64 u/ml
Cold water 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

For this assay, method of Habig et al., (Habig et al, 1974) was used with small modifications. The principle of this assay is based on formation of the conjugate product of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. In this assay, the cytosol extracted from bovine liver in our laboratory was used as the source of GST enzyme. 10 µl of this cytosol was added to the assay mixture containing 50 mM CDNB substrate, 200 mM GSH and 100 mM Phosphate buffer and 10 µl cold water extract of different concentrations with total assay mixture of 250 µl and the absorbance was measured at 340 nm using ultraviolet spectrophotometer. The control was assay mixture without cold water extract keeping the total reaction volume the same. The concentration of cold water extract used in this assay is shown in Table 12 and the reaction mixture and the procedure for this assay is illustrated in Table 13.

Table 14. Concentrations of Cold water extract used in GST assay

Dilutions	Stock Cold water extract concentration mg/ml	Final Cold water extract concentration mg/ml
1	4.8	0.192
2	1.6	0.064
3	0.53	0.0212
4	0.18	0.0072
5	0.06	0.0024
6	0.02	0.0008

Table 15. The reagent mixture used in GST assay

Reagents	Volume added (µl)	Contents
Assay buffer	200	184 µl Phosphate buffer 100 mM pH 6.5, 4 µl GSH 200 mM, 12 µl of CDNB 50 mM
cytosol	10	Source of GST
Cold water extract	10	Extract serial dilution
Phosphate buffer	30	200 mm pH 6.5
Read the absorbance at 340 nm		

CHAPTER 3

RESULTS

3.1 Extraction methods for Polyporus squamosus mushroom

In order to extract Polyporus squamosus mushroom samples, four different extraction procedures were used. Among these, cold water extraction was found to be the method with the highest yield and the highest concentration of mushroom extract as 29.5 mg dry extract/ml DMSO. The method with the lowest percent yield and the lowest concentration was ethanol as 11.6 mg dry extract /ml DMSO. (Table 16).

Based on above results, the cold water extract was preferred method of extraction. However a new cold water extract was prepared and shown to be 14.4 mg of dried extract/ml of DMSO**.** This new extract was used for the following phenol and flavonoid determination, DPPH assay as well as all the other enzyme assays.

3.2 Determination of total phenolic content (TPC) of polyporus squamousus

The total phenolic content of cold water extract of Polyporus squamosus was measured. The phenolic content was expressed as 223.60 µg of GAE/ml of extract (Table 15).

3.3 Determination of total flavonoid content (TFC) of Polyporus squamosus

The total Flavonoid content of cold water extract of Polyporus squamousus was measured. The flavonoid content was expressed as 61.25 µg of QE/ml of extract (Table 15).

Table 16. Percent Yield and Concentrations of Polyporus squamousus mushroom extracts prepared in DMSO

Mushroom Extracts	Yield %	Concentrations of extracts mg/ml
Cold water extract	29.5	29.5
Hot water extract	15.85	12.68
Methanol extract	8.26	41.3
Ethanol extract	2.9	11.6

Table 17. TPC and TFC of Polyporus squamousus mushroom extract

Mushroom Extracts	Total Phenolic Content µg GAE*/ ml	Total Flavonoid Content µg QE**/ml
Cold water extract	223.60	61.25

* 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, quercetin and gallic acid was used as positive controls. Different concentrations of these standards as well as cold water extract were used as given under methods in Table 7 and Table 8. The dose-response curve was drawn to show the ability of cold water extract for stabilizing free radical DPPH as shown as (Figure 4). It was concluded that the DPPH activity of cold water extract could form stable DPPH within relatively low range of about 35% inhibition and IC50 value was 20.55 g/L.IC50 values of quercetin and gallic acid standards were 0.031g/L and 0.0096 g/L respectively.

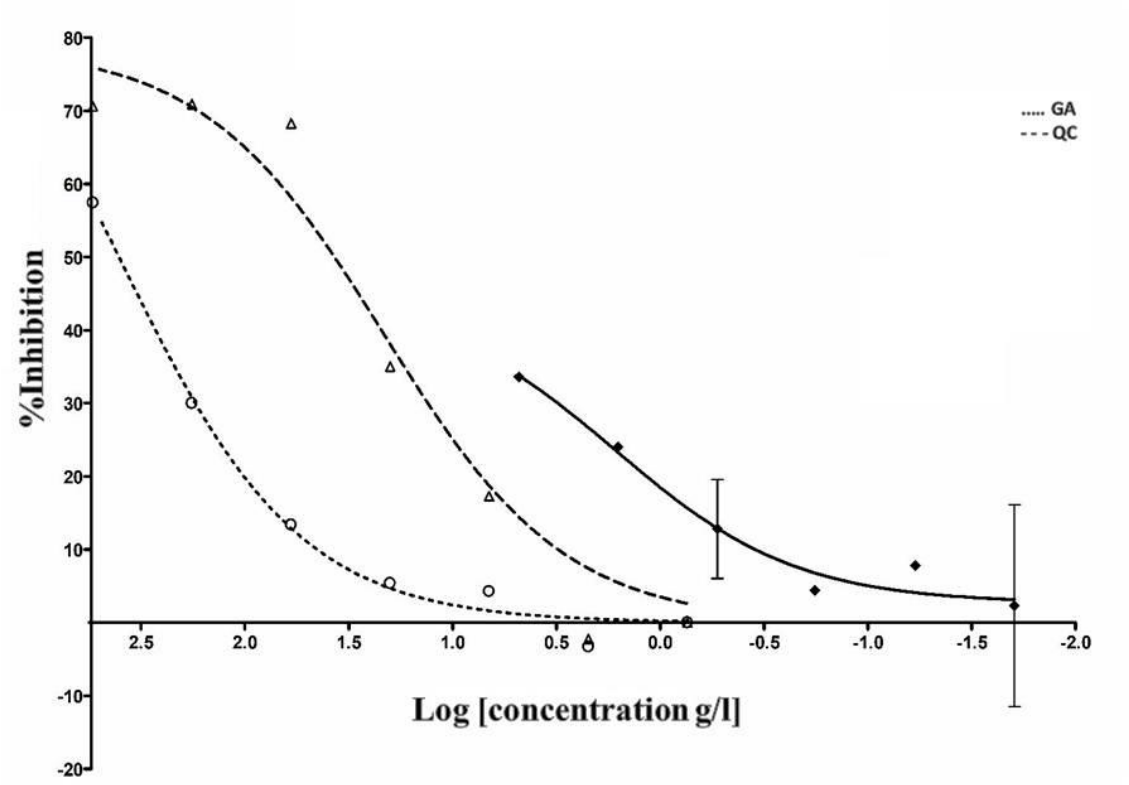

Figure 4. The effect of Polyporus squamousus extract on DPPH activity by using quercetin and gallic acid as standards

3.5 Determination of Catalase activity

In this procedure six different concentrations of cold extract were prepared as given in Table 10 under methods. The result showed that the cold water extract showed activation on catalase enzyme about 60% with respect to control and IC50 value was calculated as 2.810 g/L (Figure 5).

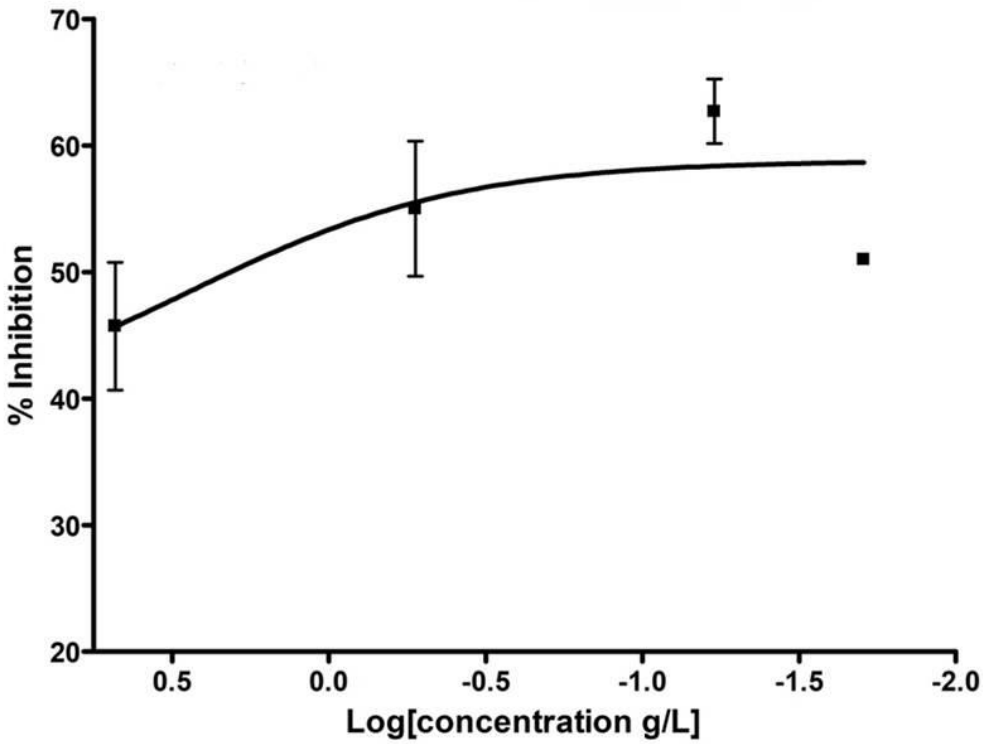

Figure 5. The effect of Polyporus squamousus extract on catalase enzyme with respect to control

3.6 Determination of SOD activity

As the source of SOD (4807 U/ml) from sigma was diluted to 3.64 U/ml and used as a stock. In this assay six different concentrations of cold water extract were used as seen in Table 12 under methods. The effect of cold water extract on SOD activity is shown in Figure 6. Surprisingly, activation of SOD was seen with all the doses used. This activation was about 95% and IC50 value was 11.94 g/L.

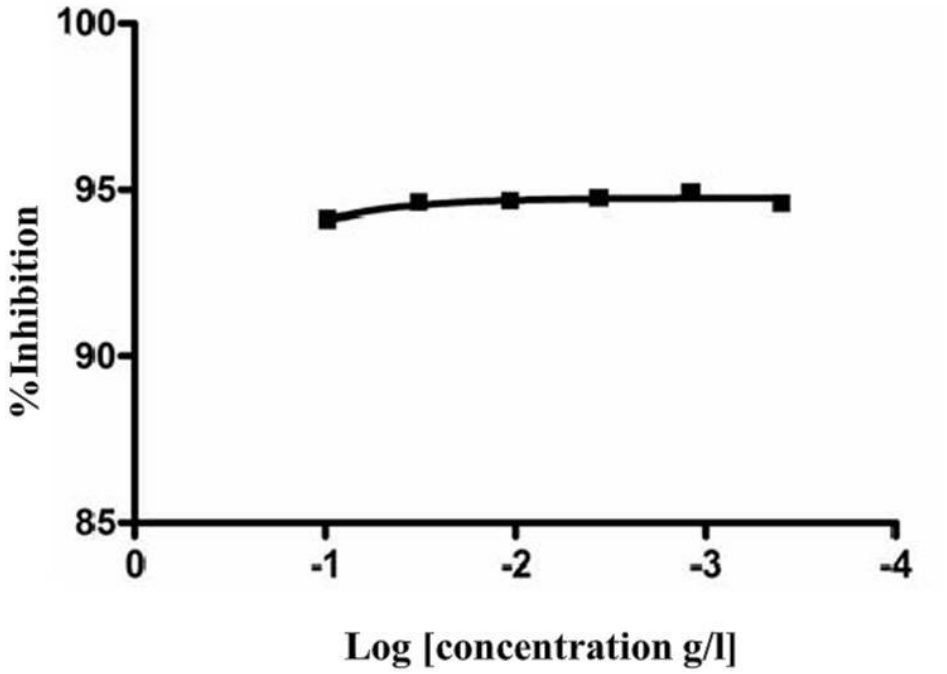

Figure 6. The effect of Polyporus squamousus extract on SOD enzyme with respect to control

3.7 Determination of GST activity

The same bovine liver cytosol with protein amount of 0.928 mg/ml was used as source of GST enzyme. The concentrations of cold water extract used in this assay were given in Table 14 under methods.

The GST activity with cold water extract is shown in Figure 7. It was shown that the concentrations of cold water extract used in this assay had nearly no effect on GST activity. The inhibitory percent with respect to control was found to just around 7.2% and IC50 value was 1.089g/L.

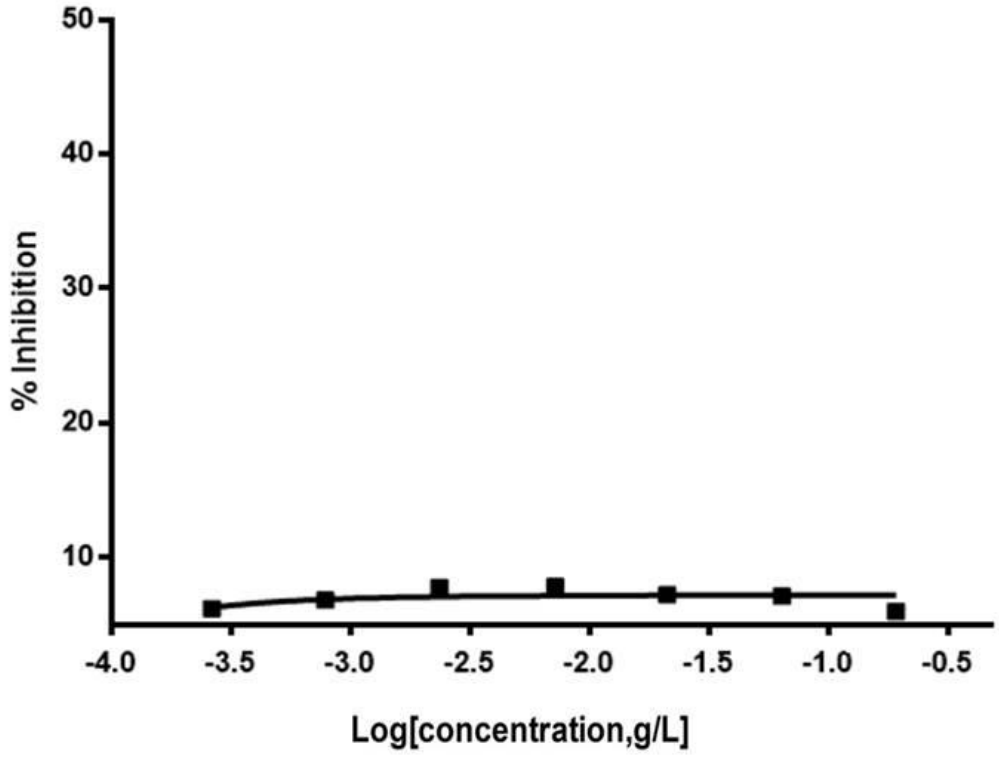

Figure 7. The effect of Polyporus squamousus extract on GST enzyme with respect to control

CHAPTER 4

DISCUSSION

In literature, there are evidences that free radicals play a dual role. They can be both deleterious and beneficial as discussed before. In all living systems consuming oxygen, energy is produced in the course of oxidative phosphorylation. Thus oxygen is vital for normal metabolism. During oxidation electrons or hydrogen are transferred from one molecule to another. Such molecules serve as antioxidants having ability to stop formation of free radicals and chain reaction preventing them to cause damages. Antioxidants are divided into two groups as exogenous from food, beverages and sunlight and endogenous from enzymatic and non-enzymatic pathways inside the body.

Recently researchers have showed that plants have antioxidants having free radical scavenging properties. Therefore they can be used as therapeutic agents in diseases including cancer. Plant extracts and Phytoconsituents found in them play role as radical scavengers. Table 18 lists different phytochemicals having antioxidant property.

Table 18. Phytoconstituents having antioxidant properties

Phytoconstituents	Example
Alkaloids	Alkaloid extract of Fumaria capreolata and Fumaria bastardii contain protopine, cryptonine, stylopine, fumariline, phtalidiisoquinoline, fumaritine, fumarafne and dehydrobenzophenanthridine possess antioxidant activity
Cartenes and Xanthophylls	Antioxidant activity of astaxanthine, α and ß carotene, lutein, lycopene, zeaxanthin, canthaxanthin were investigated.
Volatile and essential oil	Essential oil (e.g: α-terpinene, δ-3-carene, myrcene, α-pinene, p-cymene, ß-phellandrene, citronellol, trans-geraniol, α-copaene, agarospirol, globullol) isolated from Citrus reticulate and Pelargonium graveolens having activity.
Anthocyanins	Cyanidin-3-ο-ß-glucopyranoside isolated from chrysophyllum cainito, Eugenia uniflora, Myrciaria cauliflora and delphinidin-3-ο-ß-glucopyanoside was identified from Eugenia uniflora possess antioxidant activity.
Isoflavones	Isoflavones one of the important types of flavonoids having antioxidant activity.
Flavan-3-ols	Catechins possess antioxidant activity found in different plant like green tea
Flavones	Apigenin having antioxidant potential found in Thunbergia Laurifolia
Flavonols	Quercetin and isorhamnetin isolated from Haplopappus multifolius possess antioxidant activity.
Flavanones	Naringenin, a major flavanone constituent isolated from Citrus Junos possess antioxidant activities.
Coumarins	Coumarins like hernianin, O-prenyl-umbelliferone, prenyletin, haplopinol isolated from Haplopappus multifolius posses antioxidant activity.
Stilbenes	Cajaninstilbene acid from Cajanus cojan have similar antioxidant activity like the natural antioxidant reserveratrol.
Lignans	Lignans from Myristica fragrans having antioxidant potential.
Lignins	Lignins are complex phenolic polymers occurring in higher plant tissues possess antioxidant activity , example of lignins secoisolariciresinol diglycoisde.
Phenolic Acid	Phenolic acid possess antioxidant activity, Example of phenolic acid gallic acid, ellagic acid, p-coumaric acid, ferulic acid, vanillic acid, protocatecheuic acid
Triterpenoid saponins	Extract of Salvia macrochlamys contain terpenoids like monogynol A, 3ß-acetylmonogynol A, 3ß-acetyl,22ß-hydroxymonogynol A, 3ß-acetyl,21ß-22ß-dihydroxymonogynol A and extract possess antioxidant activity.
Phytosterols	Antioxidant activity of beta-sitosterol found in Morinda citrifolia investigated.
Tannins	Tannins like ellagitannins and propelargonidin isolated from Syzygium cumini fruit showed antioxidant effect.
Hydroxycinnamic acids	Hydroxycinnamic acid derivatives like caffeic acid, chlorogenic acid, sinapic acid, ferulic acid and p- coumaric acid are widely distributed in plants important for their antioxidants.
Flavonoids	Flavonoid glucosides like apigenin-7-ο-ß-glucopyranoside, luteolin7-ο-ß-glucopyranoside, luteolin-3'-ο-ß-glucopyranoside and chrysoeriol-7- ο-ß-glucopyranoside are isolated aerial parts of Verbascum salviifolium posses antioxidant activity. Flavonoids such as myricetin, quercetin, rutin, catechin, kaempferol, fisetin and naringenin also important for their antioxidant property.

In a study, 16 wild edible mushrooms were evaluated to determine their chemical compositions and antioxidant activities (ilgaz akata et al, 2012). Among these, P squamous is also included. In this study, free radical scavenging assay was carried out using DPPH and Butylated hydroxyl toluene (BHT) was used as standard (Barros et al, 2007). The assay was carried out in triplicate and the results expressed as mean values ± standard deviations. It was found that the methanol extraction of Polyporus squamosus had 17.3% extraction yield giving 3.46 mg/ml with radical scavenging activity of 95.35±0.10% as compared with standard BHT being 98.24% at 3.0 mg/ml. In our study the result of DPPH assay showed 35% of antioxidant activity scavenging DPPH radical when compared with gallic acid and quercetin as positive controls.

There are many other studies done to evaluate free radical scavenging activity and total phenolic and flavonoid contents of extracts of various flowers of Asteraceae family including O. Acabthium, C. Acanthoides, C. Arvense, C. Solstitalis. In such study, the defense enzymes such as GPx and GST inhibition activities of those plants were observed. The results showed that the highest phenolic and flavonoid concentrations were found in C. Acanthoids flowers with 90.305 mg GAE/l and 185.43 mg Q/l values respectively. The highest DPPH radical scavenging was found in C. Arvense having IC50 value 366 ng/ml.

In above study, C. Solstitalis had the maximum GPX and GST enzyme inhibition activities with IC50 values of 79 and 232ng/ml respectively. This study showed that various plants can have different content of antioxidant and different capacity to scavenge free radical or show different effects of GPx and GST enzymes. This may attribute to the structure of their contents or method of extractions. Even though some equivalence studies regarding antioxidants inhibition in mushroom species are not available. There are many studies showing the radical scavenging or antioxidant properties of mushrooms. In one study, Volvariella volvacea mushrooms showed to have high levels of antioxidants, catalase (37.37 ±0.06 µmol/min/mg), SOD (29.21±0.04 µmol/min/mg), Glutathione peroxidase (48.88± 0.07 Mmol/min/mg), Glutathione-s-transferase (12.93 0.07 Mmol/min/mg), and Glutathione reductase (27.44 ±0.03 µmol/min/mg) (Ramkumar, L., et al).

In another study, the antioxidant properties of extracts from twenty four mushrooms found in East blacksea of Turkey have been evaluated (Keleş Ali, et. al, 2011). Among these mushrooms, Polyporus squamosus was also included. In this study, the methanolic extract of the mushrooms were analysed for ferric reducing power, DPPH and total phenolic content determination assays. It was found that 92.54% dry matter of Polyporus squamosus had less than 20 mg ascorbic acid, total phenolic content of 4531.11 mg/kg, with ferric reducing power as 2242.86 µmol/g and DPPH scavenging activity of 43.30% which is similar to the result obtained in our study. The EC50 for this mushroom was not found in this study.

Again in another recent study (Dimitrijevic, M. et al, 2013), the free radical scavenging activities of ethanol extracts of 12 various mushrooms including Polyporus squamosus were examined. In this study 50% inhibition (EC50) was the main parameter for comparison among these mushrooms. It was found that the lower the EC50 value the higher activity. The EC50 values of all mushrooms extracts ranged from 10.997 to 51.159 mg/ml. The B. Regius with lowest EC50 had the highest activity of 10.997±0.842 mg/ml and C. Cibarus with the highest EC50 of 51.159±1.012 mg/ml). The result obtained for P. squamosus in this study was similar to the result obtained in the study done by Keleş as discussed above having DPPH scavenging activity of 45.33% similar to our result with activity of 35%. The EC50 was shown to be about 21.0 mg/ml which is similar to IC50 value obtained in our study as 20.55 g/l.

It can be concluded from those studies that wild mushrooms with different composition can play an important role in scavenging radicals and are considered as possible protective agents to fight against oxidative damage.

CONCLUSION

In this study, the antioxidant property of Polyporus squamosus was examined. The results showed that the ability of this mushroom to donate hydrogen to DPPH radical is relatively low with 35 % and IC50 value was 20.55 g/L. The effect of mushroom extract on Catalase, SOD and GST was also examined and it was found that the cold water extract of Poluporus squamosus had nearly no effect on inhibition of GST with The inhibitory percent with respect to control found to be just around 7.2 % and IC50 value was 1.089 g/L. The effect of cold water extract on SOD showed activation as high as 95 % and IC50 value was 11.94 g/L. and also the effect on CAT enzyme was activation of about 60 % with respect to control and IC50 value of 2.810 g/L.

As a final point, it can be concluded that Polyporus squamosus is relatively a good radical scavenger and can be used as natural antioxidant or food supplement. The fact that there was no effect on the activity of GST can be advantageous when it should act in a synergistic way with GST enzyme to fight against free radical damage. The high activation of SOD when the cold water extract of this mushroom was used can also be beneficial to enhance the effect of SOD to neutralize superoxide radical.

Based on the fact that the origin of Polyporus squamous plays an important role in chemical characteristics of composition which is directly related to the antioxidant strength can be used to determine the mushroom species with best antioxidant property.

REFERENCES

Bagchi K., Puri S, (1998) Free radicals and antioxidants in health and disease. East Mediterranean Health Jr ; 4: 350-60

Bahorun T, Soobrattee MA, Luximon-Ramma V, Aruoma OI. (2006) Free radicals and antioxidants in cardiovascular health and disease. Internet J. Med. Update. ; 1:1–17.

Banniste J, Bannister W, Rotilio G. (1987), Aspects of the structure, function and applications of superoxide dismutase. CRC crit Rev Biochem.; 22: 111-80.

Board PG,, Baker RT., Chelvanayagam G., and Jermiin LS., (1997), Zeta, a novel class of glutathione transferase in a range of species from plants to humans. Biochemical Journal, , 328, 929-935.

Cadenas, E, (1997) Basic mechanisms of antioxidant activity. Biofactors, , 6, 391-397.

Cao X, Antonyuk SV, Seetharaman SV, Whitson LJ, Taylor AB, Holloway SP, (2008) Structures of the G85R variant of SOD1 in familial amyotrophic lateral sclerosis. J Biol Chem.;, 283:16169–77.

Chang ST, Miles PG. (1992), Mushrooms biology - a new discipline. Mycol;6:64e5.

Chasseaud, L. F., (1979) The role of glutathione and glutathione S-transferase in the metabolism of chemical carcinogens and other electrophilic agents. Advances in Cancer Research, 29, 175-274.

Cheeseman, K.H., stater, T.F (1993), An introduction to free radicals chemistry. Br. Med. Bull, , 49:481-93.

Chelikani P, Fita I, Loewen PC. (2004) Diversity of structures and properties among catalasees. Cell Mol Life Sci.; 61: 192-208.

Corpas FJ, Barroso JB, del Río LA., (2001), Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci.;, 6:145–50.

Corpas FJ, Fernández-Ocaña A, Carreras A, Valderrama R, Luque F, Esteban FJ, (2006), The expression of different superoxide dismutase forms is cell-type dependent in olive (Olea europaea L.) leaves. Plant Cell Physiol.;, 47:984–94.

Cote J, Caillet S, Doyon G. 2010, Bioactive compounds in cranberries and their biological properties. Crit Rev Food Sci Nutr; 50(7):666e79.

D'Archivio M, Filesi C, Vari R. (2010), Bioavailability of the polyphenols: status and controversies. Int J Mol Sci;11:1321e42.

Dimitrijevic Marija, Jovanovic V. S. , Cvetkovic J., Mihajilov-krestev T., Gordana S. and Mitic V. (2014), Screening of antioxidant, antimicrobial an antiradial activities of twelve selected Serbian wild mushrooms, Journal of analaytical methods, 1-15

Droge W. (2002), Free radicals in the physiological control of cell function. Review. Physiol. Rev; 82:47–95.

Droge, W, (2002), Free radicals in the physiological 1’ control of cell function. Physiol. Rev., , 82, 47-95.

Dziezak JD. (1986), Antioxidants-The ultimate answer to oxidation. Food Technol;40(9):94e102.

Eisner T, Aneshansley DJ. (1999), Spray aiming in the bombardier beetle: Photographic evidence. Proc Natl Acad Sci USA.;, 96:9705–9.

Fedtke, N., Boucheron, J. A., Walker, V. E., & Swenberg, J.A, (1990) Vinyl chloride induced DNA adducts. 2 formation and persistence of 7-2'-oxoethylguanine and n2,3-ethenoguanine in rat tissue DNA. Carcinogenesisi, , 11, 1287-1292.

Fink, S.P., Reddy, G. R., & Marnett, L. J. (1997) Mutagenicity in Echerichia coli of the major DNA adduct derived from the endogenous mutagen malondialdehyde, Proc. Natl. Acad. Sci, U. S. A, , 94, 8652-8657.

Freeman, B. A., Carpo, J. D, (2004), Biology of disease: Free radicals and tissue injury. Lab invest. 1982; 47: 412-26.

Fridovich I, (1984), Overview: biological sources of $O2^{\cdot -}$, Methods Enzymol, 105, 59-61.

Frie B, Stocker R Ames BN, (1988), Antioxidant defences and lipid peroxidation in human blood plasma, Proc Natl. Acad Sci,; 37: 569-71.

Furtua S., Nishina Y, Suda I., (1997) Flurometric assay for screening antioxidant activities of vegetables. J Food Sci.; 62; 526-8.

Gaetani G, Ferraris A, Rolfo M, Mangerini R, Arena S, Kirkman H., (1996) Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood.;, 87:1595–9.

Genestra M. (2007), Oxyl radicals, redox-sensitive signaling cascades and antioxidants. Review. Cell Signal.;19:1807–1819.

Gerschman, R., Gilbert, D. L., Nye, S. W., Dwyer, P., & Fenn, W. O., (1954) Oxygen poisoning and x-irradiation-A mechaniism in common science, , 119, 623-626.

Halliwell B, Gutteridge JMC (2007). Free radicals in biology and medicine. 4th. Oxford, UK: Clarendon Press.

Halliwell B. (1995), How to characterize an antioxidant- An update. Biochem Soc Symp.; 61:73-101.

Halliwell B. (2007), Biochemistry of oxidative stress. Biochem. Soc. Trans.; 35:1147–1150.

Halliwell B. Gutteridge JMC, (1989), Free radicals in biology and medicine, 2nd ed. Oxford university Press, , 543.

Halliwell, B., Gutteridge, J. M.C. (1999), Free radicals in biology and medicine (3rd ed.), Oxford University press.

Hayes J, Flangan J, Jowsey I. (2005), Glutathione transferases. Annu rev. Pharmacol Toxicol.; 45: 51-88.

Jakobsson P. J. Morgenstern, R., Mancini, J., Ford-Hutchinson, A., Persson, B., (2000), Membrane-associated proteins in Eicosanoid and glutathione metabolism (MAPEG) A widespread protein superfamily, American Journal of Respiratory and Critical Care Medicine, 161 (supplement_1), S20-S24.

Kirk P.M. (2017), Species Fungorum (version Jan 2016).

Kovacic, P., & Jacintho, J. D, (2001), Mechanisms of carcinogenesisi; Focus on oxidative stress and electron transfer. Curr. Med. Chem., , 8, 773-796.

Lin JK, Lin CH, Ling YC, Lin-Shian SY, Juan IM. (1998), Survey of catechins, gallic acid and methylxantines in green, oolong, purej and black teas. J agric food Chem ,; J-46: 3635-42.

Manach, C., Morand, C., Crespy V., Demigne, C., Texier o., Regerat, F., and Remesy, C., (1998), FEBS Lett, , 426; 331-336.

Mannervick, B. E. N. G.T, (1985) The isoenzymes of glutathione transferase, , advances in Enzymology related areas of Molecular Biology, , 57, 357-417.

Mao, H., Schnetz-Boutaud, N.C., Weisenseel, J.P., Marnett, L. J., & stone, M.P,. (1999), Duplex DNA catalyzes the chemical rearrangement of a malondialdeyde deoxyguanosine adduct. Proc. Natl. Acad. Sci. U. S. A, , 96,6615-6620.

Marrow C. S. Smitherman, P. K, Diah, S. K, Schneider, E. Townsend, A. J., (1998), Coordinated action of Glutathion S-Transferase (GSTs) and multi drug protein 1 (MRP1) in Antieoplastic drug detoxification mechansim of GST A1-1-AND MRP1-associated resistance to chlorambucil in mcf7 breast carcinoma cells. Journal of Biological Chemistry, , 273 (32), 20114-20120.

Miller DM, Buettner, G.R, G. R. & Aust, S. D., (1990), Transition metals as catalysts of "autooxidation" reactions. Frr Radic. Biol. Med., ,8, 95-108.

Mohammad AA, Ibrahim AA, (2004) Pathological roles of reactive oxygen species and their defence mechanism. Saudi Pharmaceutical;, 12: 1-18.

Nagulendran K. Velavan S., Mahesh R., Begum VH, (2007), In vitro antioxidant activity and total polyphenolic content of Cyperus rotundus rhizomes, E- Journal of Chemistry, 4, , 440-449.

Oei P. (2003), Manual on mushroom cultivation: techniques species and opportunities for commercial application in developing countries. Amsterdam: TOOL Publications

Pacher P, Beckman JS, Liaudet L. (2007) Nitric oxide and peroxynitrite in health and disease. Physiol. Rev.;87:315–424.

Patel S, Goyal A. 2012**,** Recent developments in mushrooms as antiecancer therapeutics:a review. 3 Biotech;2(1):1e15.

Pham-huy LA, Hua He and Chuoang Pham-Huy, (2008), Free radicals, antioxidants in disease and Health, Int. J. Biomed Sci., , 4(2): 89-96

Rice-Evans CA, Diplock AT, (1993) Current status of antioxidant therapy. Free Radic Biol Med.; 15:77-96.

Sanchez C. (2004) Modern aspects of mushroom culture technology. Appl Microbiol Biotechnol; 64(6):756e62.

Sanchez C. (2010), Cultivation of Pleurotus ostreatus and other edible mushrooms.Appl Microbiol Biotechnol;85(5):1321e37.

Shafag Noori, (2012), An overview of oxidative stress and antioxdant defensive system, Open Access Scientific Reports 413, , Volume 1, issue 8.

Shi HL, Noguchi N, Niki N. (1999), Comparative study on dynamics of antioxidative action of α-tocopheryl hydroquinone, ubiquinol and α-tocopherol, against lipid peroxidation. Free radic Biol Med.; 27: 334-46.

Siems, W. G., Grune, T., & Esterbauer, H, (1995) 4-hydroxynonenal formation during ischemia and reperfusion of rat small intestine. Life Sci., , 57, 785-789.

Spahr DL. (2009). Edible and Medicinal Mushrooms of New England and Eastern Canada , Richmond, Calif: North Atlantic Books. pp. 131–35. ISBN 1-55643-795-1. Retrieved 2010-05-28).

Standtman, E. (2004), R. Role of oxidant species in aging Curr. Med. Chem, 11; 1105-1112.

Valko M, Leibfritz D, Monocol J, Cronin MTD, Mazur M, Telser J, (2007) Free radicals and antioxidants in normal physiological functions and human disease, The International Journal of Biochemistry & cell Biology, 39, , 44-84.

Valko M, Morris H, Cronin MTD. (2005), Metals, toxicity and oxidative stress. Curr. Med. Chem. ;12:1161–1208.

Valko, M.; Rhodes, C. J., Moncol, J., Izakovic, M.m & Mazur, M, (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem. Biol. Interact., , 160, 1-40.

Wang H, Cao G, Prior RL. (1996), Total antioxidant capacity of fruits. J Agric Food Chem.; 44:701-5.

Willcox JK, Ash SL, Catignani GL. (2004), Antioxidants and prevention of chronic disease. Review. Crit. Rev. Food. Sci. Nutr. ;44:275–295.

Wuerges J, Lee JW, Yim YI, Yim HS, Kang SO, Djinovic Carugo K., (2004), Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. Proc Natl. Acad Sci.; 101: 8569-74.

Yagi K. (1970), A rapid method for evaluation of oxidation and antioxidants. Agric Biol Chem;34(1):142e5.

Young I, Woodside J. (2001) Antioxidants in health and disease. J. Clin. Pathol. ;54:176–186.

Zelko I, Mariani T., Folz R. (2002), Superoxide dismutase multigene family: A comparison of the cuZn-SOD (SOD1), Mn-SOD (SOD2) and EC-SOD (SOD3) gene structures, evolution, and expression. Free radic Biol Med.; 33: 337-49.