THE EFFECT OF *DAEDALEA QUERCINA* MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYME ACTVITES

A MASTER'S THESIS

in

Chemical Engineering and Applied Chemistry

Atilim University

by

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JULY 2016

THE EFFECT OF *DAEDALEA QUERCINA* MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYME ACTVITES

A THESIS SUBMITTED TO

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

OF

ATILIM UNIVERSITY

BY

AMAL RASHUAN

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, Atılım University.

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ABSTRACT

THE EFFECT OF *DAEDALEA QUERCINA* MUSHROOM EXTRACTS ON ANTIOXIDANT ENZYME ACTVITES

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July 2016, pages 52

Recently, there were great interest in use of mushrooms and their extracts as food supplements, since they accepted to enhance immune function, and hence promote health. They are playing important roles for treatment of numerous diseases from diabetes to various types of cancers. Today, most of the clinically effective pharmaceuticals are developed from chemical entities from mushroom in the history of medicine.

In this study, total phenolic and flavonoid contents of methanol, ethanol and water extracts from *D.quercina* (L) Pers. was evaluated, for investigating their potential medicinal values against biological targets that are participating in the antioxidant defense system such as catalase (CAT), glutathione S-transferase (GST), and superoxide dismutase (SOD), and free radical scavenging activity. According to the results, the highest phenolic and flavonoid contents were found in the methanolic

extract of *D.quercina* (L) Pers as 242 µg GEA /mL and 82.562µg QE/mL values, respectively. 2, 2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging was observed with the methanol extract of *D.quercina* (L) Pers. with 50% inhibitory concentration value (IC_{50}) of 0.081 g/L. The maximum GST and CAT enzyme inhibition activities were observed with methanol extract of *D.quercina* with 50% inhibitory concentration value (IC_{50}) values of 0.05583g/L and 0.1458 g/L, respectively. The methanolic extract of *D.quercina* (L) Pers. showed less than 50% inhibitory effect on SOD enzyme activity and no IC_{50} value was available.

Keywords: *D.quercina* mushroom, radical scavenging, antioxidant enzymes, DPPH assay, Catalase, Superoxide dismutase, Glutathione-S-transferase

ÖZ

DAEDALEA QUERCINA MANTAR ÖZÜTÜNÜN ANTİOKSİDAN ENZİM AKTİVİTELERİ ÜZERİNE ETKİSİ

RASHUAN, Amal
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Danışman: Doç.Dr. S. Belgin İŞGÖR
Temmuz 2016, 52 sayfa

Son yıllarda mantarlar ve mantar özütleri özellikle bağışıklık sistemini güçlendirerek sağlık üzerine olumlu etkilerinden dolayı gıda takviyesi olarak önemli bir ilgi odağı olmuşlardır. Mantarlar, diyabetten kansere kadar çok farklı hastalığın tedavisinde önemli rol oynarlar. Tıp tarihi boyunca klinikte etkili olan pek çok ilaç mantarların kimyasal bileşiminden geliştirilmiştir.

Bu çalışmada, kuru *Daedalea quercina* (L) Pers. mantarı methanol, etanol ve su kullanılarak farklı özütleme aşamalarından geçirilerek mantara ait toplam fenol ve flavonoid içerikleri ölçülmüştür. *Daedalea quercina* (L) mantarının biyolojik hedefler için olası tıbbi potansiyelinin belirlenmesi için mantarın methanol özütünün katalaz, glutatyon-S-Trasferaz ve superoksit dismutaz antioksidan enzimleri üzerine etkinliği incelenmiştir.

Sonuçlar göstermiştir ki, en yüksek fenolik içeriği 242µg GAE/ml ve flavonoid içeriği de 82,562µg QE/ml değerleri olarak mantarın methanol özütünde tespit edilmiştir.

Mantara ait, 2-2 difenil-1-pikrilhidrazil (DPPH) radikal süpürücü etki inçelenmiş metanolde özütlenmiş mantarın radikal süpürücü etkiyi %50 oranında inhibe ettiği ve bu inhibisyon için IC50 değeri de 0.081 g/L olarak hesaplanmıştır.

Daedalea quercina (L) Pers. mantarının CAT ve GST enzimi üzerine etkileri incelenmiş mantarın maximum inhibisyon etkisi GST için 0.05583g/L, CAT enzimi için 0.1458 g/L IC50 değerlerinde gözlenmiştir. *Daedalea quercina* mantarının methanol özütü SOD enzim aktivitesini %50 den daha az inhibe etmiş olup anlamlı bir IC50 değeri hesaplanamamıştır.

Anahtar Kelimeler: *D.quercina* mantarı, radikal süpürücü, antioksidan enzimler, DPPH, Katalaz, Superoksit dismutaz, Glutathione-S-transferaz

For the beloved memory of my Mother

ACKNOWLEDGMENTS

First and foremost, all praise and thanks to my God (Allah), the lord of universe, the most beneficent, the most merciful who has blessed me throughout my life, with good health, patience, and ability to peruse and complete this study with ease.

I am forever grateful to my home country Libya for the financial support without which it would have been impossible to do this research.

I express sincere appreciation to my supervisor Assoc. Prof. Dr. Belgin İşgör for her guidance and valuable advises and encouragement during my thesis.

My appreciation goes to Assoc. Prof. Dr. Yasemin G. İşgör for her invaluable guidence and support during experiments, her friendship and kindly teaching especially for data analysis throughout this study.

I would like to thank to my lab mates, Marwa Almubrek, Ahlam İbrahim and Maiser Alsamrey for their sincere friendship and supports and providing me a friendly and relaxed atmosphere in the lab.

I wish to express my thanks to Assoc. Prof. Dr. Ilgaz Akata for providing the mushroom sample.

My sincere appreciation is extended to my parent who are always encouraging and praying for me. I am also thankful to my husband Abdul Hamid Khalil for his huge support and patient during the years of my master study contributed towards the completion of this thesis.

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LIST OF ABBREVIATION

4-AP	-	4-amino antipyrine
CAT	-	Catalase
CDNB	-	1-chloro-2, 4-dinitrobenzene
DDW	-	Double distilled water
DHBS	-	Dichloro hydroxyl benzene sulfonic acid
DMSO	-	Dimethyl sulfoxide
DPPH	-	Diphenyl-2- Picryl-Hydrazyl
GAE	-	Gallic acid equivalent
GP_X	-	Glutathione Peroxidase
GSH	-	Reduced Glutathione
GST	-	Glutathione-S-Transferase
HRP	-	Horseradish peroxidase
IC_{50}	-	Concentration of extract required for 50% inhibition of free radical
QE	-	Quercetin equivalent
ROS	-	Reactive oxygen species
RNS	-	Reactive nitrogen species

SOD	-	Superoxide dismutase
TFC	-	Total flavonoid content
TPC	-	Total phenolic content

CAPTER 1

INTRODUCTION

The occurrence and expanded human chronic illnesses include cardiovascular diseases and several types of cancer are expanding worldwide. There are many studies and researchers in this field demonstrated that the genetic inheritance factor plays an important role in disease affecting human that the genetic factors play important role in the vulnerability to the uncontrolled production of reactive oxygen species (ROS) which are continuously made in vivo. Mostly the organisms are well-protected against the damage caused by free-radicals by virtue of defensive antioxidant enzymes including glutathione peroxidase (GP_X), superoxide dismutase (SOD), and catalase (CAT) or by non-enzymatic antioxidants like polyphenols, flavonoids, glutathione (GSH), ascorbic acid (Vitamin C), and α-tocopherols (Vitamin E). Nevertheless, these systems are not inadequate to prevent damage entirely, thus an antioxidant in the human nutrition is fundamental in order to block or diminish oxidative damage. Consuming 'antioxidant-rich' foods can contribute achieving healthy life for human, although it has not proved scientifically that diet with specific antioxidant is a benefit for human body.

Recently, there has been a growing scientific interest in terms of using mushrooms and their products as supplement for human diet based on theories that they strengthen immune system and promote health. In general, mushroom contains many of the most important nutrients and minerals such as iron, chitin, proteins, zinc, fiber, essential amino acids, vitamins, and rich in trace minerals.

There are diversity of secondary metabolites, accumulating by mushrooms including phenolic compounds, terpenes, steroids, and polyketides. Studies revealed that phenolic compounds have antioxidant activity. Even amid the identified species, the ratio of well examined mushrooms is very low. Because of their natural origin, the antioxidants obtained from mushrooms are greater of benefit in comparison to artificial ones such as butylatedhydroxy anisole (BHA) and butylatedhydroxy toluene (BHT), which are being restricted due to their inherent side effects, including carcinogenicity (Amarowicz et al., 2004; Botterweck et at, 2000).

1.1 Mushroom

The kingdom of Fungi can be distributed into four different phyla (Figure 1.1) which include:

I. Basidiomycota (club fungi)

II. Ascomycota (sac fungi)

III. Zygomycota (conjugation fungi)

IV. Deuteromycota (imperfect fungi).

Species in phylum of the Basidiomycota which include species from the Basidiomycetes class which have macroscopic fruiting bodies, adequately large to be noticed by the naked eye. This phylum contains several mushrooms be like umbrellas growing from the ground. In this phylum, the most common genera are *Agaricus* (including the supermarket variety of button mushrooms), *Amanita* (including species that are delicious, deadly, or even hallucinogenic), *Boletus* (best known for the King Bolete), and *Cantherellus* (known for the beautiful and delicious Chanterelle). The species of Ascomycota phylum includes the Truffle mushroom and prized Morel mushroom. *Saccharomyces cerevisiae* is specie from this phylum that is valued because of its biotechnological applications. Black bread mold is the best known specie of Zygomycota phylum, of around 600 species, such as *Rhizopus stolonifer*. The imperfect fungi of Deuteromycota include genera such as phyla*Penicillium* (Penicillin), *Candida albicans* ("Yeast"infections), and

Trichophyton (Athlete's foot) (Alexopoulos, Mims, & Blackwell, 1996; Arora, 1986; Margulis & Chapman, 2009).

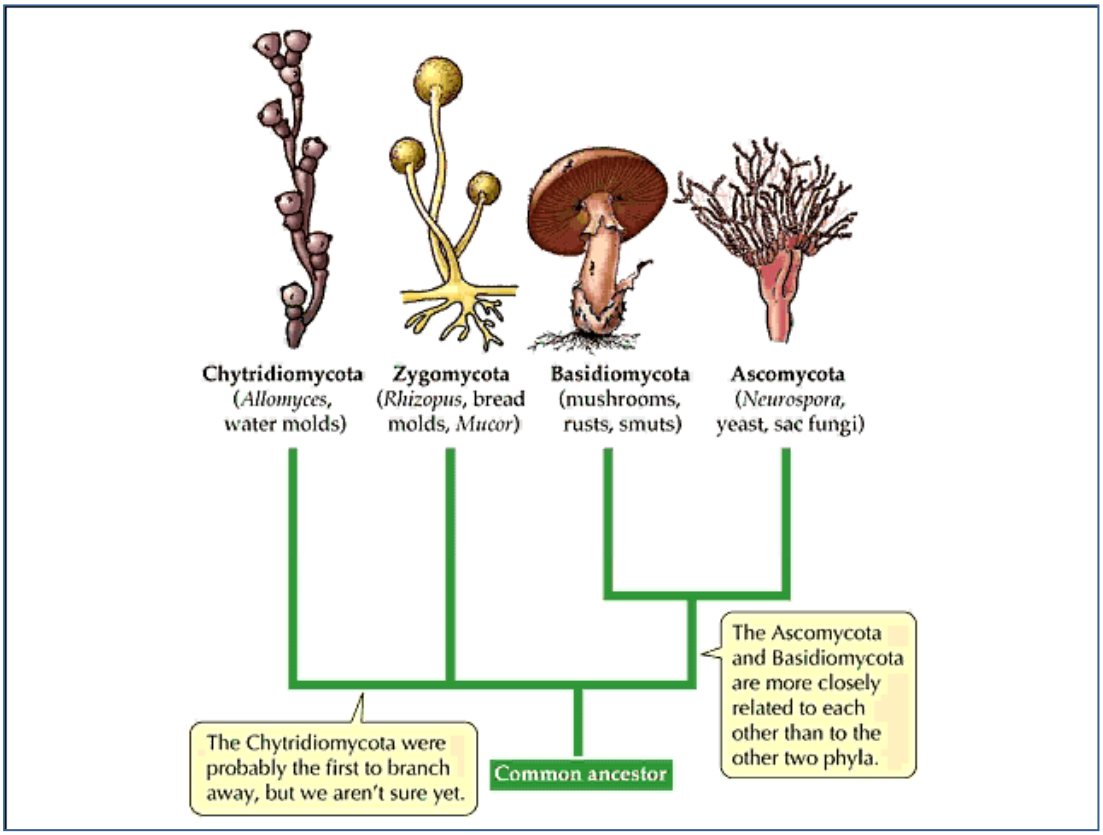


Figure 1.1 A chart of major Fungi phyla

(https://www.pinterest.com/source/wizzyschool.com)

The term “mushroom” has been used according to the definition of Chang and Miles as “a macrofungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand” (S. Chang & Miles, 1992). From a taxonomic viewpoint, mostly basidiomycetes, also some species of ascomycetes belong to mushrooms.

The number of mushroom species on the world is assessed to be 140,000. However, it is suggesting that only 10% have been discovered. Supposing that the proportion of useful mushrooms between the undiscovered and unexamined mushrooms will be only 5%, this indicates 7,000 up till now undiscovered species of potential benefit to mankind (Hawksworth, 2001). In the class of Basidiomycetes, including approximately 10,000 species from 550 genera and 80 families with macroscopic fruiting bodies. Higher Basidiomycetes contain 700 species that have been

discovered to specific important pharmacological activities (Mizuno, 1995; SP Wasser, 1995; S. P. Wasser & Weis, 1999a).

Many of Basidiomycetes mushrooms but not all, have biologically active polysaccharides in fruit bodies, culture broth, or cultured mycelium (S Wasser, 2002). The mushroom look Like plants in terms of its accumulating secondary metabolites like phenolic compounds, terpenes, polyketides, and steroids (Turkoglu et at, 2007). Moreover, the derived of mushroom which are polyphenolic and polysaccharide has been found to be an effective antioxidant, with potent free radical scavenging activity (Kim, Jung, Oh, Kim, & Shim, 2002; Wong, Chai, Tan, & Yong, 2014; Yuswan et al., 2015).

There are many species of mushroom from the Basidiomycetes class containing medicinal active compounds, that have been of great awareness in recent times (Ahad, Tanveer, & Malik, 2014; Arora & Chandra, 2011; Khatua, Paul, & Acharya, 2013; Wong et al., 2014; Yuswan et al., 2015).

Mushrooms include a giant and yet largely available source of pharmaceutical products which has not been exploited. Especially, mushroom represent unlimited source of polysaccharides with antitumor properties which stimulates the immune system.

There are many advantages of consuming mushrooms over plants as sources of bioactive compounds; 1. The fruiting body can often be produced in short time, 2. The produce optimal quantities of active product or 3. Using bioreactors, possible mass production from mycelial biomass and supernatant of submerged culture (Asatiani, Elisashvili, Songulashvili, Reznick, & Wasser, 2010; Cui & Chisti, 2003; Lung & Chang, 2011).

1.2 *Daedalea Quercina* (L) Pers.

Daedalea quercina (L) Pers. (Figure 1.2) is a species of mushroom in the Polyporales order which was established by Persoon (1801). As well as, it is considered as type of the species of the genus *Daedalea***.** It is often found on oaks (the genus *Quercus*), called as the epithet *quercina*. Their fruiting bodies annual or persistent which largely attached, bracket-like, 5–15 cm wide, upper surface convex to flat, uneven, white to pale brown or grayish brown. The pore surface is irregular, with 1 mm or wider in diameter. As well as, the walls between pores are thick and typically maze-like to nearly gill-like; the color is white to light brown at times with pinkish tones. It is in small groups or solitary. This mushroom is inedible due to its cork-like texture (Bessette, Bessette, & Fischer, 1997). In approximately most of the European countries it has been stated, followed to the distribution of oak. In addition, it was reported in Asia from Caucasus to India**,** Northern Africa **(**Tunisia**),** and in Australia (Zarzyński, 2007).

The classification of the *Daedalea quercina;*

Kingdom: Fungi

Division: Basidiomycota

Class: Basidiomycetes

Order: Polyporales

Family: Fomitopidaceae

Genus: Daedalea

Species: D. quercina

Scientific name: *Daedalea quercina* (L) pers.

Common name: Oak Mazegill

Figure 1.2 Morphology of *Daedalea Quercina* (L) Pers.

(http://www.bioimages.org.uk/html/r136996.htm)

1.3 Free Radicals

Free radicals that exist in biological systems are known as chemical species that containing an odd number of electrons in atomic orbitals (Halliwell & Gutteridge, 1999). The radicals in oxidative stress status are presented by ions or small molecules through reactions that are characterized by short lifetimes, and small activation energies. One of the most important characteristics is their small sizes which give them the capability to penetrate membranes of the cell. In general, the free radicals are always unstable and attacking quickly the nearest stable molecules, for instance lipids, carbohydrates, DNA and proteins, to capture the required electron to gain stability. Free radicals are either formed by cells through various metabolic processes or produced due to the exposure to dissimilar forms of radiations, chemicals, and environmental stress (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000). Major sources of free radicals are presented in Figure 1.3.

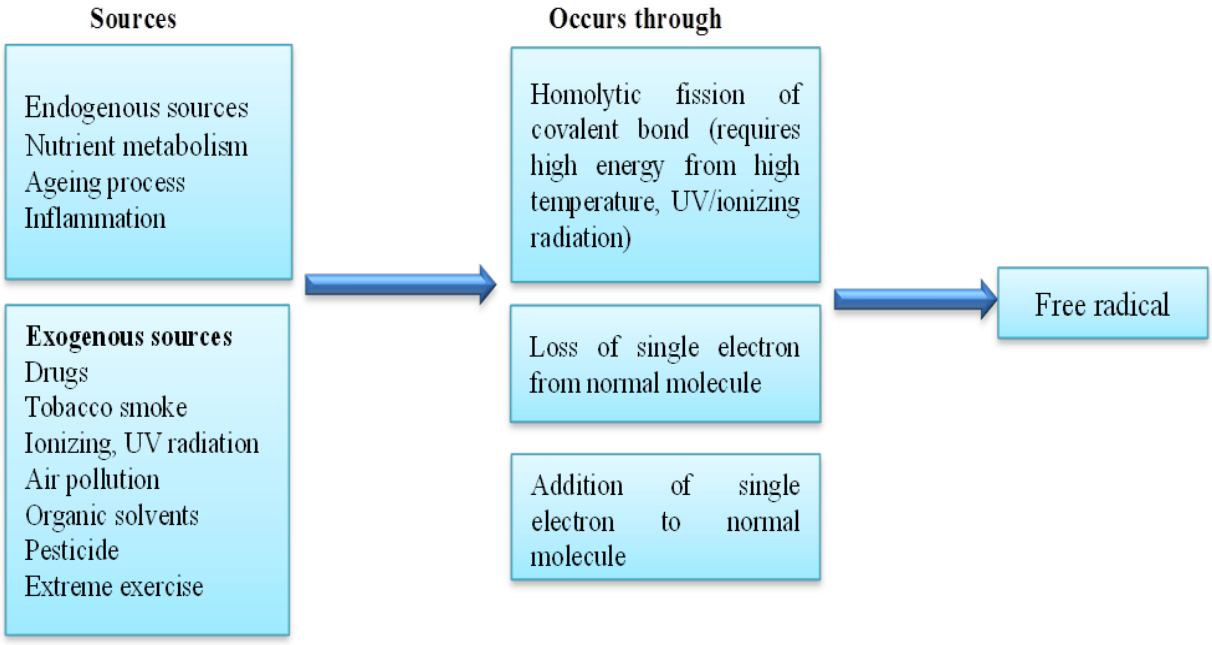


Figure 1.3 Major sources of free radical and chemistry behind the generation of free radical (Cheeseman & Council, 1993).

Aging, and initiating many illnesses such as cirrhosis, hypertension, diabetes, cancer, Alzheimer's disease, Parkinsonism, atherosclerosis are caused by free radicals (Halliwell & Gutteridge, 1985).

In terms of chemistry and biology there are different types of free radicals. Free radicals, and hence analyzed in two main groups: I. Reactive oxygen species (ROS), II. Reactive nitrogen species (RNS)

Reactive oxygen species (ROS) are chemical structures, either stable or transient, containing oxygen atoms which are highly reactive in character as a result of the occurrence of unpaired valence shell electrons, and mediate extensive biological interactions. The oxygen by excitation (O_2^-) or reduction (superoxide radicals ($O_2^{-\cdot}$), hydroperoxyl radicals ($HO_2^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), peroxyl radicals ($ROO^{\cdot}$) and alkoxyl radicals ($RO^{\cdot}$)) which are generated free radicals. However, not all ROS are free radicals. Also, there are many non-radicals which are designated as ROS and include ozone (O_3), singlet oxygen (O_2^1), hypochlorous acid (HOCl) and hydrogen peroxide (H_2O_2).

While other forms of reactive species are reactive nitrogen species (RNS). Just like ROS, not only do RNS also have free radical forms such as nitric oxide ($^{\cdot}NO$) and nitrogen dioxide radical ($^{\cdot}NO_2$), in addition to non-radicals which include peroxynitrate (O_2NOO^-), peroxynitrite (ONOO), dinitrogen trioxide (N_2O_3), nitronium cation (NO_2^+)), and peroxynitrous acid (ONOOH) (Aruoma, 1998; Halliwell & Gutteridge, 1999).

1.4 Biological Defense Systems

Exposure to internal and external sources of free radicals have been motivated the organisms to develop a series of biodefense mechanisms against oxidative damage prompted by ROS. The biodefense systems involve preventative, repair mechanisms, in addition to physical defenses, and these are dependent upon antioxidants.

1.4.1 Antioxidants

Oxidation is considered as an important and essential for most of the organisms to produce the energy to fuel biological processes. There are several mechanisms for the free radicals formation process and present antioxidant systems eliminating the radical induced damage (Kamata & Hirata, 1999).

Through the antioxidant defenses, the ROS are metabolized; some synthesized in vivo and other obtained as diet-derived (Halliwell & Gutteridge, 1999). The antioxidant defense network function is to control the ROS levels to be allowed to achieve the beneficial functions at the time of increasing the oxidative damage. John Gutteridge and Halliwe in 1999 has defined the antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell & Gutteridge, 1999). Antioxidant defenses include enzymatic defenses such as CAT, GP_x, SOD enzymes, and non-enzymatic defenses such as ascorbic acid (vitamin C), tocopherol (vitamin E), glutathione, carotenoids, flavonoids. The intracellular sources of ROS are summarized in Figure 1.4.

In normal conditions, there is a sense of balance between the activities and the intracellular levels of these antioxidants (Figure 1.5). Nevertheless, the process of producing free radicals in uncontrolled way; which leads to produce of too many radicals in relation to the available antioxidants the situation, is called oxidative stress.

There are more than hundreds of diseases which related to oxidative stress, including cardiovascular diseases, atherosclerosis, several types of cancer, cirrhosis, drug induced deafness, diabetes, neurological sicknesses (Alzheimer`s syndrome, mild cognitive impairment, Creutzfeldt-Jacob syndrome, meningoencephalitis), Parkinson`s syndrome, senile and lung diseases (Valko et al., 2007).

The different antioxidants provided defense mechanisms in contradiction to the effects caused by excessive oxidation (Antolovich et al., 2002).

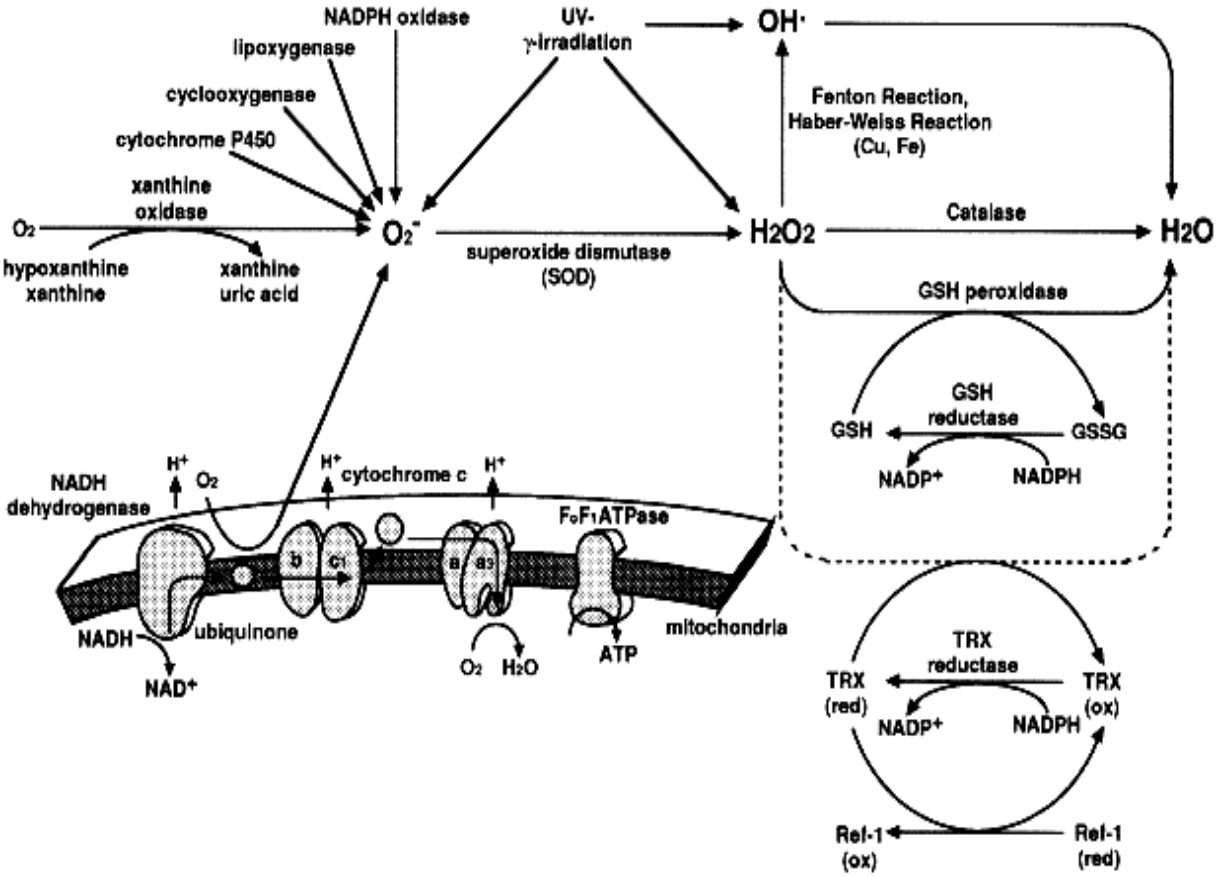


Figure 1.4 Metabolic pathways of reactive oxygen radicals generated in cells by several pathways (Kamata & Hirata, 1999).

The body has various antioxidant defensive systems in enzymatic and non-enzymatic forms which eliminate toxic species by using the subsequent mechanisms:

I. Enzymatic defense mechanism is accomplished by the endogenous enzymes. These enzymes act against the free radical action; either by enzymatic hydrolysis of ester bonds to remove fatty acid peroxides or by enzyme catalyzed reduction of peroxides, or by sequestration of transition metal ions.

II. Non enzymatic defense mechanism is additionally referred to as sacrificial mechanism where the antioxidant compound sacrifices one of its electrons and changes to a comparatively unreactive radical. The low molecular weight molecules are always performing as antioxidants such as uric acid, fatty acids, glutathione, carotenoids and flavonoids and they act as scavengers and halt the free radical propagation.

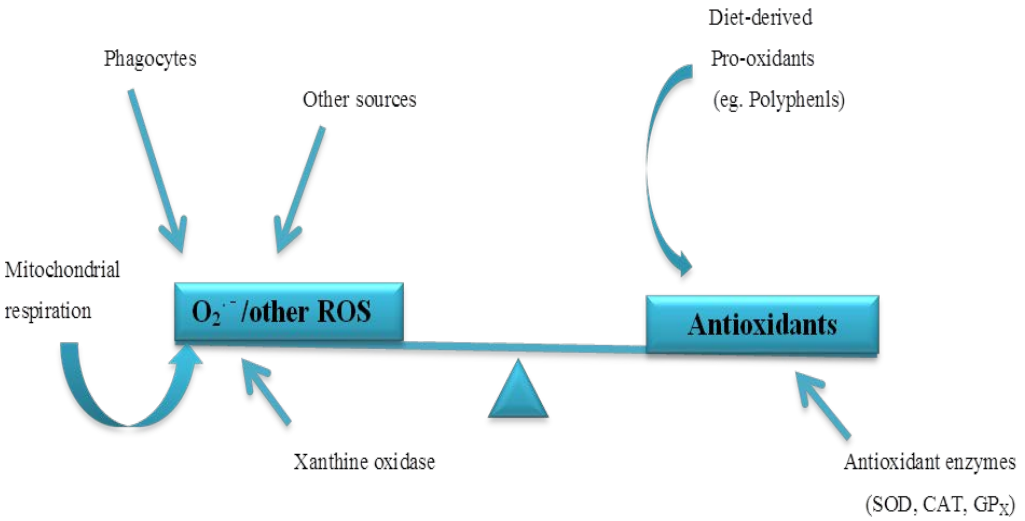


Figure 1.5 Balance of antioxidants and reactive species in vivo (Halliwell, 2008).

1.5 Antioxidant Enzymes

The non-enzymatic scavengers are critical within the defense of cellular constituents of the unavoidable injury of the greatest reactive oxidizing species. However, this protection is not adequate to deal with continuously created radicals, such that to destroy superoxide anion or hydrogen peroxides specific antioxidant enzymes which have been naturally designed. The main benefit of utilizing enzymes is that the steady-state concentration of H_2O_2 is always adjusted according to the cellular requirements. Many of the antioxidant enzymes might be activated, repressed or induced by endogenous effectors, and they play a significant role within the regulation of metabolic pathways and specific functions.

1.5.1 Glutathione-S-transferase (GST) Enzyme Family

Soluble glutathione-S-transferase, GSTs (E.C.2.5.1.18) are (homo or hetero) dimeric, abundant multifunctional proteins composed of two polypeptide subunits (Dixon, Lapthorn, & Edwards, 2002). The superfamily of GSTs is divided into two subfamilies known as cytosolic and mitochondrial GSTs. Cytosolic GSTs constructed the largest part of superfamily with distinct activities; such as thiolysis, isomerization and reduction (Hayes et al., 2005). They can bind covalently and non-covalently to non-substrate ligands and have roles in disposition of xenobiotics, and also their intracellular transport. These non-substrate hydrophobic ligands are some steroids, bilirubin, heme and lipophilic antitumor medications (Hayes & Pulford, 1995). On the other hand mitochondrial GSTs exist to protect against endogenously produced genotoxic and cytotoxic electrophiles (Harris, Meyer, Coles, & Ketterer, 1991).

Mammalian soluble GSTs are categorized in five classes that are associated with their structure and substrates as alpha, (α), mu (μ), pi (π), sigma (σ) and theta (θ). The active site in these enzymes contains two binding sites referred to as GSH-binding site (G site) and xenobiotic-binding site (H site) (Prade, Huber, & Bieseler, 1998). As well as, an insect of the specific delta class has also been outlined, and bacteria which contains a prokaryote of the specific beta class of GST (Bhat et al., 2010) (Figure 1.6). The GSTs catalyze the overall reaction shown below:

$$GSH + R\text{-}X \rightarrow R\text{-}SG + HX$$

Glutathione transferases are soluble proteins each with unique molecular weight about 50 KDa, and composed of two polypeptide subunits as illustrated in Figure 1.7. Classically, they stimulate the transfer of the tripeptide glutathione to a cosubstrate (R-X) that contains a reactive electrophilic center to create a polar S-glutathionylated reaction product (R-SG) (Dixon et al, 2000). Glutathione is important antioxidant small peptide and its reduced form so called GSH is a tripeptide (Figure 1.8) with ɣ-Glu-Cys-Gly sequence. Under oxidation GSSG is formed as 2 tripeptides bound.

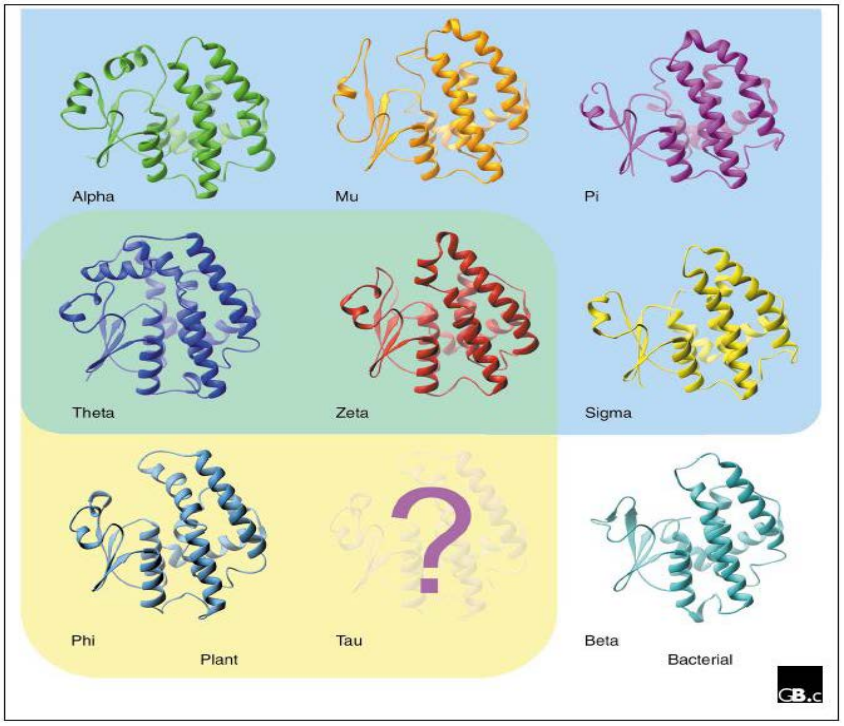


Figure 1.6 The structures of GST subunits: Blue background indicates GSTs specific to mammals, yellow background indicates plant specific ones and white background shows bacterial-specific GSTs (Dixon, Lapthorn, & Edwards, 2002).

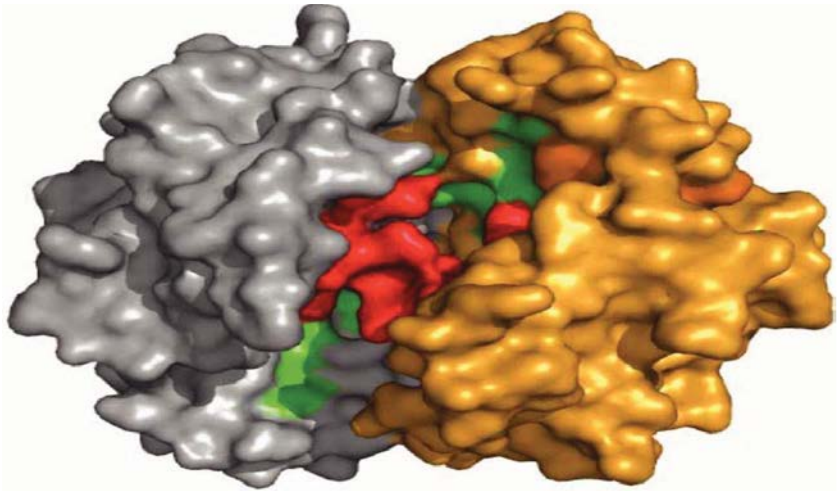


Figure 1.7 Structure of GST Enzyme: Grey, monomer 1; yellow, monomer 2; green, G-site; red, H-site (Bhat et al., 2010).

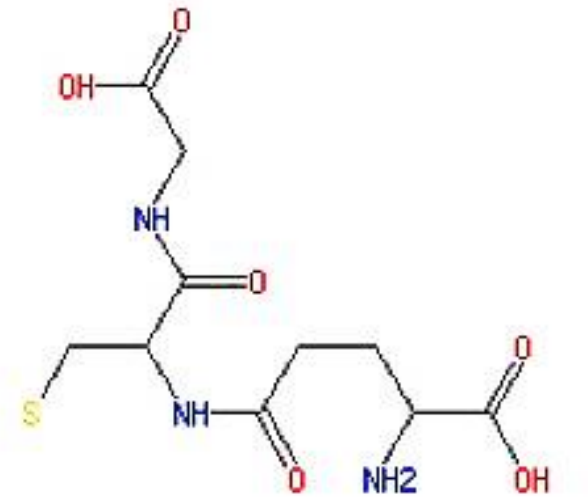


Figure 1.8 Structure of GSH Subunits.

1.5.2 Catalase (CAT) Enzyme

Catalase (E.C: 1.11.1.6) is a tetrameric hemoprotein (porphyrin containing) with a relative molecular mass of 240 KDa (Figure 1.9). Each subunit contains Fe (III) at its active site. A representative activity of catalase is that the disintegration of the H_2O_2 into water and oxygen. Furthermore, they have peroxidative functions in order to convert peroxides (ROOH) into water and alcohol (ROH). Catalase catalyzed reaction is a dismutation reaction in which one H_2O_2 is reduced into H_2O and the other H_2O_2 is oxidized to oxygen.

Below is the representation of catalase catalyzed reaction occurring in two distinctive steps:

$$\text{Catalase-Fe (III)} + H2O2 \rightarrow \text{Compound I} + H2O$$

$$\text{Compound I} + H_2O_2 \rightarrow \text{Catalase} + \text{Fe (III)} + H_2O + O_2$$

$$\text{Net: } 2\,H_2O_2 \rightarrow 2H_2O + O_2$$

The catalases are categorized into two parts; small subunit (55 to 69 KDa) enzymes containing heme b, and large subunit (75 to 84 KDa) enzymes within which the heme group have flipped through 180° and modified to heme d (Dunford, 2010). Catalase is principally confined in the peroxisomes. And also found within the cytosol of

human neutrophils and in rat heart mitochondria.

Catalase can be restrained by azide, cyanide, peroxynitrite and hypochloric acid (Halliwell and Gutteridge, 2007). The NADPH is bounded in the cells of mammalian for the purpose of catalase and the protecting the enzyme from inhibition by H_2O_2 (Kirkman & Gaetani, 1984).

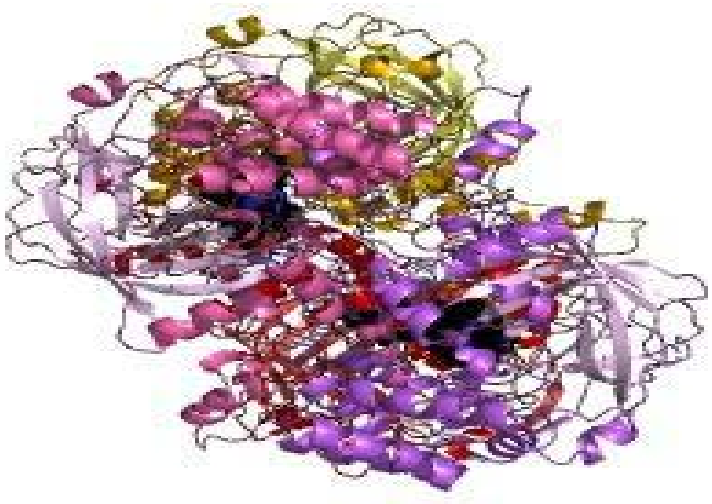


Figure 1.9 The structure of CAT (J. Bravo et al., 1995)

1.5.3 Superoxide Dismutase (SOD) Enzyme

Superoxide dismutases (E.C:1.15.1.1) are metalloenzymes, which catalyze the dismutation of superoxide to peroxide and oxygen. It has a critical role in protective cells and tissues against oxidative stress. SODs are classified into four diverse isozymes in organisms: a copper and zinc containing form (Cu-Zn SOD) which localized within the cytosol, a manganese containing form (Mn-SOD) within the mitochondria, iron containing form (Fe-SOD) within some prokaryotes and plants' outer mitochondrial membrane, and a copper-zinc containing form within the extracellular matrix (EC-SOD) (Marklund, 1982). The SODs catalyzed dismutation of superoxide possibly is written with the subsequent half-reactions:

$$M^{(n+1)+} - SOD + O_2^{-} \rightarrow M^{n+} - SOD + O_2$$

$$M^{n+} - SOD + O_2^{-} + 2H^{+} \rightarrow M^{(n+1)+} - SOD + H_2O_2.$$

Where M = Cu (n=1); Mn (n=2); Fe (n=2)

Copper-zinc superoxide dismutase (Cu-Zn SOD) is a homodimer with a distinct molecular weight of 32 kDa, localized in the cytosol. Moreover, it is found in the peroxisomes and nucleus. Cu-Zn SOD contains Cu and Zn at its active sites. Manganese superoxide dismutase (Mn-SOD), which constitutes around 10 to 15% of entire cellular SOD activity, which is a homotetramer, and their molecular weight is 88 kDa (Fridovich & Freeman, 1986). MnSOD have manganese (III) at its active site, which is localized in the matrix of the mitochondria.

Fe-SOD is localized in plants and some bacteria. Animal tissues have not yet been found to contain Fe SOD. On the other hand, some algae, yeast, and higher plants do. Mn and Fe SODs their active sites exhibit a high degree of sequence and structure similarity. These enzymes originated from a common ancestry as mentioned in a strong suggestion. EC-SOD is a secretory tetrameric Cu-Zn containing glycoprotein, with a distinct molecular weight around 135 kDa (Marklund, 1982). EC-SOD is the least abundant of the SODs in tissues, which is also the major SOD in extracellular fluids such as extracellular and matrix plasma (Maklund, 1984; Oury et al., 1994; Sandström et al., 1993).

The Fe-SOD and Mn-SOD are not inhibited by cyanide anion (CN^{-}), whereas Cu-Zn SODs are. Consequently, the inhibition by cyanide can be utilized to discriminate Cu-Zn SOD activities in tissue homogenates. Likewise, exposure to peroxide deactivates the Cu-Zn SOD and Fe SOD but not Mn SOD (Halliwell & Gutteridge, 2015). Thus, incubating the homogenates with hydrogen peroxide deactivates Fe SOD, but not Mn SOD that allowing the discrimination of these two closely related isoforms.

1.6 Non-enzymatic Antioxidants

Plants produce numbers of antioxidant compounds (Hanson, 2008). Exogenous dietary antioxidants such as vitamin C, carotenoids and polyphenols are found in plants, microorganisms, fungi, and even animal tissues. Epidemiological indication proposes that intake of an antioxidant-rich diet, such as vegetables, fruits, and tea may confer a significant influence in avoiding of chronic human disease for instance heart disease coronary (Agarwal et al., 2000; Asplund et al., 2002). The mushroom accumulates a wide range of the secondary metabolites which include terpenes, steroids, phenolic compounds, and polyketide. Phenolic compounds have been found to possess antioxidant activity. The phenolic compounds have their origins as byproducts from many intermediates in primary metabolism.

1.6.1 Polyphenolic Compounds

Polyphenolics are the compounds which have one or more aromatic rings with one or more hydroxyl groups. The Polyphenolic Compounds are existed in vegetables and fruits. As well as, in many other food sources which forming the highest part of our diets and they are considered as the greatest potent and therapeutically valuable bioactive substances. The common characteristic of phenolic compounds is at least a hydroxy-substituted aromatic ring. Currently, there are more than 8000 polyphenols and their derivatives have been specified (Andersen & Markham, 2005).

Basic polyphenolic classifications include phenolic acids, stillbenes, coumarins, tannins, and flavonoids. The foremost phenolic compounds which are commenced in mushrooms are phenolic acids.

Although phenolic compounds are non-nutritive compounds, they own specific health effects. Also, they might provide health benefits with reduced risk of chronic diseases which might be as a result of their capability to reduce agents by donating a hydrogen atom and quenching singlet oxygen (Nijveldt et al., 2001).

Furthermore, phenolic compounds play a significant role within the stability of food products, as well as in the antioxidative defense mechanisms of biological systems (Jovanovic et al., 1998).

The total efficiency of a natural phenolic antioxidant determined by participating of the phenolic hydrogen atom in radical reactions. The stability of the natural antioxidant radical made throughout radical reactions, and also the chemical substitutions current on the structure. The replacements on the structure can be the more in terms of ability of a natural antioxidant to contribute within the control of radical reactions and to form resonance-stabilized natural antioxidant radicals (Barlow, 1990).

The phenolic antioxidants (ArOH) have an important role in disturbing the chain reaction by donating of hydrogen atom to radicals according to:

$$R^{\cdot} + ArOH \longrightarrow RH + ArO$$

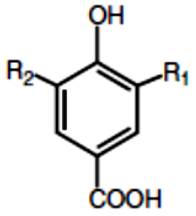
The phenoxy radical intermediates ($ArO^{\bullet}$) are a comparatively stable free radical because of resonance and hence a new chain reaction is not easily initiated. Likewise, the phenoxy radical intermediates also act as terminators of propagation rout by reacting with other free radicals “chain-breaking antioxidant” (Wrigth et al., 2001).

$$ArO + R^{\cdot} \longrightarrow ArOR$$

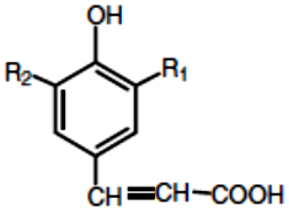
1.6.1.1 Phenolic Acids

Phenolic acids are considered as the most important member of phenolic compounds and they often classified into two major groups, hydroxycinnamic acids (e.g., ferulic acid, caffeic acid, p -coumaric acid, chlorogenic acid, and sinapic acid) and hydroxybenzoic acids (e.g., gallic acid, p –hydroxybenzoic acid, protocatechuic acid, vanillic acid, and syringic acid) (Figure 1.10), which are derivative from benzoic and cinnamic acid, respectively (Ferreira et al., 2009; Harbone et al., 2000).

In the wild mushrooms, there are variety of phenolic compounds which have been detected for instance gentisic acid, gallic acid, vanilic acid, and vanillin (Ferreira et al., 2009). Paxillus panuoides is found to contain two p-terphenyls which is potent inhibitors of lipid peroxidation (BONG-SIK et al., 2000) .



Benzoic Acid Derivatives



Cinnamic Acid Derivatives

Compound	R_1	R_2	Compound	R_1	R_2
p-Hydroxy benzoic acid	H	H	*p*-Coumaric acid	H	H
Vanillic acid	H	OCH_3	Ferulic acid	H	OCH_3
Syringic acid	OCH_3	OCH_3	Sinapic acid	OCH_3	OCH_3
Dihydroxybenzoic acid	OH	H	Caffeic acid	OH	H
Gallic acid	OH	OH			

Figure 1.10 The category of Phenolic acids.

1.6.1.2 Flavonoids

Flavonoids have represented a large group of phenolic compounds with antioxidant activity, which occur as expected in plants and are presented in flowers, fruits, grains, vegetables, barks, roots, stems, and derived products such as tea and wine. The risk of major chronic diseases have been linked to these compounds in order to diminish their severity (Liu, 2004). They are characterized as for the carbon skeleton C6–C3–C6. The compounds primary structure contains 2 aromatic rings (A and B rings) which connected by a three carbon chain that is typically in an oxygenated heterocycle ring, or C ring (Figure 1.11) (Iwashina, 2000).

According to the basis of differences in the generic structure of the heterocycle C ring there are variety of flavonoids and can be categorized into six subgroups: flavones (luteolin and apigenin), flavonols (quercetin, kaempferol, and myricetin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanins and isoflavonoids (genistein) (Nijveldt et al., 2001) are common flavonoids in the diet (Liu, 2004).

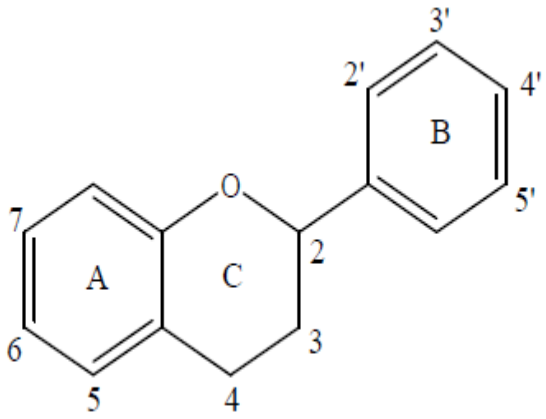


Figure 1.11 The general structure of flavonoids.

The defensive effects of flavonoids in biological systems are recognized according to their capacity to transfer electrons to free radicals, activate antioxidant enzymes, chelate metal catalysts, reduce alpha-tocopherol radicals, and inhibit oxidases and anti-inflammatory, anti-proliferative activities. The flavonoids have greatly effective scavengers of most forms of reactive molecules, including the singlet oxygen and numerous free radicals (Bravo, 1998), that are probably involved in DNA damage and tumor promotion (Wright et al., 2001) .

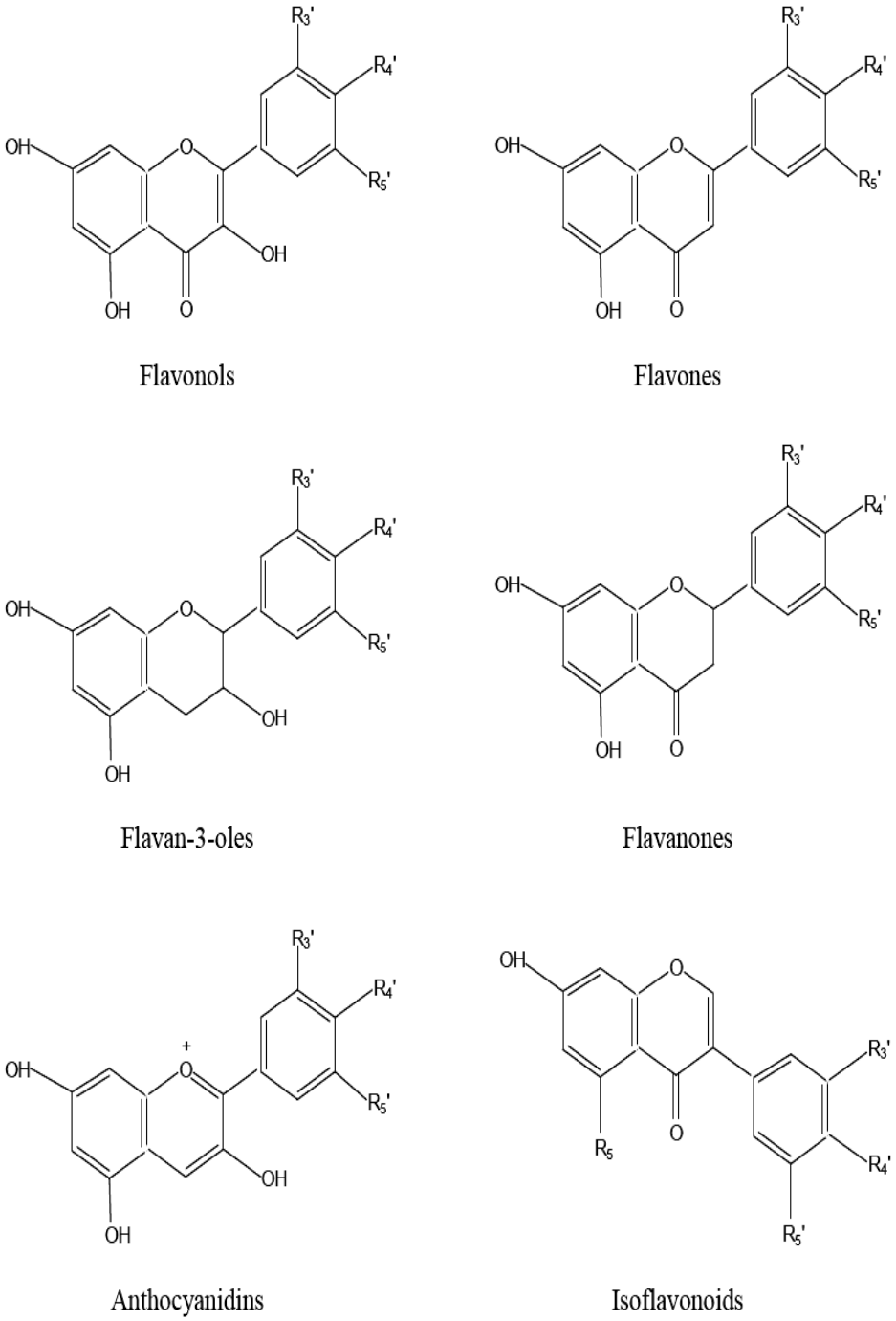


Figure 1.12 Chemical structures of the main flavonoid subclasses.

1.7 Scope of the Study

The aim of this study is to evaluate total phenolic and flavonoid contents of methanol, ethanol, and water extracts of *Daedalea qurecina* (L) Pers., their free radical scavenging activity, their effect on the antioxidant defense system components such as CAT, GST, and SOD to estimate their potential medicinal value.

Chapter 2

MATERIALS AND METHODS

2.1. Materials and Instrumentations

Gallic acid $C_7H_6O_5$ (Merck), quercetin dehydrate $C_{15}H_{10}O_7.2H_2O$ (Sigma), folin-ciocalteu's phenol reagent 2N (Sigma), 99%Mehanol, 96% ethanol (J.T.Baker), Dimethyl sulfoxide (DMSO), sodium acetate CH_3COONa (Sigma), potassium phosphate monobasic KH_2PO_4 (Riedel-de Hae), potassium phosphate dibasic K_2HPO_4 (Riedel-dehen), aluminum chloride anhydrase $AlCl_3$ (Merch), sodium carbonate $NaCO_3$ (Fisher scientific), 1-chloro-2,4-dinitrobenzene CDNB (Fluka analytical), reduced glutathione GSH (Sigma), hydrogen peroxide H_2O_2, Sodium azide NaN_3 and 2,2-diphenyl-1-pichrylhidrazyle DPPH was obtained from Calbiochem company.

Rotary evaporator (Büchi B-490), double beam UV-vis spectrophotometer (HP 8453), Magnetic stirrer-heater (Heidolph Mr Heine), ultra-pure water apparatus (Millipore). During the experiments chemicals kept in refrigerator (Ata, Van Den Bosch, Harwanik, & Pidwinski), ice machine (Scotsman AF80), multimode microplate reader (Spectra Max M2) and microplates (Brandplat), Vortex (Heidolph).

2.2 Methods

2.2.1 Preparation of Mushroom Extracts

The mushroom *Daedalea quercına* (L) pers. was collected from Belgrade Forest (41°11′40″ N 28°57′05″ E), Istanbul, Turkey. Mushroom was authenticated by Assoc. Prof. Dr. Ilgaz Akata, Department of Botany, Ankara University, Ankara, Turkey.

The dried mushroom sample was prepared by cutting in small pieces and crushed into powder in a mortar with liquid nitrogen. The powder was stored at 4°C. Then the sample was extracted by using ethanol, water (cold and boiling water), and methanol with two different extraction protocols by using Me1 and Me2 short symbolizations which will be used to distinguish the two extracts throughout the study (where Me1 was obtained in totally of one round of extraction of the same ground material, and Me2 was obtained in totally of three rounds of extraction of the same ground material) with a sample to solvent ratio of 1:10 (w/v). The extraction steps are summarized in Table 2.1. The obtained sample was dissolved in DMSO, then concentration of the sample was calculated and then the soluble mushroom extracts were divided into 1 mL fractions and stored at -20 °C until used for further experiments. The concentration was expressed as mg extract per mL of solution (mg/mL).

Table 2.1 Etraction steps of mushroom extracts

STEP I				**STEP II**			
Extracts*	Soaking time and temperature	Centrifugation (speed, time,temp.)	Filtering through Whatman No 1 filer paper. (solvent and residue collected separately)	Residue**	Centrefugation (speed, time)	Filtering through Whatman No 1 filer paper. (solvent collected; residue discarded)	The solvents collected from step I and step II are combined and evaporated by using a rotary evaporator at 40 ° C.
Boiling water♣	95° C for 2 h	6000 rpm , 25° C for 10 min		Extracted twice by the same method (boilig water)	6000 rpm , 25°C for 10 min		
Cold water♦	RT for 24 h with stiring	–		Extracted twice by the same method (cold water)	–		
Ehanolic extract♠	4° C for 24 h	6000 rpm , 4° C for 10 min		–	–		
Methanolic extract• (Me 1)	4° C for 24 h	6000 rpm , 4° C for 10 min		–	–		
Methanolic extract°° (Me 2)	RT for 24 h with stirring	–		Extracted twice by the same method (mehanolic extract)	–		

*for methanolic extracts, Me1 and Me2 short notations will be used to distinguish the two extracts
**residue is a solid part which is obtained through the filtration
♣ Oyetayo et al., 2009
♦ Yeh et al., 2011
♠ Koc et al., 2015
• Coruh et al., 2007
°° Yeh,Hsieh, Wu and Tsai, 2011

2.2.2 Determination of Phenolic Contents of Mushroom Extracts

Total phenolic contents of extracts were carried out by using the Folin-Ciocalteu's reagent method (Cicco et al., 2009) with slight modifications. The optimized concentrations of mushroom extracts were found to be the concentration range of 2.443 to 12.215 mg/mL. The assay mixture for this method was given in Table 2.2.

Table 2.2 The reaction components for the Folin-Ciocalteu's reagent method

Stock solution	Volume added
Each extract solutions	0.1 mL
(1:10 diluted with distilled water) Folin-Cioculteu's reagent	1 mL
Incubation for 5 minutes	
2% (w/v) sodium carbonate	1 mL
Incubation for 1 hour at room temperature and read at 750 nm	

The absorbance of reaction mixtures was measured at 750 nm against a blank solution (DMSO) with double beam UV/Visible spectrophotometer. Gallic acid was used as standard. The concentrations of standard were 0, 50, 100, 200, and 400µg/mL in DMSO.

The total phenolic content was expressed as µg Gallic acid equivalent per milliliters of mushroom extract (µg GAE/mL). The total phenolic content for extract was calculated based on the prepared standard calibration curve of Gallic acid. Assay mixture only containing DMSO was used as a blank.

2.2.3 Determination of Flavonoid Content of Mushroom Extracts

Total flavonoid content in extracts was estimated by using the aluminum chloride colorimetric method (Chang et al., 2002). In this method, 96% ethanol, 10% aluminum chloride and 1 M sodium acetate solution were used. Quercetin was used as standard and the concentrations were 0, 25, 50, 100, 200 µg/mL. The optimized concentration of mushroom extracts within the range of 2.443 to 12.215 mg/mL was used. Assay mixture for this method was given in Table 2.3.

Table 2.3 The reaction components for the aluminum chloride colorimetric method

Stock solution	Volume added (µL)
Each mushroom extracts	250
96% ethanol	750
10%(w/v) aluminum chloride	50
1M sodium acetate	50
DMSO	1000
Incubation for 30 minutes and read at 415 nm	

The absorbance of reaction mixtures was measured at 415 nm against a blank solution (96% ethanol) with double beam UV/Visible spectrophotometer. Total flavonoid contents were expressed as µg of quercetin equivalent (QE)/milliliters of mushroom extract (µg QE/mL). Assay mixture only containing ethanol (96%) was used as a blank.

2.2.4 Effect of Mushroom Extract on Glutathione-S-Transferase (GSTs) Enzyme Activity

The change in total GST activities against CDNB (1-chloro-2,4-dinitro benzene) substrate (ε = 0.0096 $\mu M^{-1}cm^{-1}$), was measured spectrophotometrically by monitoring thioether (GSH-CDNB conjugate) formation at 340 nm and adjusted at room temperature (Habig et al., 1974; Isgor et al., 2010). The mushroom extract with various concentrations used in this assay was given in Table 2.4. The reaction components of incubation mixture for GST enzyme assay are shown in Table 2.5. The bovine liver cytosol (19.287mg/mL) was used as source of enzyme. Assay mixture without the enzyme source was used as blank.

Table 2.4 The concentrations of mushroom extract were used in GST assay.

Mushroom extract (stock concentrations) (mg/mL)	Mushroom extract (assay concentrations) (mg/mL)
24.34	0.12215
16.287	0.0814
12.215	0.0611
9.772	0.04886
8.140	0.0407
4.886	0.0244
3.490	0.0175

Initial rates of enzymatic reactions were determined as micromoles of the conjugation product of GSH and reported as µmole/minute/mg protein.

Table 2.5 The reaction components for single reading of GST enzyme assay

Stock solution	Volume added
Assay buffer	
50mM GSH	0.4 mL
200mM phosphate buffer, pH(6.5)	10 mL
Double distilled water	6.6 mL
Added amount in 1 mL cuvette	
Assay buffer	895 µL
20mM CDNB	50 µL
Mushroom extract	5 µL
Cytosol	50 µL
Read at 340 nm	

2.2.5 Effect of Mushroom Extract on Catalase (CAT) Enzyme Activity

The CAT activity was measured by using previously described method (Weydert et al., 2010) with some modifications for microplate applications (Isgor et al., 2013). using purified CAT (100 unit/mL) from bovine liver cytosol (Sigma) as the enzyme source, against 10 mM the hydrogen peroxide substrate, in 50 mM potassium phosphate buffer (pH 7.0). The change in CAT activity was monitored at 520 nm, after addition of chromogen solution supplemented with horse radish peroxidase (HRP). The assay mixture containing mushroom extract with various concentrations was presented in Table 2.6. The assay conditions for determining CAT enzyme activity were given in Table 2.7. The assay mixture containing all components except mushroom extract was measured as control.

Table 2.6 The concentrations of mushroom extract were used in CAT assay.

Mushroom extract (stock concentrations) (µg/mL)	**Mushroom extract (assay concentrations) (µg/mL)**
5670	3.022
1890	1.070
630	0.336
210	0.112
70	0.037
23	0.012

Table 2.7 The reaction components for single reading of CAT enzyme assay.

Stock solution	Volume added
Chromagen (in total amount 5 mL)	
10 mM DHBS (dichloro hydroxy benzene sulfonic acid)	1 mL
1.25mM 4 AP (4-amino antripyrine)	1 mL
150 mM Phosphate buffer, pH (7.0)	3 mL
For each 5 mL Chromagen 5 µL HRP (horseradish peroxidase) was added	
Assay mixture	
Mushroom extract	4 µL
50 mM Phosphate buffer, pH (7.0)	26 µL
100 U/mL Catalase	20 µL
10 mM Hydrogen peroxide	50 µL
Incubation for 2 minutes	
15 mM Sodium azide NaN_3	50 µL
Incubation for 3 minutes	
(above mix) + chromagen	5 µl + 255 µL
Incubation for 40 minutes and read at 520 nm	

2.2.6 Effect of Mushroom Extract on Superoxide Dismutase (SOD) Enzyme Activity

The SOD activity was measured by using previously described method (Bruce & Winge, 1984) with some modifications for micro plate applications (B. S. Isgor et al., 2013). The bovine liver cytosol was used as enzyme source (0.928 mg protein/mL) with the existence of 0.3 mM Xanthine, 2 U/mL XOD, 25 mM nitro blue tetrazolium (NBT), in 200 mM sodium carbonate buffer (pH,10) containing 10 mM EDTA. After adding the XOD the change in absorbance at 550 nm for 30 min was monitored as SOD activity. The assay mixture containing mushroom extract with various concentrations was presented in Table 2.8. The assay conditions for determining SOD enzyme activity were given in Table 2.9.

The assay mixture containing all components except mushroom extract was measured as control.

Table 2.8 The concentrations of mushroom extract were used in SOD assay.

Mushroom extract (stock concentrations) (µg/mL)	**Mushroom extract (assay concentrations) (µg/mL)**
5670	0.305
1890	0.102
630	0.034
210	0.011
70	0.004
23	0.001

Table 2.9 The reaction components for single reading of SOD enzyme assay.

Stock solution	**Volume added (µL)**
Assay buffer	
25mM NBT (Nitro blue tetrazolium)	3
0.3mM Xanthine	150
200 mM sodium carbonate buffer (pH 10) containing 10 mM EDTA	75
Assay mixture	
Assay buffer	213
cytosol	10
DDW(double distilled water)	57
Mushroom extract	5
XOD (xanthine oxidase)	5
Incubation for 30 minutes and read at 550 nm	

The antioxidant activity of the mushroom extract was estimated by using the 2, 2-DiPhenyl-1-PicrylHydrazyl (DPPH) free radical scavenging method. This method was originally described by Brand-Williams et al. (1995). Then, it is modified by Sánchez-Moreno et al. (1998) and is one among the most widely used antioxidant assay since it allows high-throughput screening of potential antioxidant capacity. The 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$, Figure 2.1) is stable organic nitrogen radical, which has deep purple color and commercially available. This assay determines the reducing capacity of antioxidants toward $DPPH^{\bullet}$. Upon reduction, the color of $DPPH^{\bullet}$ solution diminishes and this color change is appropriately monitored spectrophotometrically at 517 nm. Thus, test compounds with high antioxidant activity result in a rapid drop in the absorbance of the $DPPH^{\bullet}$ (Amarowicz et al., 2004; Antolovich et al., 2002).

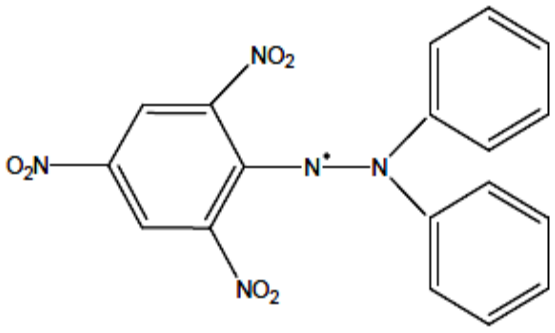


Figure2.1 Chemical structure of 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$).

when a solution of $DPPH^{\bullet}$ is added to a substance that may a donate hydrogen atom, the reduced form of the radical is created supplemented by loss of color (Ali et al., 2008) (Figure 2.2). The assay was performed in a 96-well microplate reader (Koc et al., 2015). The protocol was as follows: 6µL from several concentrations of methanolic extract were mixed with 144µl of 96% ethanol and 50 µl of DPPH (200µM). The mixture was shaken well and left to stand for 25 min in the dark at 25°C.

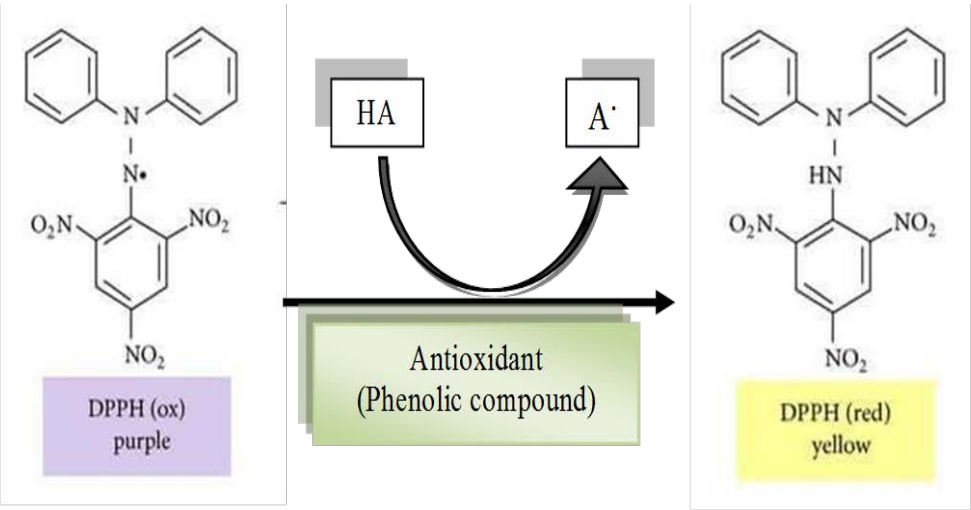


Figure2.2 DPPH· Free radical conversion to DPPH by antioxidant compound.

The reduction of the DPPH radical was determined by measuring the absorbance at 517 nm. The optimized concentrations of mushroom extract were given in Table 2.10. Galic acid and quercetin were used as standards. The final standard concentrations of gallic acid 0.741, 2.222, 6.67, 20, 60, 180, and 540 µg/mL for quercetin were 0.247, 0.741 , 2.222, 6.67, 20 , 60, 180 µg/mL. The DPPH radical scavenging activity of each sample was expressed as IC_{50} value and calculated from the dose–response curve.

Table 2.10 Concentrations of mushroom extract were used in DPPH assay.

Mushroom extract (stock concentrations) (µg/mL)	**Mushroom extract (assay concentrations) (µg/mL)**
18.41	0.5520
6.14	0.1840
2.05	0.0614
0.68	0.0205
0.23	0.0068
0.08	0.0023
0.03	0.000758

CHAPTER 3

RESULTS

3.1 Mushroom Extracts

Each extract was prepared by dissolving 1 g of dry samples in 10 mL solvents methanol, ethanol, and water. The extraction yields of *Daedalea qurecina* (L) Pers. respect to dry mass were measured in between 2.8 and 5.5 % and concentrations of extracts are presented in Table 3.1.

Table3.1 The percent (% w/w) yield and concentration of *Daedalea qurecina* (L) Pers. extracts

Extracts*	**Yield %(W/W)**	**Concentration (mg dry extract/mL)**
Methanol (Me1)	2.8	18.67
Methanol (Me2)	5.5	22
Ethanol	4.3	28.67
Boiling water	5.32	2.4
Cold water	4.09	2.56

*Me1extracted for one time and Me2 extracted for three times

3.2 Determination of Phenolic Contents of Mushroom Extracts

The phenolic contents of the mushroom were determined by using spectrophotometrical methods. Total phenolic contents (TPC) of extracts were measured within ranging from 65.91 to 260.885 µg GAE/mL of mushroom extract. According to the results the highest values of total phenolic contents were found in the methanol (Me2) extract with 260.885 µg GAE/mL of mushroom extract. Cold water extract, whereas, showed the lowest TPC (65.91 µg GAE/mL) (Table 3.2).

Table3.2. Total polyphenolic contents (TPC) of mushroom extracts.

Extracts*	**TPC (µg GAE/mL)**
Methanol (Me1)	242.92
Methanol (Me2)	260.89
Ethanol	176.24
Boiling water	133.76
Cold water	65.91

*Me1extracted for one time and Me2 extracted for three times

3.3 Determination of The Flavonoid Content of Extracts

Total flavonoid contents (TFC) were measured varied from 23.47 to 119 µg QE/mL of mushroom extract (Table 3.3). The best flavonoid contents were achieved by methanolic (Me2) extract with 119 µg QE/mL of mushroom extract. Cold water extract showed the lowest flavonoid content as 23.47 µg QE/mL of mushroom extract.

Table 3.3 Total flavonoid contents (TFC) of mushroom extracts

Extracts *	**TFC (µg QE/mL)**
Methanol (Me1)	82.92
Methanol (Me2)	119.00
Ethanol	75.06
Boiling water	73.25
Cold water	23.47

*Me1extracted for one time and Me2 extracted for three times

Mushroom that has high phenolic contents are normally well correlated with antioxidant activity (Cheung et al., 2003). The result showed that methanolic (Me1) extract was considered to be the best solvent for extraction of TPC and TFC compared to other solvents so methanolic (Me1) extract is used in further studies.

3.4 Determination of Effect of Mushroom Extract on Enzyme Activity

The data analysis has been applied with the Graphpad Prism 6.0 program (GraphPad Software, San Diego, CA). The kinetic results were expressed as inhibition of enzyme activity with respect to control (inhibition as % of control). IC_{50} values were gotten from dose–response curves that were constructed.

3.4.1 Effect of Mushroom Extract on Glutathione-S-Transferase (GSTs) Enzyme Activity

The final concentration of methanolic extract used in the assay was optimized within the range of 0.0175-0.1222 mg/mL. The inhibitory effect of methanolic extract of *Daedalea qurecina* (L) Pers. on GST activity was about 60 % and IC_{50} value was calculated as of 0.05583 g/L (Figure 3.1).

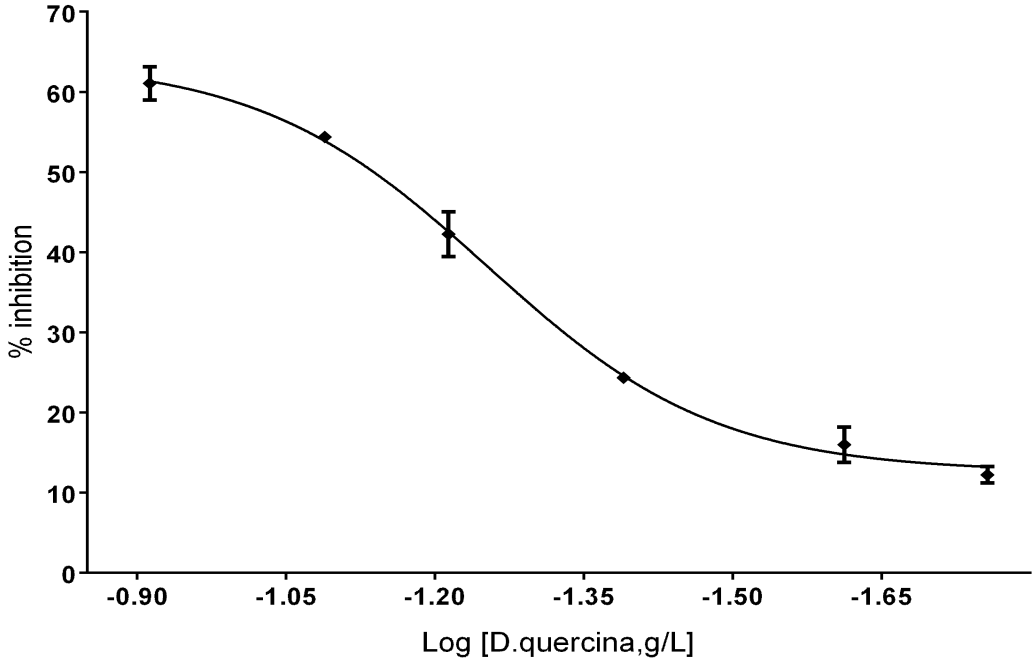


Figure 3.1 Percent GST inhibitory activity of methanolic extract of *D.quercina* (L) Pers.

3.4.2 Effect of Mushroom Extract on Catalase (CAT) Enzyme Activity

The final concentration of methanolic extract used in the assay was optimized within the range of 0.0124-3.0220 µg/mL. The inhibitory effect of methanolic extract of *Daedalea qurecina* (L) pers. on CAT enzyme activity was about 85 % and IC_{50} value was calculated as of 0.1458 g/L (Figure 3.2).

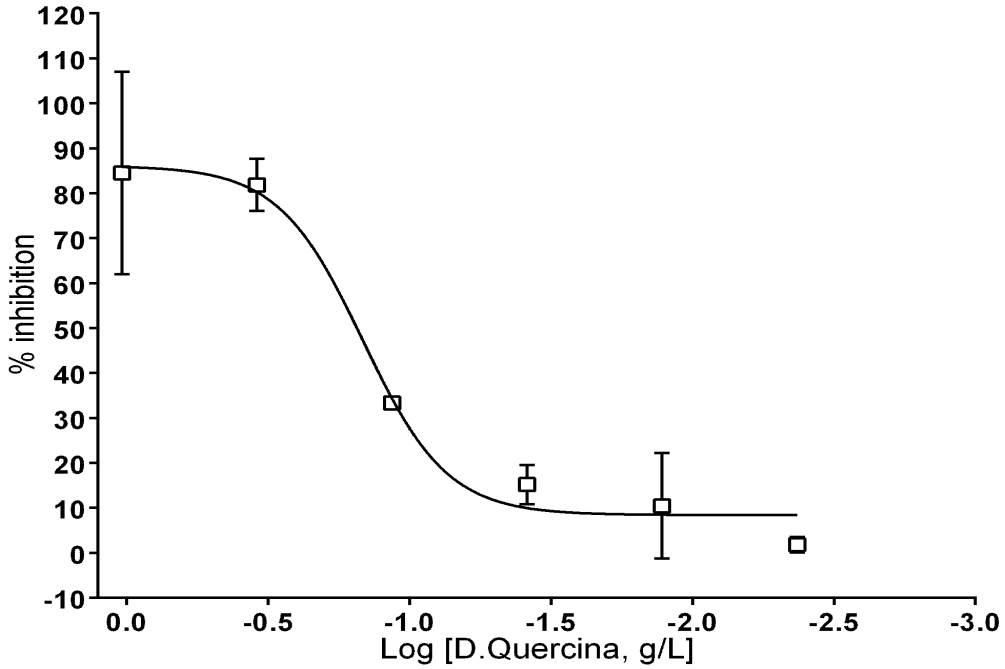


Figure 3.2 Percent CAT inhibitory activity of methanolic extract of *Daedalea qurecina* (L) Pers. with IC_{50} value of 0.1458 g/L.

3.4.3 Effect of Mushroom Extract on Superoxide Dismutase (SOD) Enzyme Activity

The final concentration of methanolic extract used in the assay was optimized within the range of 0.001255-0.305mg/mL. The inhibitory effect of methanolic extract of *Daedalea qurecina* (L) Pers. on SOD enzyme activity was less than 50% and no IC_{50} value was available (Figure 3.3).

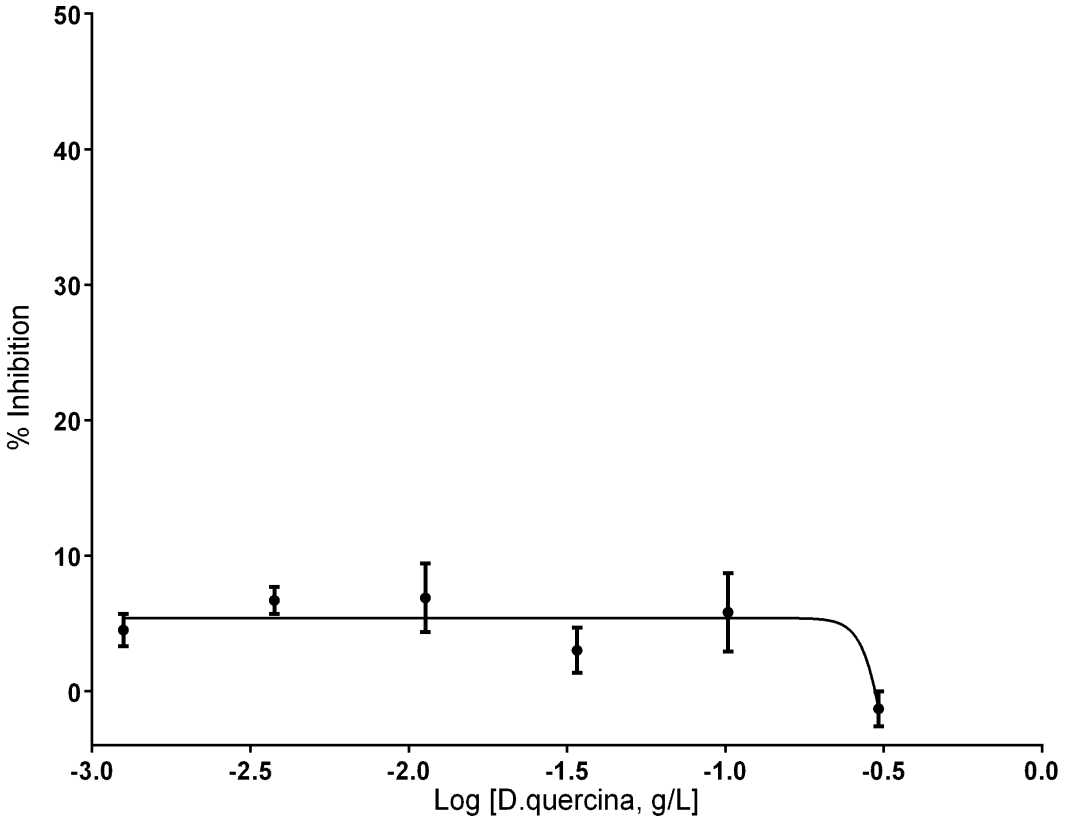


Figure 3.3 Percent SOD inhibitory activity of methanolic extract of *Daedalea qurecina* (L) Pers.

3.5 Scavenging Effect on 2,2-diphenylhydrazyal Radicals (DPPH)

Free radical scavenging activity of extract was determined by utilizing the DPPH radical scavenging method and results are presented as radical scavenging activity of phenolic and flavonoid concentrations of methanolic extract (g/L). The best free radical scavenging activity was gotten by phenolic concentration of extract with 0.081 g/L IC_{50} value (Figure 3.4). IC_{50} values for Gallic acid and quercetin solutions which were used as a positive control were 0.0096, and 0.031g/L, respectively (Figure 3.4).

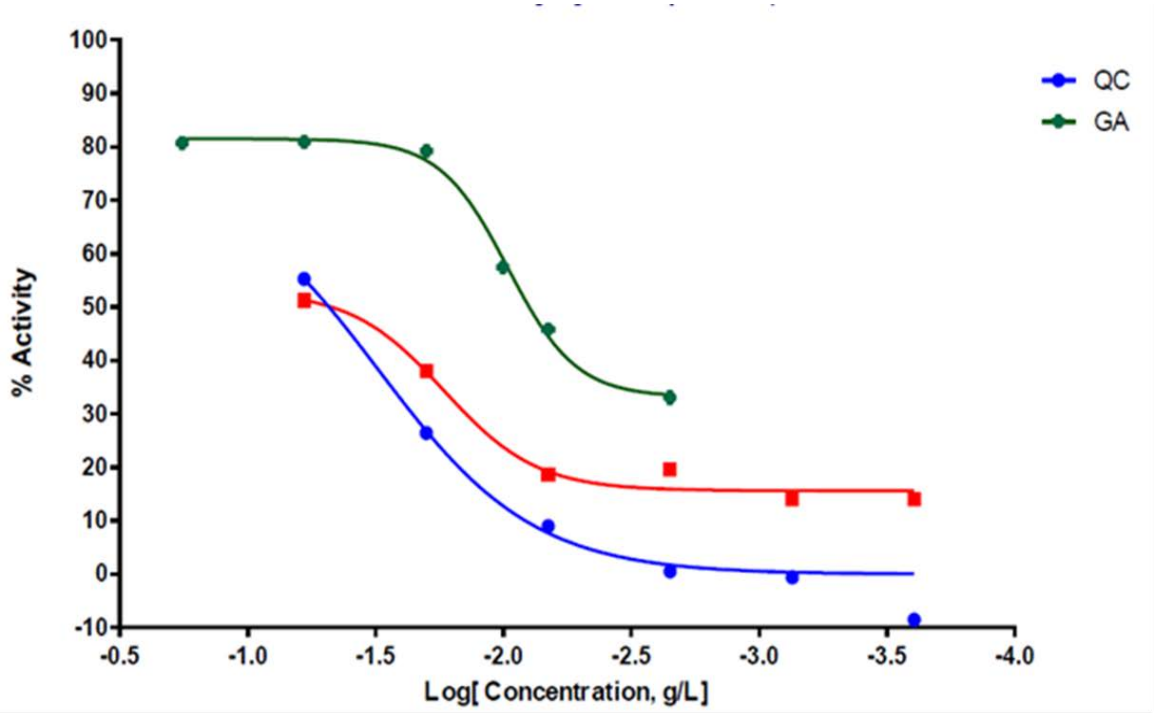


Figure 3.4 Percent free radical scavenging activity of gallic acid and quercetin solutions with IC_{50} values 0.0096 and 0.031 g/L, respectively, and free radical scavenging activity of methanolic extract of *Daedalea qurecina* (L) Pers. with IC_{50} value of 0.081 g/L.

DISCUSSION

In this study, three solvents namely methanol, ethanol and water were used to determine the best extraction of phenolic profiles. Methanol and ethanol solvents are commonly used for phenolic extraction, as they provide the highest yield of total extract even though they are not highly selective for phenols (Spingo et al., 2007). However, there is no equivalent data have been reported for this mushroom. From the results, methanol was regarded as the best solvent for extraction of phenolic compounds compared to ethanol and water. These results were in agreement with previous studies in edible wild mushrooms, *Vateria indica* and *Macrosolen parasiticus* (L) Danser reported by Gupta et al. (2012) and Seng Yim et al (2009).

Daedalea qurecina (L) pers., also known as oak mazegill, belongs to an inedible mushroom of the Fomitopidaceae family. Previous studies reported that some bioactive components from *Daedalea qurecina* (L) pers. have been isolated and characterized (Gebhardt et al., 2007; Manzoni et al., 2001; Rösecke et al., 2000). The first described triterpene derivatives isolated from this mushroom by Rösecke et al. (2000) but there was no studies of biological properties of these triterpene derivatives. Gebhardt et al. (2007) isolated active compound quercinol [(-)-(2S)-2-hydroxymethyl-2-methyl-6-hydroxychromene 1] from fractionation extracts of *Daedalea qurecina* (L) pers. mycelial culture. They demonstrated that this compound showed a broad anti-inflammatory activity against cyclooxygenase 2 (COX-2), xanthine oxidase (XOD), and horseradish peroxidase (HRP) at micromolar concentrations.

Milinaric et al. (2005) emphasized that extracts obtained using methanol and dichloromethane from *Daedalea qurecina* (L) pers. exhibited less than 40% inhibitory effect on HIV-1 reverse transcriptase activity in vitro, using a non-radioactive assay.

In the present study, it was concerned by the estimation of phenolic and flavonoid contents of mushroom extract on the GST, SOD and CAT enzyme activities. Phenolic compounds possess at least one or more aromatic rings with one or more hydroxyl groups attached (Fresco et al., 2006). Many phenolic compounds are stated to possess potentials of antioxidant antiatherosclerotic, antibacterial, antiviral, anticancer, and anti-inflammatory effects (Valko et al., 2007). Flavonoids are phenolic compounds existed through the plant kingdom. They have been reported to possess a diversity of biological activities in organisms. Several flavonoids have anti-proliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis, antitumor, and antioxidant activities (Ren et al , 2003; Zeng et al., 2009). From the results, the highest total phenolic and flavonoid contents were found in methanolic (M1) extract of *Daedalea qurecina* (L) pers. as 242 µg GEA /mL and 82.562µg QE/mL values, respectively. Veligodska et al. (2015) reported that ethanolic extract of *Daedalea qurecina* (L) pers. mycelial culture contained little amount of phenolic contents (9 mg/g of dry mass).

Glutathione-S-transferase enzyme is one of the phase II enzymes (Ata et al., 2007) and plays a critical role in the detoxification and metabolism of many xenobiotic compounds. The physiological activities of the enzyme have been involved in development of resistance by cancer cells against chemotherapeutic agents (Udenigwe et al., 2007). For that reason, the inhibition of GST activities is important in the improvement of anticancer drugs. In this study it has been concluded that the methanolic(Me1) extract of *Daedalea qurecina* (L) pers. showed some inhibitory effect on GST activity. In this study, the effect of *D. quercina* (L) Pers. extract on the GST enzyme activity was shown first.

In living tissues, are endowed with unique antioxidant defense, such as the presence of the enzymes CAT and SOD. SOD is the enzyme that catalyzed the dismutation of superoxide into hydrogen peroxide and oxygen. the previous studies revealed that SOD has the potential to induce apoptosis via the generation of H_2O_2 (de Haan et al., 1996; Ho et al., 2001). Catalase is a very important enzyme of living organisms which catalyzed the decomposition of hydrogen peroxide to water and oxygen.

Previous studies suggest that antioxidant activity is impaired in lung cancer(Jaruga et al., 1994; Melloni et al., 1996).
In this study, it has been concluded that the methanolic (Me 1) extract of *Daedalea qurecina* (L) pers. showed inhibitory effect on CAT but not on SOD activity. The effect of *D. quercina* (L) Pers. extract on the primary antioxidant enzymes activity was shown first.

From Basidiomycota phyla, this study with *A. fuscosuccinea* (white var.) shown that the mushroom extract possess high SOD activity (2.10 U/mg) and the highest polyphenolic content among the variety of extracts of the mushroom (Lin et al., 2013). However, the extracts were not shown to exhibit any possible effect on mammalian antioxidant defense enzymes.

In our study, the antioxidant activity of the methanolic extract was analyzed by DPPH radical scavenging method. It is one of widely used method, since it is easy, rapid and sensitive analysis tool to determine the antioxidant activity (Huang et al., 2005; Koleva et al., 2002; Sanchez-Moreno et al., 1998). In this study, the result suggests that the methanolic (Me1) extract exhibited a moderate radical scavenging capacity, with IC_{50} of 0.081 g/L value. This result is close to Asatiani et al. (2010) findings where DPPH free-radical scavenging activity of the water extract obtained from the submerged *Daedalea qurecina* (L) Pers. mycelia is 49% with IC_{50} value of 0.7 mg/mL.

CONCLUSION

In this study, for the first time, it has considered the biological potential of *D. quercina* (L) Pers. on the antioxidant defense system namely SOD, GST and CAT enzymes. It has been concluded that the methanolic (Me1) extract of *D. quercina* (L) Pers. has showed inhibitory effect on GST, CAT enzyme activities and has shown no inhibitory effect on SOD enzyme activity. These activities seem to be partly associated to the phenolic and flavonoid contents. Besides, it has found that the methanolic (Me1) extract of *D. quercina* (L) Pers. has free radical scavenging potential and antioxidant activity.

These results concerning on the antioxidant enzymes and antioxidant treatment in vitro and provide insights into the antioxidant nature of *D. quercina* (L) Pers., which might be useful for therapeutic purposes to prevent ROS disorders and explain their use in the treatment of various inflammatory disorders. However, the result cannot be extended directly to *in vivo* systems which are more complex and may differ from *in vitro* systems.

Although there have not been studies found in the literature related to the effect of mushroom extract on the antioxidant enzymes, in this study the effect of *D. quercina* (L) Pers. methanolic (Me1) extract on the important antioxidant enzymes activities were shown first.

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