THE EFFECT OF PANUS CONCHATUS MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYMES

A MASTER'S THESIS

in

Chemical Engineering and Applied Chemistry Department

by

AHLAM IBRAHIM

JULY 2016

THE EFFECT OF *PANUS CONCHATUS* MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYMES

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OF

ATILIM UNIVERSITY

BY

AHLAM IBRAHIM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

APPLIED CHEMISTRY

AT

CHEMICAL ENGINEERING AND APPLIED CHEMISTRY DEPARTMENT

JULY 2016

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

_____

(Prof. Dr. İbrahim AKMAN)

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

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This is to certify that we have read the thesis " The Effect of *Panus conchatus* Mushroom Extracts on Antioxidant Enzymes" submitted by "Ahlam Ibrahim" and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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ABSTRACT

THE EFFECT OF *PANUS CONCHATUS* MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYMES

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July 2016, 53 pages

Many studies were proven the antioxidant effect of several mushroom families by protecting the body from free radicals and hence increasing the human life span. However only few recent researches evaluated the antioxidant bioactivity of *Panus conchatus*. In addition, the effect of *Panus conchatus* on anti-oxidant enzymes has not been studied so far. Therefore, for the first time in this study, antioxidant effect of *Panus conchatus* mushroom extract was analyzed by measuring its total phenolic and flavonoid contents, determining free radical scavenging activity of mushroom, and finally, measuring the effect of mushroom on the activity of the antioxidant enzymes; glutathione-S-transferase, catalase and superoxide dismutase. The mushroom extracts were prepared by different methods so called hot water, cold water, methanol, and ethanol extraction methods. The total phenolic and flavonoid contents of these different extracts were compared, then, the extract with highest total phenolic compounds was used for further analyses.

The results showed that hot water extract has highest total flavonoid and phenolic contents, however the ability of mushroom to inhibit the free radical 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) was 100 % with half maximal inhibitory concentration value (IC_{50}) of 0.02214g/L.

. The inhibitory effects of mushroom on both catalase (CAT) and superoxide dismutase (SOD) activities were less than 25%, whereas, Glutathione-S-transferase (GST) inhibition was less than 50 % with half maximal inhibitory concentration value (IC_{50}) of 0.3744g/L.

Keywords: *Panus conchatus* mushroom, radical scavenging, antioxidant enzymes, DPPH assay, Catalase, Superoxide dismutase, Glutathione-S-transferase

ÖZ

PANUS CONCHATUS MANTAR ÖZÜTÜNÜN ANTİOKSİDAN ENZİMLER ÜZERİNE ETKİSİ

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Temmuz, 2016, 53 sayfa

Günümüzde pekçok çalışma, farklı mantar ailesine ait antioksidan etkinin, vücudu serbest radikallerden koruyarak yaşam örünü artırdığı yönünde etkisini ispatlar niteliktedir.

Panus conchatus mantarına ait literaturde çok az sayıda çalışmada mantarın antioksidan özelliğinden bahsedilmiştir. Ancak mantarın antioksidan enzimler üzerine etkisini gösteren bir çalışmaya rastlanmamıştır.

Bu çalışmada ilk olarak *Panus conchatus* mantarının antioksidan enzimler üzerine etkisi çalışılmıştır. Mantara ait toplam fenol ve flavonoid içeriklerinin belirlenerek radikal süpürücü etkinliği tespit edilmiş ve mantar özütünün GST,CAT ve SOD gibi antioksidan enzimler üzerine etkisi incelenmiştir.

Panus conchatus mantar özütü, sıcak su, soğuk su , metnol ve etanol çözücüleri kullanılarak hazırlanmış ve özütlere ait toplam fenol ve flavonoid içerikleri kıyaslanmıştır.

Sonuçlara göre, sıcak suda hazırlanan mantar özütü en yüksek toplam fenol ve flavonoid içeriğe sahip olup, tüm analizler bu özüt kullanılarak gerçekleştirilmiştir.

Mantarın 2,2-Difenil-1-Pikrilhidrazil (DPPH) serbest radikalini süpürücü etkinliğini %100 den az bir inhibisyonla önlediği gözlenmiş, IC_{50} degeri ise 0.02214 g/L olarak hesaplanmıştır. *Panus conchatus* mantar özütünün KAT ve SOD aktivitelerini sırasıyla %25 den daha az önlediği ancak GST enzim aktivitesini yaklaşık olarak %50 önlediği bulunmuştur. GST enzimi için *Panus conchatus* mantar özütü IC_{50} degeri 0.3744 g/L olarak hesaplanmıştır.

Anahtar Kelimeler: *Panus conchatus*, radikal süpürücü, antioksidan enzimler, DPPH , Katalaz, Superoksit dismutaz, Glutathione-S-transferaz

ACKNOWLEDGEMENT

I would prefer to express my feeling and sincere thanks to all who gave me the likelihood to finish this thesis. First I am deeply grateful to almighty god who gave me health, patience and also the strength to be ready to finish this work.

I would prefer to give thanks to my Supervisor: Assoc. Prof. Dr. S. Belgin İşgör for her great support and valuable advices, also for her constant inspiration, encouragement and guidance throughout my thesis.

I appreciate to Assoc. Prof. Dr. Yasemin G. İşgör for her priceless guidance and support throughout experiments, her friendly relationship, kindly teaching and particularly for her guidence in the analysis throughout this study and corrections.

I would like to express my whole gratitude to the Head of the Department Prof. Dr. Atilla Cihaner for his valuable suggestions and corrections, faculty members, and my biochemical laboratory-mates.

Words not enough to tell how grateful and thankful I am to my husband for his supporting, his sacrifice and patience throughout all my studies.

Thank you for everything, my mother and my father and thanks for your prayers. Finally, thanks for my brothers, my sisters, and my daughters.

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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
MAPEG	-	Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism
HRP	-	Horse Reddish Peroxidase
DHBS	-	Dichlorohydroxy Benzene Sulfonic Acid
4-AP	-	4-Amino Antipyrine
EDTA	-	Ethylene Diamine Tetra acetic acid Disodium Salt Dihydrate
NBT	-	Nitro Blue Tetrazolium Chloride
QE	-	Quercetin Equivalent
GAE	-	Gallic Acid Equivalent
H_2O_2	-	Hydrogen Peroxide
TFC	-	Total Flavonoid Content
TPC	-	Total Phenolic Content
DMSO	-	Dimethyl Sulfoxide
XOD	-	Xanthine oxidase
IC_{50}	-	The half maximal inhibitory concentration
ROS	-	Reactive Oxygen Species
Q_{10}	-	Coenzyme ubiquinone

CHAPTER 1

INTRODUCTION

The presence of antioxidants in human body is good and required considering their ability in protecting human body from the harmful effects of oxidative agents, and their ability in assisting body to reduce or resist aerobic injury. Recently, a large number of natural antioxidants have been isolated from different parts and species of plants such as oil seeds, fruits, leaves, roots, vegetables, spices cereal, crops and herbs (Osawa, Ochi, Kawakishi, Ramarathnam, 1995). For thousands years, the Chinese herbs have been used for medical purpose. A number of them are supposed to display the activities of vital antioxidant (Kim et al., 1994 and Su, 1992). As well as, mushrooms are used commonly in Chinese medication and also consumed for nutritional support.

In addition, some mushrooms may enhance the effect of some drugs like anticancer drugs by attacking antioxidant enzymes and drug connect with GSH, this leads to increase its penetration to nucleus. (Backos, Franklin, and Reigan, 2012).

1.1 Free Radicals:

Free radicals are electrically charged molecules and need to be stabilized. Having unpaired electron, causes them to hunt out and catch the electrons from different materials for such stabilization or neutralization. Though, the free radical atom is created by the initial attack to be neutralized, but other radical is created within the same reaction, leading to a sequence of radical forming reactions to happen. Thousands of free radical reactions occur within a few seconds within the first reaction (initial attack) until they are deactivated.

Oxygen may be an extremely reactive atom that can be capable of changing into a portion of the potentially harmful molecules referred to as reactive oxygen

species (ROS) or free radical. It is found that 5 % or more of the O_2 which enter the body during inspiration process is changed again to ROS such as hydrogen peroxide, superoxide, and hydroxyl radicals through the O_2 univalent reduction (Uday Bandyopudya et al., 1999). Therefore, cells that live in aerobic situation are continually vulnerable to ROS attack. So, it is important for cell to be prepared for ROS by extremely strong antioxidant system. This antioxidant system contains, nutrient-derived antioxidants (e.g., tocopherols and tocotrienols, vitamin C, lipoic acid and glutathione, carotenoids), antioxidant enzymes (e.g.,super oxide dismutase, SOD; glutathione peroxidase, GPx; catalase, CAT and glutathione-S-transferase, GST), metal binding proteins (e.g., albumin, lactoferrin, ferritin, and ceruloplasmin), and various different forms of the antioxidant phytonutrients.

There are variety of food sources are rich in these antioxidants. The oxidative stress is resulted when the balance that existed between antioxidant defense and the ROS production is lost, that over a series of events deregulates the cellular functions resulting in varied pathological situations (Trevor F, Slater, 1984 and Chitra K.P., K.S.Pillai, 2002).

1.2 Reactive Oxygen Species (ROS):

Reactive Oxygen Species are extremely reactive, oxygen containing molecules, together with free radicals. ROS are superoxide anion radical, nitric oxide radical, peroxides, singlet oxygen, hydroxyl radical, hypochlorite radical, and numerous lipid peroxides.

The cells produce most of the oxidants due to:

- The ordinary aerobic metabolism,
- The oxidative burst by the phagocytes (white blood cells) by which the microorganism are killed, and antigens are denatured.
- Xenobiotic metabolism by which the detoxification of harmful materials takes place. Moreover, ROS can be internally generated during the course of various diseases, including chronic inflammation and infections, during the medical treatment or during exposure to allergens, toxin like smoke, pollution, insecticides, and pesticides and all contribute to increase the body's ROS concentration.

The types of ROS targets in cells are generally the biomolecules such as nucleic acids, proteins and unsaturated fatty acid (Figure 1.1), as well as carbohydrates.The properties of the intrinsic membrane will be altered by these reactions which include liquidity, ion transport, protein synthesis, damage of deoxyribonucleic acid, enzyme activity loss, eventually leading to necrobiosis (Uday Bandyopudya et al., 1999). There are numerous human disorders caused by free radical like metabolic disorders, arthritis, upset, hemorrhagic shock, neurodegenerative illness, gastrointestinal ulcer genesis, cystic fibrosis and AIDS. Some diseases that are caused by ROS specifically are Alzheimer's disease, Parkinson's disease, Down's syndrome, lipoprotein oxidative modification in cancer, atherosclerosis, and ischemic reperfusion damage in several tissues like brain, kidney, liver and heart. Among those, atherosclerosis and ischemic injury in heart and brain that are produced by ROS were studied extensively (Uday Bandyopudya et al., 1999 and Chitra K.P., K.S.Pillai, 2002).

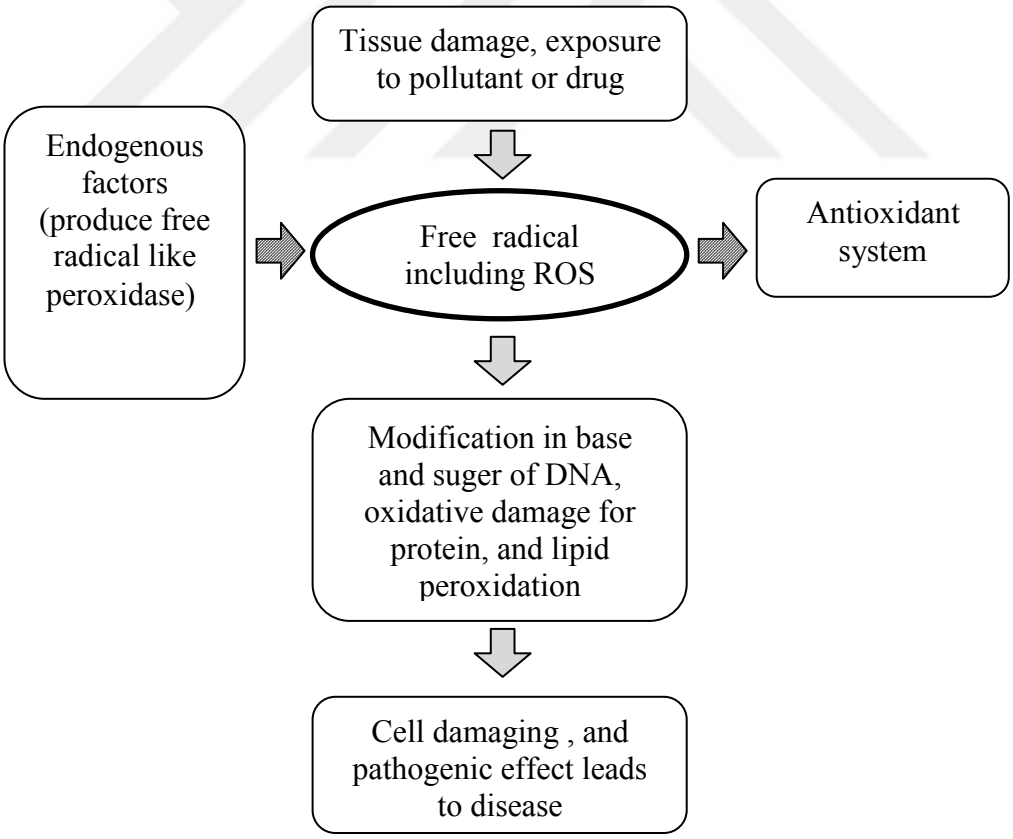

Figure 1.1: Metabolism of ROS and mechanism of cellular damage that causes diseases

1.3 Antioxidant Protection System:

Humans have an extremely fine and sophisticated antioxidant protection and defense system. It involves a wide range of constituents, which are endogenous or exogenous in origin, both act synergistically to neutralize free radicals (Table1) (Mark Percival, 1998).

Table 1: ROS and their corresponding protective neutralizing agent

ROS	Antioxidants
Hydroxyl radical	Vit C, Flavonoids, Lipoic acid, Glutathione
Hydrogen peroxide	Vit C, flavonoids, lipoic acid, vit E, Glutathione
Lipid peroxides	Flavonoid, Vit E, Beta-carotene, ubiquinone

These protective antioxidants can be classified into

a) Endogenous antioxidants that reduce free radical by electron donors are:
   - Bilirubin
   - Thiols, e.g., glutathione, lipoic acid, N-acetyl aminoalkanoic acid.
   - NADPH and NADH.
   - Ubiquinone (coenzyme Q10)
   - Uric acid
   - Enzymes which catalyze ROS removal for example GST, GPX, SOD and CAT.

b) Exogenous dietary antioxidant that can also reduce the free radical are:
   - Vitamin C and vitamin E (Halliwell B., J., Gutteridge(Eds) 1999)
   - Beta carotene and alternative carotenoids and oxycarotenoids,
   - Polyphenols, e.g, flavonol's, flavonoids, flavones, and proanthocyanidins.

c) Metal binding proteins that can bind pro-oxidant metals as copper and iron, and these are:
    - Albumin (copper).
    - Ceruloplasmin (copper)
    - Metallothionein (copper)
    - Ferritin (iron)
    - Myoglobin (iron)
    - Transferrin (iron).

1.3.1 Endogenous Antioxidants (Antioxidant Enzymes):

The body depends on many endogenous defensive techniques which assist in the protection against the cellular radical injury produced by the endogenous free radicals other than the antioxidant. The antioxidant enzymes are superoxide dismutase, SOD; heme oxidase; catalase, CAT and glutathione peroxidase, GPx. They metabolize oxidative toxic and harmful intermediates and need certain cofactors like metallic elements such as zinc, copper, iron for optimal catalytic action in detoxification reactions. They may also require Glutathione, GSH a vital soluble antioxidant, which is synthesized from the amino acids cysteine, glycine and glutamate. Glutathione directly quenches ROS like lipid peroxides, and plays a significant role in the metabolism of xenobiotics.

The metabolic detoxification system inside the body converts xenobiotics such as drugs and environmental pollutants, or endobiotics like hormones from lipid insoluble substances, into water soluble, less toxic substances that can be excreted from the body via kidneys. This system consists of phase I and phase II metabolizing enzymes in which their activities lead to biotransformation of xenobiotics.

In phase I biotransformation, the xenobiotics are converted into hydrophilic metabolites by the action of Cytochrome P450 (CYP), and these metabolites can bind to proteins and nucleic acid that cause alteration in their functions. Also in phase I, their toxicity can be reduced by microsomal epoxide hydrolase into dihydrodiol that can be excreted. Moreover, metabolites produced in phase I biotransformation can be

combined with the glutathione and the reactivation is catalyzed by phase II biotransformation enzymes that is known as Glutathione –S-transferases (GSTs). GSTs are families of enzymes which convert phase I metabolites into more water soluble and less toxic compounds to be eliminated and excreted through bile duct and kidneys.

The detoxification metabolism occurs mainly in the liver (Murray GI., Barnes TS. et al., 1988 and DeWaziers I., Cugnenc PH. et al., 1990) and it also takes place in kidneys, intestine, and lungs (Anttila S., Hukkanen J. et al 1997; Murray GI., Barnes TS. et al., 1988 and DeWaziers I., Cugnenc PH. et al., 1990).

1.3.1.1 Glutathione S-Transferase Enzyme:

Glutathione S-transferase (GST; EC2.5.1.18) isoenzymes are naturally found in various organisms such as microbes, insects, plants, fishes, birds, and mammals (Hayes and pulford, 1995). The GSTs possess numerous activities and participate in many different kinds of reactions.

Glutathione S-transferase are considered a complex large groups of proteins. There are two entirely dissimilar super families which have the transferase action (Strange and Hayes, 2000). The first GST family was characterized as soluble or cytosolic enzyme (Boyland and Chasseaud, 1969 and Mannervik, 1985). To date, a minimum of sixteen members of this family are found in human (Hayes and strange, 2000, Board et al., 1997, 2000). All soluble GSTs have been divided into eight classes or families depending on their degree of sequence identity and called as alpha (α), kappa (κ), mu (μ), pi (π), omega (ω), sigma (σ), theta (Θ) and zeta (ξ) (Board et al.,1997; 2000; Meyer et al.,1991; Mannervik et al., 1985; Meyer and Thomas, 1995 and Pemble et al., 1996). The second GST family is consisting of microsomal transferases, and has been identified as membrane-associated proteins in glutathione and eicosanoid metabolism (MAPEG). Which is a group having at least six members in human (Jakobsson et al., 1999a and Jakobsson et al.,2000).

The soluble GSTs seem to be concerned mostly within the foreign chemicals metabolism, for example environmental pollutants, carcinogens, and cancer

chemotherapeutical medicine, also have roll in the detoxification of damaging endogenously derived reactive composites (Hayes and Pulford, 1995). So that the transferases have antioxidant function (Mclellan and Hayes, 1999; Mannervik 1986). A little group of soluble GSTs are concerned within the prostaglandins structure and inactivation.

Classically, GST enzymes have essential role in phase II of drug- metabolism. These enzymes catalyze the reaction when reduced glutathione conjugated with electrophilic compounds produced in phase I of drug-metabolism. The reaction leads to the formation of a thioether bond between the sulphur atom of GSH and the substrates, and they become to be eliminated readily from the cell (Chasseaud, 1979 and Mannervik 1985). Besides, many of GST isoenzymes exhibit alternative GSH-dependent catalytic activities in the reduction of organic hydro peroxides (Ketterer et al., 1990) and isomerization of unsaturated compounds (Habig and Jakoby, 1980; Benson et al., 1977).

In chemotherapy, GST overexpression in tumors results in resistance to the therapeutics that targets DNA and this causes problem to patient rather than benefit (Morrow et al., 1998).Therefore, GST inhibition may be a therapeutic benefit for such cases.

The process of aerobic respiration will result in formation of reactive oxygen species which cause lipid peroxidation (Trevor F., Slater, 1984), and this harmful effect can also be prevented by action of soluble GSTs that reduce the free radicals (Hayes and Mclellan, 1999).

GSTs are found in the nucleus, cytoplasm, mitochondria and endoplasmic reticulum. (Sherratt et al., 1998).

Cytosolic GST has two domains which are C-terminal and N-terminal domain. C-terminal domain consists of α-helixes and N-terminal thioredoxin-like domain characterized by βαβαββα topology (Figure 1.2).

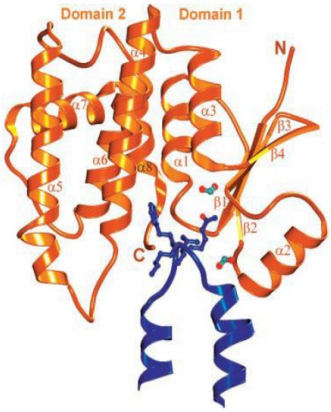

Figure1.2: The structure of glutathione S-transferase (Wolf K, Backer A *et al*., 2003).

1.3.1.2 Catalase (CAT) Enzyme:

Catalase (EC 1.11.1.6, H_2O_2:H_2O_2 oxidoreductase) has been known as antioxidant enzyme and it has another name, hydro peroxidase. It is a protective catalyst that has been found in nearly all animal cells and plant cells. The enzyme is a tetrameric protein consisting of four similar tetrahedral subunits of 60 kDa each and it has one group of ferriprotoporphyrin as a prosthetic group per subunit. The molecular mass of enzyme is 240 kDa (Aebi HE., 1978).

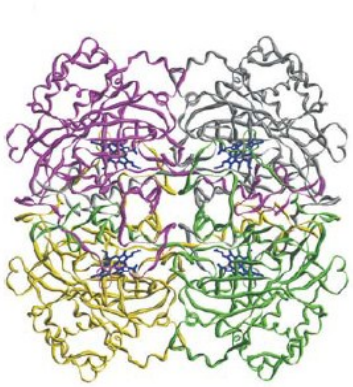

Figure 1.3.: A Large Tetrameric Catalase Subunit. (Evans S., 1993).

Three categories of proteins that are not depended on the sequence basis but according to structure that gives the effectiveness of the important enzyme. The class which is the most spread naturally and it consists of monofunctional, heme-

containing enzymes grouped into large (less than75 kDa) and small (less than 60 kDa) subunits. The analyses of phylogenetic have been proposed the presence of two special small subunit enzymes subgroupings and one large subunit enzymes subgroup between the monofunctional catalases (KlotzM.G., Klassen G.,1997). The second class, less widespread category consists of bi functional, heme-containing catalase-peroxidases which almost similar to plant peroxidases in structure and sequence. The first two classes are present in aerobic organisms and, their structure and biochemical reactions have been extensively known (Koller, F. and Zamocky, M., 1999). The third one of enzyme classes includes the nonheme or Mn-containing catalases. This class is found in the most of microorganisms in microaerophilic environment, which includes mesophilic lactic acid bacteria e.g., Lactobacillus plantarum (Kono, Y., Fridovich, I., 1983).

The enzyme is important in detoxification of hydrogen peroxide (H_2O_2) where CAT reacts with two molecules of H_2O_2 to produce water and oxygen. In addition, this enzyme reacts with hydrogen donors (formic acid, phenols, ethanol or methanol) with the activity of peroxidase

.

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

$$ROOH + AH_2 \xrightarrow{CAT} H_2O + ROH + A$$

H_2O_2 is detoxified by glutathione peroxidase; GPX and catalase; CAT enzymes in animals. Catalase enzyme works on protecting the cells from peroxides which are produced by cell itself. However, under the normal circumstances, catalase enzyme is not important for a few cell varieties. catalase enzyme plays a vital role to gain tolerance against oxidative stress in the cells response. Survival of rats that exposed to 100 % O_2 was redoubled once liposomes having CAT and SOD were injected before and through the exposure intravenously (Turrens JF., Crapo JD. et al., 1984).

Catalase can be additionally acts as a regulatory protein, by releasing $NADP^+$ once the cell is under peroxidative stress leading to increasing detoxification of hydrogen peroxide through the glutathione reductase-glutathione oxidase

mechanisms. (Figure 1.4). Since, the first step of this mechanism depends on the level of unbound $NADP^+$ (Kirkman, H. N., et al.,1980).

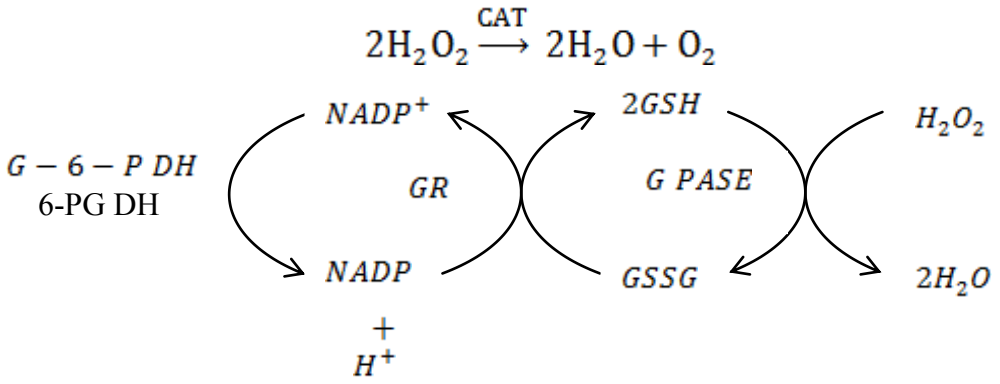

Figure 1.4: Mechanisms of hydrogen peroxide detoxification CAT: Catalase enzyme, G-6-P DH: Glucose -6- phosphate dehydrogenase, 6-PGDH: 6-Phosphogluconate dehydrogenase, GR: Glutathione reductase, G PASE: Glutathione peroxidase, GSH: Reduced glutathione, GSSG :Oxidized glutathione, NADPH : Nicotinamide adenine dinucleotide phosphate

1.3.1.3 Superoxide Dismutase Enzyme (SOD Enzyme):

SOD (EC 1.15.1.1) has been isolated from bovine blood as green copper protein in the first time (Mann and Keilin, 1938). The catalyst is present, being widely distributed among O_2-consuming organisms, aero tolerant anaerobes, and some obligate anaerobes (Fridovich, 1986). All superoxide dismutases, regardless of supply, are multimeric metalloproteins that have scavenging effect on the superoxide radical. The Cu/ZnSODs, as most organism Mn-SODs and FeSODs, are dimeric, whereas the MnSODs from mitochondria and some thermophilic microorganism are tetrameric.

Unrelated three enzymes are addressed by their capacitiy of converting two superoxide molecules to at least one molecule of oxygen and hydrogen peroxide, with the ingestion of two equivalents of H^+. One family of SODs utilizes a Ni particle to interpose the chemistry. Other utilizes the Cu ion complexed with a Zn to try the same action.

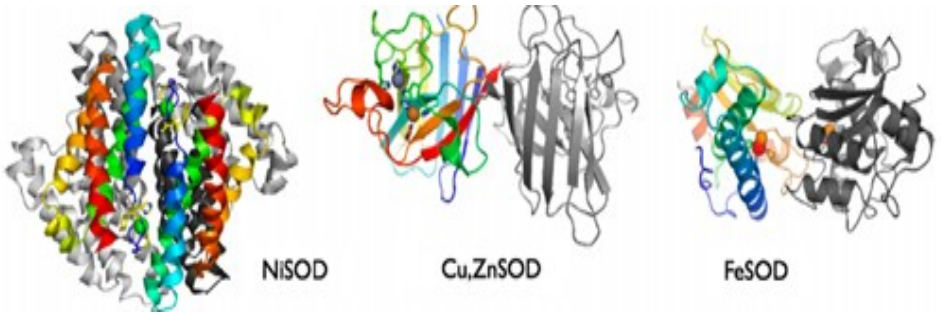

Figure 1.5: Superoxide Dismutase Enzyme (Lah, M.S., Dixon et al., 1995 and Ramirez, D.C. et al. 2009)

The third family comprises enzymes which use Manganese, Mn or iron, Fe. In addition, as enzymes which will use either various families of SODs vary not only with relation to the metal particle that supporting the effectiveness. They may also vary with relation to the fold of super molecule.

1.3.2. Mushrooms as Exogenous Antioxidant:

Mushrooms are group known as fungi which are a separate kingdom from plants, higher basidiomycetes, and they do not have chlorophyll therefore they cannot make their foods. Fungi are fleshly, spore bearing fruiting bodies that live on soil or on their food sources. They are used by humans as food or as drug since a lot of drugs are extracted from mushroom (Law, S.k. and T.B.N.G., 2001) and these mushrooms are called medicinal mushrooms.

Macro fungi are divided ecologically to three groups: parasites, saprophytes and symbiotic (mycorrhizal) species. The most terrestrial macro fungi are symbionts or saprobes but some of the fungi are pathogens or saprobes like species that are fruited on wood substrate. (Mandeel QA. et al., 2007).

Mushrooms live in darkness and wetting places in forests, so they need to protect themselves from microbes by producing natural biologically active compounds. Recent scientific researches explain how compounds that are extracted from mushrooms affect positively on human health (Zaidman et al., 2005).

In the past, fungi were used in traditional medicine in far east. Especially in treatment of cancer that attack gastro intestinal tract and lung as in China, Japan, Korea, Russian, Siberia (Wasser et al., 1999). In addition, many pharmaceutical compounds have been extracted from mushroom recently. There are a lot of studies proposed that higher basidiomycetes mushrooms are probiotic because they help the body to be healthy by increasing the resistance to disease, maintaining physiological hemostasis, and restoring the balance of the body. Medicinal mushrooms are known as Host Defense Potentiators because they enhance the immune system so that they are used with anticancer drug.(Tomatis et al.,2001).

Mushrooms have several biological activities such as antibacterial, antifungal, antioxidant, antiviral, antitumor, cytostatic, immune suppressive, antiallergic, hypoglycemic, anti-inflammatory and hepato protective (Wasser et al., 1999).

1.3.2.1 Mushrooms as Food Supplement:

Numerous artificial antioxidants will effectively improve defense mechanisms but they have adverse effects under some conditions. The natural compounds were preferred to be used as sources for natural, antioxidants instead of artificial compounds. These natural antioxidant present in plants like that present in edible mushrooms so that antioxidant potential and chemical composition of the mushrooms have been studied deeply. For the purpose of improving antioxidants defenses, It can be used the edible mushrooms directly through dietary supplements to reduce oxidative stress. Cultivated or wild mushroom are both associated with important antioxidant properties because of their bioactive components, like minerals, carotenoids, vitamins, polysaccharides, and polyphenols. The reasons of using the edible mushroom as an useful food are to have delicious taste and the antioxidant properties which lead to the health benefits owned. The foods which contain antioxidant and the supplements of antioxidants work to facilitate the oxidative damage against microorganism. Furthermore, antioxidants are widely used in producing foods, packing which use in the cosmetics production, anti-aging and healthcare. Antioxidants materials market are categorized according to the type of product to natural antioxidants and artificial antioxidants.

Natural antioxidants are grouped into plant and plant extracts, flavonoids, ubiquinol (reduced form of molecule Q_{10}), selenium(Se), glutathione, carotenoids, Zink (Zn), spices (nutmeg, thyme, clove, basil, marjoram, pepper, cinnamon, oregano, sage, and rosemary), tocotrienols and tocopherols and vitamin C (Brewer, M.S.,2011). Synthetic phenolic antioxidants as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) et al. e.g., propyl group gallate, tert-ethoxyquin (EQ), tert-butylhydroquinone (TBHQ), (Venkatesh, R., Sood D., 2011). Nevertheless, harmful multiple effects are caused by some of artificial antioxidants under some conditions (Kozarski, M.S., Klaus, A.S., 2014 and Ferreira, I.C.F.R. et al.,2009). BHA, that is usually used as additive in food trade, will have negative effects on organizing of the effectivness of mitogen-activated protein kinase enzyme (MAPK) according to the dose (Yu, R. et al.,1997 and Kozarski, M.S., Klaus, A.S., 2014). Recently, the edible mushrooms have been attracted as food source of antioxidants (Khatua et al., 2013; Ferreira, I.C.F.R., Barros, 2009 and Kozarski, M.S., Klaus, A.S., 2014). Besides the distinctive flavor of mushrooms, which are owned, they are considered to be the foods with low fat level. As well as, mushrooms contain high percentage of unsaturated fatty acids (PUFA), creating them similar temperament for low calorie diets. Edible mushroom provides a nutritionally significant content of vitamins (B1, B2, B12, C, D, and E) (Finkel, T., Holbrook, N.,2000 and Kozarski, M., Klaus, A. et al., 2015). Furthermore, the glycemic index of the mushroom is weak and also it contains a large amount of mannitol, so it is a useful food for the patients who have diabetes. Moreover, the concentration of sodium is very low in mushroom, so it is considered a useful food for hypertensive patients (Chang, S.T., Wasser, S.P., 2012). In Asia, mushrooms are used so far in home treatments against many diseases caused by stress, which produces ROS (Khatua, S., Paul, S., Acharya, K., 2013)

Mushrooms have many biological activities besides their antioxidant advantages like antitumor, antidiabetic, anti-coagulant, antiviral, hypolipidemic, hepatoprotective, immunostimulant. Due to their activities mushrooms are used in waste water management, the cosmetics industry, bio-medicine, nutrition and environmental protection. (Van Griensven, L.J.L.D., 2009; Loria-Kohen, V., Lourenco-Nogueira, T., 2014 and Kozarski, M.S., Klaus, A.S., 2014).

1.3.2.2 Antioxidant Compounds in Mushrooms:

Many edible mushrooms have been reported to have antioxidant effectiveness, and the compounds of antioxidant which found in their fruit mycelium, bodies and broth are polysaccharides, flavonoids, phenolics, ergothioneine, ascorbic acid, tocopherols, carotenoids, and glycosides (Kozarski, M., Klaus, et al, 2015; Chen, S. Y., et al, 2012 and Klaus, A., Kozarski, M., et al, 2011).

There are two main varieties of antioxidants in mushroom, the first group are primary and they are called chain breaking, free radical scavengers (Brewer, M.S., 2011; Ferreira, I.C.F.R. et al., 2009; Kozarski, M.S., Klaus et al., 2014; Kozarski, M., Klaus et al., 2015 and Klaus, A., Kozarski, M. et al, 2011). The second group are secondary or preventive antioxidants which are the consequence of deactivation of metals, inhibition or breakdown of lipid hydro peroxides, singlet oxygen ($^1O_2$) extinguishing. Some mushroom which have antioxidant activity function as inducers of cell signals, leading to changes in gene expression, that result in the enzymes activation which detoxify ROS (Ferreira, I.C.F.R., Barros et al., 2009, and Chang, H.Y., Ho et al., 2007).

1.3.2.2.1 Polyphenols:

Food contains a large variety of polyphenols, which generated different biological activities (Johnson, I.T., Scalbert et al., 2005; Vujovic, D. et al., 2015 and Kuntz, S., Wenzel, U., Daniel, H., 1999).

The phenolic acids are the major phenolic structure that were found in mushrooms (Ferreira, I.C.F.R., Barros et al., 2009). Phenolic acids can be classified into main teams, hydroxybenzoic acids and hydroxycinnamic acids, which are derived from the non-phenolic compounds benzoic and cinnamic acid, respectively (Ferreira, I.C.F.R., Barros et al., 2009; Manach, C., Scalbert et al., 2004, and Choi, D.Y., Lee, Y.J. et al., 2012). The hydroxyl cinnamic acids are further prevalent than the hydroxybenzoic acids and involve primarily of ferulic, sinapic, p-coumaric, and caffeic acids.

These acids are rarely found in unstructured form except in processed food items that are subject to the freezing, fermentation or sterilization. The bound forms are glycosylated derivatives or esters of shikimic acid, tartaric acid and quinic acid (Manach, C., Scalbert et al., 2004, and Choi, D.Y. et al., 2012). The greatest prevalent carboxylic benzoic acid derivatives that been found in mushrooms which are stated to be gallic, p-hydroxybenzoic, veratric vanillic, protocatechuic, 5-sulphosalicylic, gentisic, homogentisic, syringic, and vanillin (Ferreira, I.C.F.R. et al., 2009). The work as antioxidant in the phenolic antioxidant compounds by promote self-anti-oxidant capability or ending the free radical types. Furthermore, some polyphenols stimulate synthesis of endogenous antioxidant molecules in cells (Finley, J.W., Kong, A.N. et al., 2011 and Scalbert, A., Johnson et al., M., 2005).

Flavonoids are mostly found in nature within the variety of esterification or glycosylate form of conjugates. They can additionally exist as aglycones in food, particularly as a result of food processing. Flavonols are the greatest abundant flavonoids which occur in foods (Farkas, O., Jakus, J., Heberger, K., 2004 and Amic, D., Davidovic-Amic et al., 2007). Generally, it can be assumed that animals do not have the ability to assemble and supply flavonoids compounds, while the plants have the ability to do (Ferreira, I.C.F.R. et al, 2009).

According to the presence of a C_2-C_3 double bond within the heterocyclic pyrone ring and also the degree of hydroxylation, flavonoids are divided into thirteen categories (Sánchez-Moreno, C., 2002), the most vital categories are isoflavones, flavonols, flavones, flavanols, flavanones, oranthocyanins and anthocyanidins (Scalbert, A., 2000).

The antioxidant action mechanisms of flavonoids are direct scavenging of RS, chelating of trace metal ions interested in RS formation, inhibition of enzymes, e.g., lipoxygenases (LOXs) and xanthine oxidase, involved in producing RS, and membrane-bound antioxidants regeneration like α-tocopherol (Renaud, S.C., de Lorgeril, M., 1992). In general, it can be thought that. the first mechanism of the oxidizing agent scavenging of flavonoids is the contribution of hydrogen atom. (Farkas, O., Jakus, J., Heberger, K., 2004, and Amic, D., Davidovic-Amic et al., 2007). Also, flavonoid can act through the signaling pathways modulation and gene

expression, and this could conjointly donate to protecting the flavonoids characteristics (Manach, C., Scalbert, A. et al., 2004). The position and the existing of many hydroxyl groups in their structure controls the ability of flavonoids to inhibit ROS.

1.3.2.3 *Panus conchatus*:

Panus Chonchatus is mushroom which belongs to polyporaceae family that has a lot of medical use in traditional medicine and recent research proved a lot of pharmacological effect of many genera that follow this family.

The classification of *Panus conchatus* mushroom is:

Kingdom	Fungi
Phylum	Basidiomycetes
Class	Agaricomycetes
Order	Polyporales
Family	Polyporaceae
Genus	*Panus*
Species	*Panus conchatus*

Since, there were a lot of mushrooms that were used in history of medicine as drug such as those from genera *Ganoderma, Lentinus, Flammulina* and other genera that belong polyporaceae family which recent research reported that they have significant medical properties (Wasser S.P., 2002). For example, *Gandorma lucidum* mushroom used in China and Japan in treatment of hepatitis, nephritis, arthritis, bronchitis, gastric ulcer, and recent study on Indian *Gandorma lucidum* proved that they have antimutagenic, and antioxidant effect in vitro (Lakshmi, P.,Ajith et al., 2003).

In addition, there were compounds that isolated from this family have antimicrobial effects such as Steroids like 5a-ergosta-7,22-dien-3b-ol or 5,8-epidioxy-5a,8a-ergosta-6,22-dien-3b-ol, isolated from *Ganoderma applanatum* which are weakly active against a number of gram positive and gram-negative microorganisms (Smania Jr. A., Delle Monache F., 1999).

Moreover, small molecular weight compounds extracted from Ganoderma species have antiviral activity such as ganoderic acid B inhibits protease of human immunodeficiency virus type 1 (El-Mekkawy S., Meselhy MR., 1998)

Panus conchatus that present in Tanzania forest have antioxidant effect and DPPH scavenging effect (Hussein J. M., Tibuhwa D. D. et al., 2015) and another research reported that it has anticancer effect by inhibiting the growth of malignant (Zaidman et al., 2005).Also, laccase enzyme which is polyphenol peroxidase was isolated from *panus conchatus* mushroom and has been used in pulp bleaching (Aniwor et al., 2003, Mo et al 2006).

Figure 1.6: *Panus conchatus* Mushroom (Photo © John Plischke III**)**

1.4 Scope of Study:

The objective of the study is to predict the medical use of *Panus conchatus* mushroom by evaluating its effect on antioxidant defense enzymes which are GST, CAT and SOD, and also by its free radical scavenging capacity.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials:

Panus Conchatus Mushroom, 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 phosphate (Riedel.dehaen), Reduced form of Glutathione (Sigma-Aldrich), 1-Chloro-2,4-Dinitrobenzene (CDNB) (Fluka), Bovine Liver Cytosol was extracted in our laboratory from the bovine liver was brought from slaughter house in Kazan-Ankara, 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) (Calbiochem), Hydrogen Peroxide (H_2O_2) (J.T.Baker), Dichlorohydroxy Benzene Sulfonic Acid (DHBS) (Sigma), 4-Amino Antipyrine (AP) (Acros), Horse Reddish Peroxidase (HRP), Sodium Azaide (Acros), Catalase was purchased from its company 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 Methods of *Panus Conchatus* Mushroom:

Panus Conchatus was collected from Ankara, Turkey by Assoc. Prof. Dr. Ilgaz Akata, Department of Botany, Ankara University, Ankara, Turkey. and It was obtained in dried form.

The dried mushroom was grinding by using liquid nitrogen into powder for extraction. Four methods were used in order to extract *P.conchatus* mushroom. The

extraction methods used in this study were hot water extraction, cold water extraction, methanol extraction and ethanol extraction method (Table 3).

All dried extracts that were obtained from extraction methods, were dissolved for further analysis. Since the dried extracts that obtained by ethanol and methanol extraction methods were dissolved in DMSO, while dried extract that prepared by cold water extraction method was dissolved in water, and dried extract that produced from hot water extraction method was dissolved in 20.668 % DMSO. Finally, dissolved extracts are kept at -20 ^{o}C to use them for further analysis.

Table 2: Concentrations of Prepared Extracts

Method	**Concentration (mg of dried extract / mL of solvent)**
Hot water extraction	26.86
Cold water extaction	29.33
Ethanol extraction	12.00
Methanol extraction	12.47

Table 3: Extraction Methods

	Extraction Methods	**Step I**					**Step II**		**References**
			Centrifugat-ion	Filtration	Times of Extraction		Filtration	Evaporation	
1	Hot Water	1g of dried powder was boiled with 35 mL of distilled water for 1 hour	3000 rpm for 45 minutes	_	Two times	The water extracts were collected	Under pressure	by using rotary evaporator at 60 ^{o}C with 80 rpm to dryness.	Yeh, J. Y., (2011)
2	Cold Water	1 g of powder was stirred with 16mL distilled water at room temperature for 24 hours	_	Under pressure			_		
3	Methanol	1g powder /10 mL alcohol for 30 min and kept it in refrigerator for 24 hr**.**	at 4 ^{o}C, using 6000rpm for 10 minute	_	_	_	Filtration Without pressure	The filtrate was evaporated in rotary evaporator at 45 ^{o}C	Coruh et al., 2007
4	Ethanol								

2.2.2 Determination of Total Phenolic Content (TPC) of *Panus chonchatus*:

Phenolic contents of *P.conchatus* mushroom were measured by using the Folin–Ciocalteu assay (Slinkard and Singleton, 1977). Since total phenolic content of four extracts were measured by using calibration curve of standard gallic acid which was prepared from different concentrations (0-200 µg/mL) dissolved in DMSO. The calculated concentrations of prepared extracts and the standards were given in Table 4. The total phenolic content in extracts were expressed as µg of gallic acid equivalent (GAE)/mL of mushroom extracts solutions.

The reaction components of the phenolic content determination assay was given in Table 5. In tha assay 1mL of the diluted Folin–Ciocalteu agent (1:10) was added to 100 µL of mushroom extracts solutions, and incubated in dark for 5 minutes. After incubation period was finished, 1mL of 2 % (w/v) of $Na_2CO_3$was mixed with previous mixture and then the mixture was incubated for 1 hour in dark at room temperature. The blank contains DMSO instead of mushroom extract. The absorbance was measured at 750 nm, at the end total phenolic components of four extracts were measured by using calibration curve of standard gallic acid which prepared from different concentrations (0-200) µg/mL dissolved in DMSO.

Table 4: Concentrations of the four mushroom extracts and gallic acid control that used in phenolic determination

Dilutions	**Hot Water Extraction (mg/mL)**	**Cold Water Extraction (mg/mL)**	**Methanol Extraction (mg/mL)**	**Ethanol Extraction (mg/mL)**	**Gallic Acid Control (µg/mL)**
1	26.86	29.33	12.47	6.00	50.0
2	13.43	14.67	6.23	3.00	100.0
3	_	7.33	_	_	200.0

Table 5: The reaction components of phenolic contents determination

Stock Components	Added Volumes
Hot water extract	100 µL from each concentration that is given in Table 4
Cold water extract	
Methanol extract	
Ethanol extract	
Gallic acid	
Folin–Ciocalteu's reagent (1:10 diluted with distilled water)	1mL
Incubation for 5 minutes in dark	
a 2 % (w/v) sodium carbonate solution	1mL
Incubation for one an hour in dark at room temperature	
Read at 750 nm	

*The reaction mixture without mushroom extract was read as a blank.

2.2.3 Determination of Total Flavonoid Content (TFC) of *Panus chonchatus*:

The total contents of flavonoids in extracts was measured by using the aluminum chloride colorimetric method (Chang et al., 2002). The standard curve was prepared from quercetin solutions with different concentrations 0–200 µg/mL that were mentioned in Table 6. The calculated mushroom extracts were mentioned in Table 6. The total flavonoid contents of the extracts were expressed as µg of quercetin equivalent (QE) / mL of mushroom extract (µg QE/mL). Ultraviolet-Visible spectrophotometer was used to measure TFC.

The reaction conditions were given in Table 7 in which 250 µL of mushroom extracts were mixed with 750 µL of absolute ethanol, 50µL of 10 % (w/v) aluminum chloride, 50µL of 1M sodium acetate, and 1ml of DMSO, then the mixture was incubated for 30 minutes in dark. The absorbance was read at 415nm. The assay mixture that contains 250µL of DMSO instead of mushroom extract was used as a blank.

Table 6: Concentrations of the four mushroom extracts and quercetin control that were used in flavonoid contents determination assay

Dilution	Hot Water Extraction (mg/mL)	Cold Water Extraction (mg/mL)	Methanol Extraction (mg/mL)	Ethanol Extraction (mg/mL)	Quercetin Control (µg/mL)
1	6.72	29.33	12.47	12.00	25.0
2	3.36	14.67	6.23	6.00	50.0
3	_	_	_	_	100.0
4	_	_	_	_	200.0

Table 7: The reaction components of flavonoid contents determination

Stock Components	Added Volumes
Hot water extract	250 µL from each concentration that mentioned in Table 2.5
Cold water extract	
Methanol extract	
Ethanol extract	
Quercetin standard	
Absolute ethanol	750 µL
10 % (w/v) Aluminum chloride 1M Sodium acetate DMSO	50µL 50µL 1 mL
Incubation for 30 minutes in dark	
Read at 415 nm	

*The reaction mixture without mushroom extract was read as a blank

All assays were conducted with hot water extract. All enzymes assay except GST were done by using multi-mode plate reader (Figure 2.1), since GST enzyme activity was measured by using UV spectrophotometer.

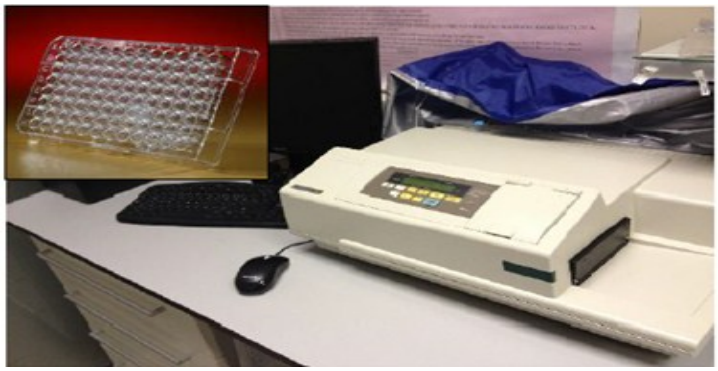

Figure 2.1: Multi mode plate reader (Molecular Devices Spectramax M2) and its 96 well plate

2.2.4 The Effect of *Panus chonchatus* on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity:

It is spectrophotometric assay depends on the stable radical chemical agent (DPPH) where the antioxidant effect of hot water *P.conchatus* mushroom extract depends on transferring of electron or proton from mushroom into DPPH (radical scavenging effect of DPPH free radical) that leads to bleaching the purple color of alcoholic solution of DPPH. The absorbance was measured at 517 nm by using a micro plate reader spectrophotometer depending on the methods of Sharma and Bhat (2009) with some modifications. Different concentrations of quercetin, and gallic acid standards were used as positive control and they were mentioned in (Table 8), And different concentrations of hot water extracts were used, and they were shown in Table 9.

The DPPH free radical scavenging assay component were given in (Table 10). Where 6 µL of each mushroom concentration was mixed with 144 µL of methanol and 50 µL of 200 mM of DPPH, then the mixture was incubated for 25 minutes in the dark at 21-25 ^{O}C. The reaction components without mushroom extract was used as control. The DPPH radical scavenging activity of mushroom was expressed as IC_{50} value and calculated from the dose–response curve.

Table 8: Concentrations of Gallic Acid, and Quercetin that were used in DPPH assay

Serial Dilutions	Stock Quercetin Concentrations (µg/mL)	Final Quercetin Concentrations (µg/mL)	Stock Gallic Acid Concentrations (µg/mL)	Final Gallic Acid Concentrations (µg/mL)
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 9: Concentrations of mushroom extract that used in DPPH assay

Dilutions	Stock Mushroom Concentrations (mg/mL)	Final Mushroom Concentrations (mg/mL)
1	24.735	0.742
2	8.245	0.247
3	2.748	0.082
4	0.916	0.027
5	0.305	0.009
6	0.102	0.003
7	0.034	0.001

Table 10: The DPPH free radical scavenging assay component

Stocks	Volumes
Different standards /mushroom extract	6 µL
Methanol	144µL
DPPH (200 µM)	50µL
Incubate for 25 minutes in the dark at 21-25 ^{O}C	

*DPPH assay mixture without mushroom extract was used as control

2.2.5 The Effect of *Panus chonchatus* on Catalase Enzyme Activity:

This assay is used for monitoring the CAT enzyme activity. This assay relies on the measuring the hydrogen peroxide substrate remaining once the action of the enzyme was stopped by sodium azide, and the remaining H_2O_2 was determined by colorimetric method that depends on the formation of red quinoneimine dye (Aebi, 1984; Bai et al., 1999; Fossati et al., 1980). The assay was miniaturized for micro plate application (Isgor et al., 2008) and The absorbance was measured at 520 nm. The concentrations of mushroom used in assay were given in Table 11. CAT enzyme was supplied from its company sigma. Finally, the reaction components for single reading of CAT enzyme assay was explained in Table 12. Since 4 µL of mushroom extract was mixed with 20 µL of catalase 100 U/mL, 50 µL of 10 mM H_2O_2, 26 µL of 50 mM Phosphate buffer respectively. The mixture was incubated for 2 minutes in dark, after that the reaction was stopped by adding 50 µL of 15 mM NaN_3 and then the solution was incubated for 3 minutes in dark. 255 µL of chromogen with horse reddish peroxidase(HRP) was added to 5 µL of previous mixture and the final mixture was incubated for 40 minutes in dark. The absorbance was read at 520 nm. (Chromogen was prepared by mixing 1 mL of 10 mM Dichloro Hydroxyl Benzene Sulfonic Acid (DHBS) with 150 mM Phosphate Buffer and 1 mL of 1.25 mM of 4 amino antipyrine 4 AP). For each 5 mL chromogen, 5 µL of HRP was added. The enzyme activity was followed by measuring the remaining H_2O_2 which was calculated from calibration curve constructed in range of 0.00192-0.12308 mM of hydrogen peroxide. The reaction mixture without enzyme was read as blank.

Table 11: Concentrations of mushroom extract that used in catalase assay

Dilutions	**Stock Mushroom Concentrations (mg/mL)**	**Final Mushroom Concentrations (mg/mL)**
1	5.582	0.223
2	1.861	0.074
3	0.620	0.025
4	0.207	0.008
5	0.069	0.003
6	0.023	0.001

Table 12: The reaction components that used in a single reading of CAT assay

Stock Solution	**Added Amount**
Chromogen (in total amount of 5mL)	
4 amino antipyrine (4 AP),1.25 mM	1 mL
Dichloro Hydroxyl Benzene Sulfonic Acid (DHBS), 10 mM	1mL
Phosphate buffer,150 mM	3mL
For each 5 mL chromogen, 5 µL HRP was added	
Assay mixture	
Mushroom extract	4 µL
100.1U/ml Catalase	20 µL
Hydrogen peroxide, 10 mM	50 µL
Phosphate buffer, 50 mM	26 µL
Incubation for 2 minutes	
Sodium azide, 15 mM	50 µL
Incubation for 5 minutes	
Above mixture +Chromogen	5 µL+255 µL
Incubation for 40 minutes and read at 520 nm	

*the reaction mixture without enzyme was read as H_2O_2 blank (enzyme control).

*DMSO control was without mushroom extract and had 100% NaN_3 activity.

2.2.6 The Effect of *Panus chonchatus* on Superoxide Dismutase (SOD) Enzyme Activity:

The principle of SOD activity depends on the inhibition of nitroblue tetrazolium (NBT) reduction by using method of Isgor, B.S., 2013. Since the reduction of NBT results from the action of superoxide radicals to blue colored formazan. The measurement was carried out at 560 nm. The concentrations of mushroom are showed in (Table 13), and reaction components in SOD assay was explained in (Table 14), in which 213 µL of assay mixture was mixed with 10 µL of cytosol which was used as source for SOD enzyme, 62 µL of deionized water (DDW), and 5µl of Xanthine Oxidase respectively. The mixture was incubated for 30 minutes in dark, then the absorbance was read at 560 nm. (Assay mixture was composed of 3µL, 25mM NBT (nitroblue tetrazolium), 150 µL,0.3 mM xanthine, and 75 µL, 200 mM sodium carbonate buffer with 10 mM EDTA)

Table 13: The concentrations of mushroom extract that used in SOD assay

Dilutions	**Stock Mushroom Concentrations (mg/mL)**	**Final Mushroom Concentrations (mg/mL)**
1	16.747	0.289
2	5.582	0.096
3	1.861	0.032
4	0.620	0.011
5	0.207	0.004
6	0.069	0.001

Table 14: reaction components in SOD assay

Reagents	Added Volumes (µL)	Contents
Assay Buffer	213	3µL, 25mM NBT(nitroblue tetrazolium), 150 µL,0.3 mM xanthine, 75 µL, 200 mM sodium carbonate buffer with 10 mM EDTA
Cytosol	10	From stock directly
Deionized water(DDW)	62	180ohm,22micro filtered
XOD	**5**	2U/mL
Incubation for 30 minutes		

*****Control was without mushroom, (DDW) was added instead of mushroom extract

2.2.7 The Effect of *Panus chonchatus* on Glutathione-S-Transferase (GST) Enzyme Activity:

The reaction depends on the determination of the conjugation product of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) according to Habig et al., (Habig et al, 1974) with small modification. The cytosol that was extracted from bovine liver in our laboratory was used as GST enzyme source and ultra violet spectrophotometer was used to follow GST enzyme assay. The concentrations of mushroom used in the enzyme assay was given in Table 16. The components of assay mixture was described in Table 15.

In the assay mixture 50 µL of 20 mM CDNB substrate was mixed with different volumes of *P.conchatus* extract. The reaction was started by adding 50 µL of bovine liver cytosol (Table 17). The total volume of assay was 1mL and the absorbance was measured at 340 nm. Initial rates of enzymatic reactions were determined as micro moles of the conjugation product of GSH and reported as micro mole/min.µL.

Table 15: The components of assay mixture that used in GST enzyme assay

Components	Added Volumes
Reduced Glutathione GSH (50Mm)	0.8 mL
0.2 M K_2PO_4 PH 6.5	20.0 mL
Deionized water	13.2 mL

Table 16: The added volumes and the concentrations of hot water mushroom extract in GST assay

Added Volumes (µL)	Mushroom Concentrations (mg/mL)
50	1.343
75	2.015
100	2.686
125	3.358
150	4.029
200	5.372

Table 17: The reaction mixture of GST assay

	Assay Mixture	**CDNB (20 µm)**	**Hot Water Extract (26.86 mg/mL)**	**Bovine Liver Cytosol**
Added Volumes (µL)	850	50	50	50
	825	50	75	50
	800	50	100	50
	775	50	125	50
	750	50	150	50
	700	50	200	50

*****The blank was assay mixture without enzyme, instead of enzyme 50 µL of 0.2 M K_2PO_4 was added.

*****The Control was assay mixture without mushroom extract.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Extraction methods for *Panus chonchatus* mushroom:

Four different extraction protocols were followed to extract the mushroom samples.These methods are hot water extraction, cold water extraction, methanol extraction, and ethanol extraction. The method that produced highest percent of the yield was cold water extraction, and the extract that has highest concentration was cold water extract (Table 18). While the method that resulted lowest percent of the yield was methanol extraction, together with the ethanol extraction both of them had lowest calculated concentrations (Table 18).

Table 18: Concentration and Yield % of *Panus conchatus* mushroom extracts

Mushroom Extracts	**Yield %**	**Concentrations of Extracts (mg/mL)**
Hot Water Extract	25.96%	26.86
Cold Water Extract	26.40%	29.33
Methanol Extract	1.87%	12.46
Ethanol extract	3.00%	12.00

3.2 Determination of Total Phenolic Content (TPC) of *Panus chonchatus*:

Since mushroom with antioxidant phenolic compounds have ability to scavenge reactive free radical and can reduce oxidative damage. The method that resulted extract with highest concentration of phenolic content was the hot water extraction method, and its extract was used to measuring the effect of mushroom on

antioxidant enzymes and used in DPPH assay. Since phenolic content was 195.55 µg of gallic acid equivalent (GAE) /ml of extract and Total phenolic of all *P.conchatus* mushroom extracts are given in Table 19.

3.3 Determination of Total Flavonoid Content (TFC) of *Panus chonchatus*:

Also the extract that has highest flavonoid content was hot water extract and TFC are equal to 120.8 µg of querceten equivalent(QE) /mL of extract. TFC of all extracts were calculated in Table 19.

Table 19: TPC and TFC of *P.conchatus* mushroom extracts

Mushroom Extracts	Total Phenolic Contents (µg/mL)*	Total Flavonoid Contents (µg/mL)**
Hot Water Extract	195 55	120.80
Cold Water Extract	154.32	42.21
Methanol Extract	169.50	16.92
Ethanol extract	94.00	19.25

*Total phenolic contents expressed as µg of gallic acid equivalent/ml of extract
**Total flavonoid contents expressed as µg of querceten equivalent/mL of extract.

Since the hot water extract has the highest phenolic and flavonoid contents, all further analysis were followed by using it, hot water extract of *Panus chonchatus*.

3.4 The Effect of *Panus chonchatus* on 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Scavenging activity:

In this assay 2,2-Diphenyl-1-Picrylhydrazyl was used as free radical, and Different concentrations of quercetin, and gallic acid standards were used as positive control (Table 8). Also, different concentrations of hot water extract were used and they mentioned in (Table 9). The effect of mushroom to stabilize the free radical DPPH was given in inhibition curve (Figure 3.1).It was concluded that the free radical (DPPH) scavenging activity of *P.conchatus* was 100 %, and IC_{50} was

0.02214g/L. IC_{50} values of quercetin and gallic acid standards were 0.031 g/L and 0.0096 g/L respectively.

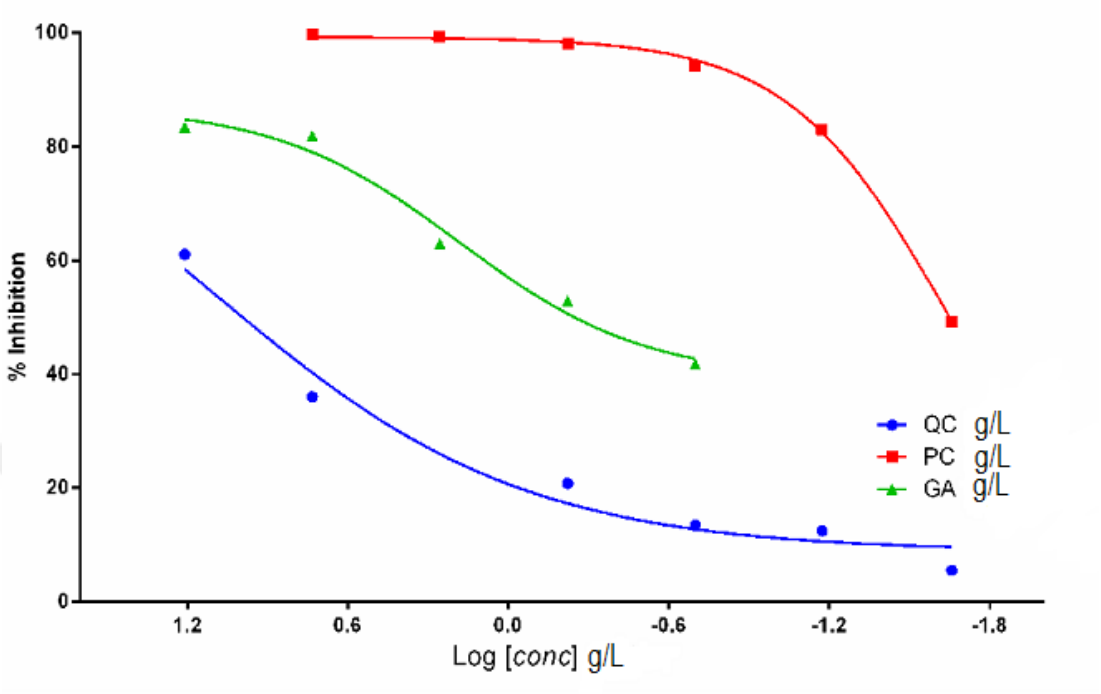

Figure 3.1: Effect of *P.conchatus* mushroom extract to stabilize 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging Activity

3.5 The Effect of *Panus chonchatus* on Catalase (CAT) Enzyme Activity:

The commercially available catalase enzyme was used in the assay which was purchased from sigma company and hydrogen peroxide as substrate. In the assay six concentrations of mushroom extract were used to measure the effect of mushroom on catalase enzyme activity which was mentioned under Method (Table 11).

The *Panus conchatus* mushroom inhibited the catalase enzyme activity less than 25 % of the control. Since the effect was insignificant the IC_{50} value of the extract was not detected. (Figure3.2).

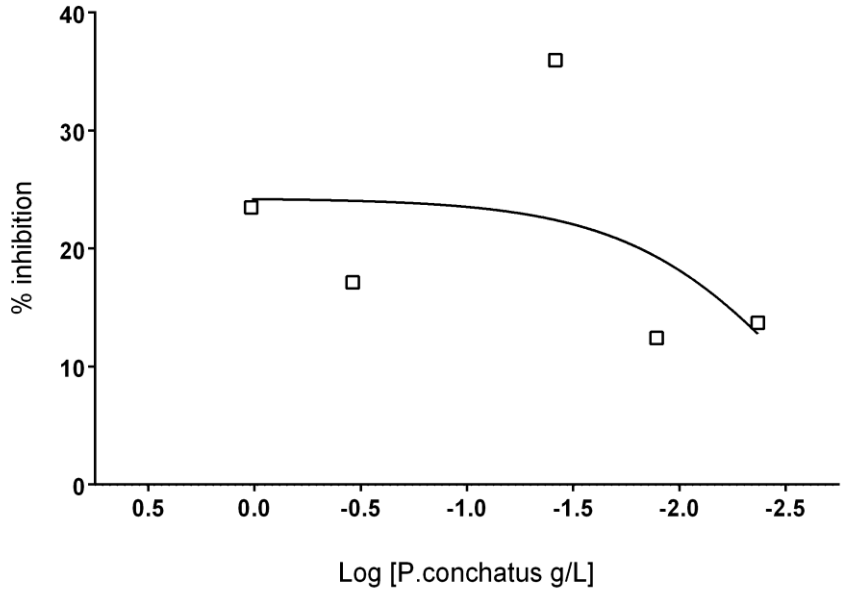

Figure 3.2: Effect of *Panus conchatus* mushroom on catalase enzyme activity with maximum 24 % inhibitory effect with respect to control.

3.6 The Effect of *Panus chonchatus* on Super Oxide Dismutase (SOD)Enzyme Activity:

The bovine liver cytosol with the protein amount 0.928 mg /mL was used as source for superoxide dismutase enzyme assay and six different concentrations of mushroom were used to measure the effect of mushroom on enzyme activity as it was mentioned under Method (Table 13).

The effect of *P.conchatus* mushroom on superoxide dismutase activity was given in Figure3.3. Since the maximum inhibitory effect of extract was less than 25 % of control, it was not possible to determine the IC_{50} value from dose-response curve on figure. Hence, the extract is said to have no significant effect on superoxide dismutase activity.

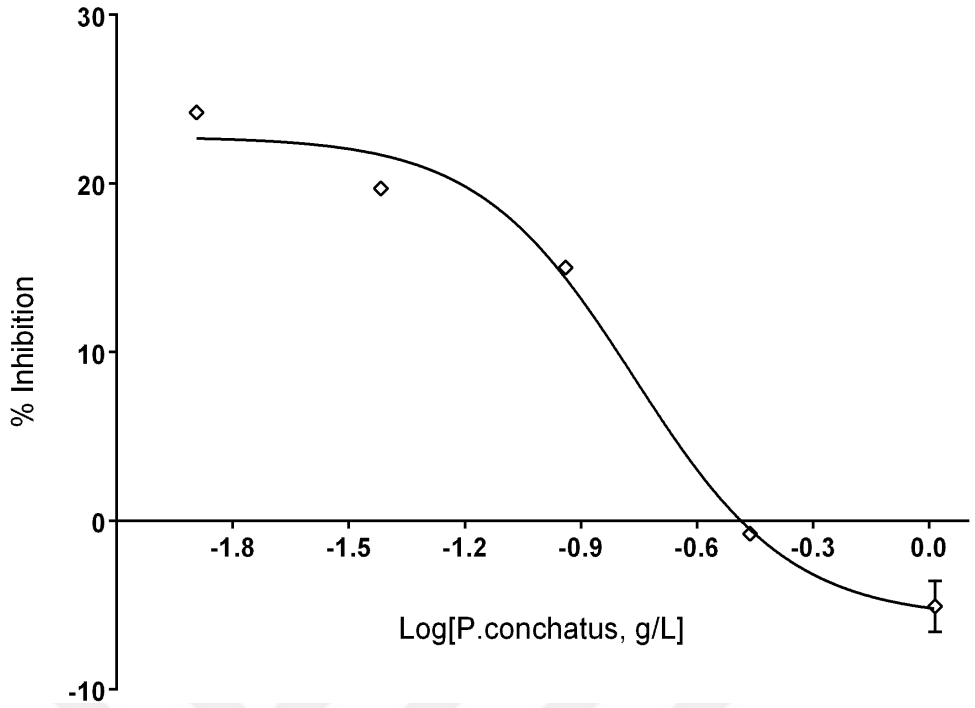

Figure 3.3: Effect of *Panus conchatus* mushroom on superoxide dismutase enzyme activity with maximum 23 % inhibitory effect with respect to control

3.7 The Effect of *Panus chonchatus* on Glutathione-S-Transferase (GST) Enzyme Activity:

Liver bovine cytosol (0.928 mg protein /mL) was used as source for glutathione-S-transferase enzyme. The reaction components without mushroom extract was taken as control and it has maximum enzyme activity between 0.0023 and 0.0024 Micro mole/min.µL. The concentrations of *P.conchatus* mushroom used in the assay was given in Table 16 under Method.

The effect of *Panus conchatus* mushroom on glutathione-S-transferase enzyme activity towards its substrate is given in Figure 3.4. The figure showed that the enzyme activity was inhibited by six different concentrations of mushroom extract. Since Effect of *Panus conchatus* mushroom on glutathione-S-transferase enzyme activity has maximum 47 % inhibitory effect with respect to control, and IC_{50} was 0.3744g/L.

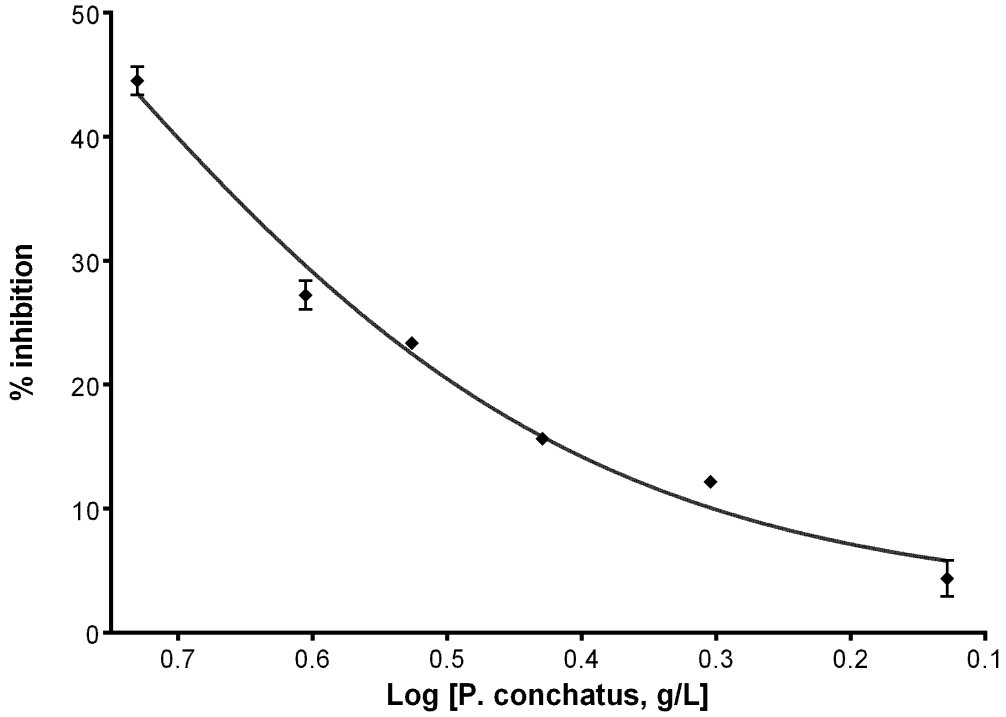

Figure 3.4: Effect of *Panus conchatus* mushroom on glutathione-S-transferase enzyme activity with maximum 47 % inhibitory effect with respect to control

The data analysis was applied with the Graph Pad Prism 6.0 program (Graph Pad Software, San Diego, CA). The kinetic results were expressed as inhibition of enzyme activity with respect to control (inhibition as % of control).

DISCUSSION

In recent years, mushrooms have attracted attention because of their natural medicinal properties. Generally mushrooms rich in various polyphenolic and flavonoid compounds are recognized as antioxidants (Cheung, LM,,Cheung PC. et al.,2003). Studies on 5 totally different species of fungus genus (Agaricus) have conjointly showed the presence of antioxidant compounds like phenol, flavonoids ascorbic acid, ß-carotene and carotenoid and vital antioxidant activity (Barros L., Falcão et al., 2008). In addition, Wong and Chye (2009) and Heleno et al., (2012) also reported the antioxidant activity of mushroom to be absolutely related to the content of their phenolic compounds.

In this study four different extraction methods were used to extract the phenolic and flavonoid contents from *Panus conchatus* which was collected from Ankara region. Among the methods the hot water extract showed highest total phenolic content (195.55 µg of Gallic acid equivalent /mL of extract), and it also has the highest total flavonoid content (120.80 µg of quercetin equivalent /mL of extract). Due to the highest phenolic and flavonoid content of hot water extract all further analysis was done by using this extract. Among the methods the ethanol extract has lowest TPC (94.00 µg of GAE / mL of extract) and methanol extract has lowest TFC (16.92 µg of QE / mL of extract).

In the literature it has shown that *Panus conchatus* that were collected from Tanzania forest, with phenolic (273.51 mg GAE100 g^{-1}) and flavonoid (7.49 mg QE100 g^{-1}) contents, has lower DPPH scavenging activity that was about 46.53 % (Hussein J. M., Tibuhwa D. D.2015), when it is compared with the *Panus conchatus* mushroom which collected from Ankara and used in this study (phenolic content was 195.55µg GAE / ml of extract), (flavonoid content was 120.855µgQE / mL), its scavenging activity was 100 %.

Free radical scavenging could be a well-known mechanism simply saying that antioxidants inhibit lipid oxidation (Cheung et al., 2003). In general, other specieses like Pleurotus species showed some ability to stabilize, 1-diphenyl-2-picrylhydrazyl (DPPH), which redoubled as the concentration of fungus extract redoubled (Fu et al., 2002 and Yang et al., 2002). The mushrooms tested by Fu et al., (2002), *P. ostreatus and P. eryngii* had low scavenging ability, starting from 14.9% to 29.1%, While Yang et al. (2002), found that *P. ostreatus* scavenging ability was high, about 80%. like scavenging ability of *Panus conchatus* mushroom that was measured in this study was high too (100 %).

In general, there were not researches that studied the effect of mushrooms on antioxidant system, where they studied the level of antioxidants inside mushroom itself such as L. Ramkumar, t. Ramanathan and j. Johnprabagaran in 2012 showed that other species which is the *Volvariella volvacea* (dried Mushroom) has high levels of antioxidants; Catalase (37.37±0.06 µmol/min/mg), Superoxide Dismutase (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)when these mushroom were cultured with calcium carbonate. So, *V. volvacea* can be considered as a source for natural antioxidant. In contrast, the effect of *Panus conchatus* on antioxidant system was measured in this study and no effect of mushroom extract was shown on antioxidant enzyme. Since it showed less than 50% inhibition effect on glutathione-S-transferase enzyme, inhibition effect on both catalase and superoxide dismutase enzymes were less than 25 %. Again in the literature, it has shown that, *Panus conchatus* mushroom has anticancer activity due to presence of two epoxy compounds derivatives of quinones (panepoxydone and cycloepoxydon) that are NF-κB inhibitors, since NF-κB, is involved in tumor growth (Zaidman, B., Yassin, M. et al., 2005; Umezawa K., 2006). Moreover, laccase in *Panus conchatus* has been used in pulp bleaching (Aniwor et al.,2003, Mo et al., 2006).

CONCLUSION

In this study the flavonoid and phenolic content of *Panus conchatus* were extracted by using different extraction methods. That are hot water extraction, cold water extraction, methanol extraction, and ethanol extraction. The total phenolic and flavonoid contents of four extracts were compared. Since hot water extract has highest TPC and TFC, all assays were done by using this extract.

From results, it has been showed that ability of *P.conchatus* to stabilize free radical DPPH is too high (100 %). So that, The mushroom may protect the body from free radical that are exogenous like drug or endogenous like super oxide that generated inside the body. So it can be concluded, This mushroom may be useful to be used as food supplement Moreover, the experimental results in this study showed that the inhibition effect of *P.conchatus* on GST enzyme that is the main enzyme in phase II of drug biotransformation was about 47 %, it may be advantages to use this mushroom to gather with anti-cancer drug especially with patient whose cancer cell developed resistance to chemotherapy. It has to be mentioned that further studies are required to study the effect of chemotherapy in absence and presence of this mushroom on cancer cell because *P.conchatus* may potentiate the effect of anticancer drug. It was found that there was no significant inhibitory effect of *Panus conchatus* on both enzymes catalase and superoxide dismutase (less than 25% of controls). That are responsible for protect the body from oxidative damage.

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