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To the Graduate Council:

I am submitting herewith a thesis written by Sharon Rose Jean-Philippe entitled "Antioxidant Properties of Some Edible Fungi in The Genus *Pleurotus*." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

Karen Hughes, Major Professor

We have read this thesis and recommend its acceptance:

Beth Mullin, Jay Whelan

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Anne Mayhew

Vice Chancellor and
Dean of Graduate Studies

(original signatures are on file with official student records)

ANTIOXIDANT PROPERTIES OF SOME EDIBLE FUNGI IN THE GENUS PLEUROTUS

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Sharon Rose Jean-Philippe
August 2005

Acknowledgements:

I would like to thank my major professor Dr. Karen Hughes, for her undying support and guidance throughout this enlightening process. Furthermore, I would like to thank my other committee members, Drs Beth Mullin and Jay Whelan for there invaluable input and help throughout my research.

A number of exceptional opportunities was allotted while in graduate school at The University of Tennessee. The Department of Botany helped fund a trip to Thailand. The Bridges program at Vanderbilt allowed me to participate in research at their university. Teaching allowed me to grow as a person in addition to giving me the satisfaction of being able to have the opportunity share what I knew with others. I would also like to thank the following graduate students and laboratory technicians for their continued friendship: Edward Grand, Brook Nelson, Ngoz Ali, Susan Farmer, Sarah Noble, Jana, and Ed Lickey.

Lastly, I thank my family who have supported me during the happy and sad times, I do not know what I would do without you.

Abstract

Antioxidant activity, of several species within the Genus *Pleurotus* was studied. Sequencing of the internal transcribed spacer (ITS) region of the ribosomal genes ITS1-5.8S-ITS2 confirmed species identification. Free radical scavenging ability of several *Pleurotus* species was demonstrated by the DPPH assay. Two lipid model systems were used to test for oxidation of stripped corn oil; the first was an oil-in-water emulsion method which measured a primary product formed from lipid peroxidation, hydroperoxides. The second method was thiobarbituric acid (TBARS) which measured one of the secondary breakdown products of lipid peroxidation, malonaldehyde (MDA). Phenol, protein, and carbohydrate content were measured to elucidate relationships these compounds might have with antioxidant activity. In general all species sampled showed ability to scavenge free radicals. In particular *P. drynius*, had high scavenging ability (71-75%). Pearson correlation coefficients were calculated to test if there was any significant relationship between levels of protein, phenol, or polysaccharide with respect to antioxidant activity. The ability of *Pleurotus* species to inhibit lipid oxidation using the thiobarbaturic acid assay was significant ($P<0.04$). Phenol, protein and carbohydrate levels varied between and within species of *Pleurotus.* Protein and polysaccharide were significantly correlated with antioxidant activity measured by the TBAR assay ($P<0.04$) . There was no significant correlation between free radical scavenging activity and protein, polysaccharide, and phenol levels.

Protein and polysaccharide levels were significantly correlated with each other across *Pleurotus* species ($P<0.04$).

Table of Contents

Chapter	Page
I. Introduction	1
Overview	1
The Genus *Pleurotus*	3
Medicinal Uses of *Pleurotus*	4
Oxidative Damage and Antioxidants	5
Formation of Oxygen-Derived Species	6
Antioxidant Defenses	7
Antioxidant Properties of Mushrooms	7
Phenolics in Mushrooms	8
Polysaccharides in Mushrooms	9
Methods for Testing Lipid Peroxidation	10
Confirmation of Species Designation by Sequencing of the Ribosomal ITS Region	11
II. Methods and Materials	13
Establishment and Maintenance of Cultures	13
Growth and Sacrifice of Cultures	13
Fruitbody and Cultures of Lentinula edodes	13
Fruitbody and Cultures of Agaricus bisporus	15
DNA Extractions	15
PCR of the Ribosomal ITS Region	16
DNA Sequencing	17
Sequence Alignment Procedures	18
Phylogenetic Analyses	18
Determination of Protein Content	19
Bradford Protein Assay	19
Bradford Stock Solution	19
Bovine Serum Albumin Protein Standards	19
Fungal Protein Determination	21
Tests for Polysaccharides	22
Anthrone Reagent	22
Glucose Standard Curve	22
Fungal Polysaccharide Determination	23
Test for Fungal Phenolics	25
Phenol Standard Curve	25
Determination of Phenolics	26
DPPH Assay for Free Radical Scavenging Activity	27
Scavenging Effect on the DPPH Radicals	27
DPPH Stock Solution	27
Determination of Free-Radical Scavenging Activity	28
Oil-in-Water Emulsion for Determination of Lipid Peroxidation	29
Extraction and Incubation of Fungal Extracts	29

TBARS Method for Measuring Lipid Peroxidation …..…… 30
Fungal Extraction …… 31
Oxidation of Stripped Corn Oil …… 32
Antioxidant Screening …… 32
Statistical Methods …….. 33

III. Results

Phylogeny of *Pleurotus*…….. 34
Protein Levels in *Pleurotus* Species……. 34
Polysaccharide Levels in *Pleurotus* Species……... 37
Phenolic Levels in *Pleurotus* Species.……..... 37
Free-Radical Scavenging Activity of *Pleurotus* Extracts……. 40
Antioxidant Activity of *Pleurotus* extracts in Oil-in-Water Emulsion……. 40
TBARS Assay …… .. 40
Statistical Analyses…… 45

IV. Discussion and Conclusions…… 47

Literature Cited …… 56

Appendix…… 64

Vita……. 68

List of Tables **Page**

Table 1. Fungal Collections…… 14
Table 2. Protein Stock Solution…… 20
Table 3. Glucose Stock Solution…… 23
Table 4. Preparation of Samples…… 28
Table 5. Protein levels in Pleurtous…… 36
Table 6. Polysaccharide levels in Pleurotus…..…… 38
Table 7. Phenolic levels in Pleurotus……..…… 39
Table 8. Free-radical Scavenging Activity…..…… 41
Table 9. Antioxidant Activity in Oil-in-Water Emulsion…… 43
Table 10. Thiobarbituric Acid Assay…… 44
Table 11. Correlations…… 45
Table 12. Mauchly's test of Sphericity…… 46

Figures **Page**

Figure 1. Protein Standard Curve…….. 21
Figure 2. Glucose Standard Curve…… 24
Figure 3. Phenolic Standard Curve…… 26
Figure 4. Structure of Malondialdehyde…… 31
Figure 5. Phylogenetic Tree…… 35
Figure 6. Antioxidant Activity in Oil-in-Water Emulsion…… 42

Chapter I

Introduction

Overview

Since ancient times throughout the world, higher *Basidiomycete* mushrooms have been used in folk medicine. Medicinal mushrooms useful against cancer are known in China, Russia, Japan, Korea, as well as the United States and Canada (Wasser, 1999). Some species of edible higher *Basidiomycetes* have been found to noticeably inhibit the growth of different kinds of tumors. There are about 200 species of higher *Basidiomycetes* that have been found to possess this activity (Lucas *et al*., 1957). A large number of mushroom-derived compounds, both cellular components and secondary metabolites, have been shown to effect the immune system and could be used to treat a variety of diseases (Chihara *et al*., 1982; Jong *et al*., 1991). However, problems evaluating data published by different investigators working with even the same "species" of mushroom exist because identification of species is often not accurate. The overall objective of this study was to evaluate the antioxidant activity of representative species within the genus *Pleurotus* using three assays, a DPPH assay and two assays which measured lipid oxidation. Secondary goals were: 1) to accurately identify *Pleurotus* species used in this study using the ribosomal internal transcribe spacer genes as an identification tool; 2) to compare protein, polysaccharide, and phenolic content between species; and 3) to determine if there

was a correlation between the phylogenetic distribution of *Pleurotus* species in relation to their antioxidant activity.

Recently, the study of oxidative stress (free-radical peroxide oxidation of lipids [POL]), particularly in human body, has become a subject of great interest and practical significance (Badalyan, 2003). In the presence of different oxidants, the balance between lipid oxidation and antioxidant activity is disrupted, which promotes the propagation of many diseases such as cancer, cardiovascular pathology, and atherosclerosis. Agents that suppress oxidation reactions are antioxidants. Natural antioxidants are found in plants and different groups of microorganisms (bacteria, yeast, filamentous fungi), particularly in producers of melanin and other phenolics substances (Badalyan, 2003).

Many fungi have antioxidant properties (Badalyan, 2003; Fu et al., 2002**)**. Previous researchers have investigated antioxidant properties of fungi in lipid systems but did not accurately identify the fungal species used in their investigations. Further, comparisons between fruit bodies and mycelium with respect to ability to inhibit lipid oxidation and scavenge free radicals were not investigated. Finally, no studies have examined antioxidant activity across an entire genus and examined results within phylogenetic context. In this study, antioxidant activity of several *Pleurotus* species was evaluated. Accurate identification of species was confirmed by sequencing of the ribosomal DNA internal spacer region and a comprehensive analysis was done to correlate the phylogenetic distribution of *Pleurotus* species with their antioxidant activities. Techniques used to evaluate antioxidant activity were, 1) an oil-in water emulsion

system, 2) the thiobarbituric acid (TBA) assay, and 3) reduction of 1,1-diphenyl-1-picrylhydrazyl (DPPH) assay. Carbohydrate, protein and phenol levels for fungal extracts were obtained to determine if levels of these compounds in fungal tissue correlated with antioxidant activity. Vegetative states, specifically fruit body and mycelium were compared between selected species to give a general idea about the difference between these two stages.

The Genus *Pleurotus*.

Species of *Pleurotus,* commonly called oyster mushrooms, consist of gilled mushrooms that have an eccentric or lateral stem or are laterally or dorsally attached and sessile (Thorn *et al*. 2000). *Pleurotus* species are wide-spread, saprophytic mushrooms that grow on wood, usually on dead standing trees or on fallen logs. *Pleurotus* species are distributed throughout the temperate and tropical hardwood forests of the world (Gunde-Cimerman, 1999). Fungal populations are established and developed in nature through both sexual and asexual reproduction (Cohen *et al*., 2002).

The genus *Pleurotus* is one of the most diverse groups of cultivated mushrooms that have important economic and medicinal value (Cohen *et al*., 2002). One of the reasons for their success is that oyster mushrooms are by far the easiest and least expensive to grow of all industrially cultivated edible mushrooms (Gunde-Cimerman 1999) and they grow on a number of different plant substrates. As food, the oyster mushrooms are a good source of nonstarchy carbohydrates, have high content of dietary fiber, contain moderate quantities of

good quality proteins and most of the essential amino aids, minerals, and vitamins (Gunde-Cimerman 1999).

Medicinal Uses of *Pleurotus*.

A number of medicinal properties have been attributed to *Pleurotus* species. *Pleurotus* spp. have been shown to modulate the immune system, have hypoglycemic activity and antithrombotic effects, lower blood pressure and blood lipid concentrations, and inhibit tumor growth, inflammation, and microbial activity (Chang, 1993, 1996; Eisenhut and Fritz, 1991). Lectin and lovastatin are therapeutic compounds isolated from *Pleurotus* species. Lectins are carbohydrate containing-proteins of non-immune origin that agglutinate cells, or precipitate polysaccharides or glyconjugates (Liener *et al*., 1986). Kaneko et al., (1993) examined hemaggutinating activity in crude extracts prepared from four fungal developmental stages; vegetative mycelium, primordium, immature fruit body, and mature fruit. Lectin activity was not seen in vegetative mycelium but increased through the other three fungal developmental stages. Kues & Liu 2000 reported that lectin produced by *P. corncucopiae* has a potential application for haemagglutination.

Lovastatin is a potent hypocholesteraemic agent. This low-molecular-weight substance is a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG CoA reductase), the key enzyme in cholesterol metabolism that catalyses the reduction of HMG CoA into mevalonate (Wasser & Weis, 1999). The best known organism for the potential production of lovastatin

from edible higher basidiomycete mushrooms are species of the genus *Pleurotus* (*P. ostreatus*, *P. cornucopiae*, *P. erygnii*, and *P. sapidus*: Cimerman and Cimerman, 1995; Gunde-Cimerman *et al*. 1993 a, b). The highest content of lovastatin was found in fruiting bodies of *P. ostreatus* (Wasser and Weis 1999).

Oxidative Damage and Antioxidants.

Oxidation is vital to living organisms for the production of energy to fuel biological process. However, oxygen-centered free radicals and other reactive oxygen species that are continuously produced *in vivo*, result in cell death and tissue damage (Halliwell & Gutteridge, 1984). Oxidative damage caused by free radicals may be related to aging and disease. A free radical is any species that contains one or more unpaired electrons and is capable of independent existence (Halliwell *et al*., 1995). The term reactive oxygen species (ROS) and oxygen derived species are used to include both oxygen radicals ($O^{\cdot}{}_2^{-}$,•OH, LOO•, and LO•) and nonradical oxygen-containing reactive agents (H_2O_2, $^1O_2$) (Halliwell *et al*, 1995). Tissues contain several compounds called antioxidants that inhibit free radicals (Thomas, 1995). The reason antioxidants are important to an organism's physical well being comes from the fact that oxygen is a potentially toxic element since it can be transformed by metabolic activity into more reactive forms such as the superoxide anion, hydrogen peroxide, singlet oxygen and the hydroxyl radical (Hampson & Svoboda, 1999). Almost all organisms are well protected from free radical damage by enzymes such as superoxide dismutase and catalase, or

compounds such as ascorbic acid, tocopherols, glutathione and flavenoids (Niki *et al*., 1994).

Formation of Oxygen-Derived Species.

Xanthine oxidase is one of the main enzymatic sources of reactive oxygen species (ROS) *in vivo* (Sanchez-Moreno, 2002). Xanthine oxidase in normal tissue is a dehydrogenase enzyme that transfers electrons to nicotinamide adenine dinucleotide (NAD^+) as it oxides xanthine or hypoxanthine to uric acid. Under certain stress conditions, such as oxidative stress, the dehydrogenase is converted to an oxidase enzyme by oxidation of essential thiol groups or by limited proteolysis (Sanchez-Moreno, 2002). Upon this conversion the enzyme reacts with the same electron donor, by reducing oxygen instead of NAD^+, thus producing superoxide and hydrogen peroxide and contributing to the initiation and progression of a number of pathological processes (Halliwell *et al*., 1995). Hydroxyl radicals are generated *in vivo* by the homolytic fission of O-H bonds in water, driven by continuous exposure to background ionizing radiation (Halliwell *et al*., 1995). Singlet molecular oxygen ($^1O_2$) is an electronically excited oxygen which is formed in biological systems via photosensitization reactions. This pathway is thought to be important in light-exposed tissue. O_2 can interact with target molecules either by transferring its excitation energy to another molecule or by chemical combination (Stahl & Sies, 2002).

Antioxidant Defenses.

Living organisms have evolved a number of mechanisms that are protective against lipid peroxidation or oxidant stress. Some are enzymatic, blocking the formation of reactive compounds, others may scavenge for reactive compounds or act in reducing oxidant stress by unknown mechanisms.

Superoxide dimutases (SODs) remove $O_2^{\cdot -}$ by greatly accelerating its conversion to H_2O_2 and H_20 (Britigan et al., 1986). Catalases in the peroxisomes convert H_2O_2 into water and O_2 and help to dispose of H_2O_2 generated by the action of oxidase enzymes located in these organelles (Halliwell *et al*., 1995). However, the most important H_2O_2 – removing enzymes in human cells are glutathione peroxidases (GSHPX). GSHPX enzymes remove H_2O_2 by using reduced glutathione (GSH) as an electron donor, generating oxidize glutathione (GCCG) (Chance *et al*., 1979). Tocopherols delay lipid peroxidation by reacting with chain propagating peroxyl radicals faster than these radicals can react with proteins or fatty acid side-chains (Burton & Ingold, 1986). Ascorbic acid prevents aqueous oxidants from attacking and oxidizing LDL's which protects isolated human low-density lipoproteins against lipid peroxidation (Retsky *et al*., 1993).

Antioxidant Properties of Mushrooms.

A number of studies have focused on the search and development of antioxidants of natural origin, including fungi (Fu *et al*., 2002). Among various naturally occurring substances, mushrooms may prove to be one of the useful

candidates in the search for an effective antioxidant with free radical scavenging activity (Liu *et al*., 1997). Free radical scavenging is a generally accepted mechanism for phenolic antioxidants to inhibit lipid oxidation (Bors & Saran, 1987). Mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, and secrete substances into culture broth. Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various allogeneic and syngeneic tumors, prevent metastasis (Wasser *et al*., 2002), and have antioxidant properties (Liu, et al., 1997).

Phenolics in Mushrooms.

The antioxidative effect of phenolics is determined by their chemical structures. Free radical scavenging activity increases with the number of hydroxyl groups and their location in the molecules involved (Dziedric & Hudson 1984) and is due to the ability of these molecules to donate an electron. Studies with the mushroom *Agarcius bisporus* indicated that this species, at least, contained significant amounts of phenolic amino acids (tyrosine, L-glutaminyl-4-hydrobenzene, 3,4,dihydroxyphenylalinine and L-glutaminyl-3,4,dihydroxy benzene; Choi & Sapers 1994). Free radical scavenging activities were correlated with total phenolics in a variety of mushrooms including *Agaricus bisporus*, *Flammulina* sp. and *Pleurotus* (Fu *et al*., 2002).

Polysaccharides in Mushrooms.

Mushrooms in general contain dietary fibers, including β-glucans, chitin and pectinous substances (Cohn *et al*., 2002). Oyster mushrooms contain approximately 55% insoluble and 10-15% soluble β-glucans (Bobek *et al*., 1997). These compounds, homo- and heteroglucans with beta 1→3, beta 1 →4 and beta 1→6 glucosidic linkages, are supposed to play a key role in health promoting properties of mushrooms such as activation of a nonspecific immune stimulation (Yoshioka *et al*. 1973; Ikekawa, Ikeda *et al*., 1982; Ikekawa, 1995), reduction of blood cholesterol and blood glucose levels, and free radical scavenging activity (Huang, 2000; Liu *et al*., 1997). Mushroom polysaccharides prevent oncogenesis, show direct antitumor activity against various allogeneic and syngeneic tumors, and prevent tumor metastasis (Wasser, *et al*., 2002). Polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host. Free radical scavenging activity of polysaccharide extracts was shown by Liu *et al* 1997, where various mushroom species quenched superoxide and hydroxyl radicals. Assays used for polysaccharides required an alcohol extraction, which denatures and inactivates enzymes. Therefore, any antioxidant activity observed in these studies was due to non-enzymatic factors.

Mau *et al* (2002) did not find appreciable levels of the antioxidants ascorbic acid or β-carotene in several widely divergent fungi including the basket stinkhorn *Dictophors indusiata* (a stinkhorn), *Grifola frondosa*, *Hericium erinaceus* (lion mane fungus, a basidiomycete) and *Tricholoma giganteum*

(matsutaki) but *P. ostreatus* and *P. eryngii* have been shown to have high levels of ascorbic acid. In this study these compounds were not examined, nor was there an attempt to assay for tocopherols which are found in some fungi.

Methods for Testing Lipid Peroxidation.

The extent of lipid peroxidation can be determined by a number of methods which can be divided between those that measure primary products of lipid peroxidation such as hydroperoxides and those that measure the secondary breakdown products of lipid hydroperoxides such as malondiadehyde, 4-hydroxynonenal and volatile hydrocarbons (Punchard and Kelly, 1996).

Huang *et a*l, (1994) measured the primary products of lipid peroxidation using an oil-in-water system. Lipids such as stripped corn oil (without tocopherols) oxidize more rapidly in an emulsion than in bulk oil (Huang *et a*l., 1994). Oxidation was assayed by following the formation of hydroperoxides, which were determined with a colorimetric assay by absorbance at 500 nm. In Huang's study, the control (no extract added) obtained a peroxide absorbance value of 0.4 A_{500} after 3 days of storage at 60° C but with ethanol extracts of mushrooms, the peroxide values were less than 0.4 indicating that the fungal extracts inhibited lipid peroxidation. Using similar protocols, Fu *et al* (2002), found that mushroom extract antioxidant activity was highest in *Agarius bisporus* and lowest in *Lentinula edodes*. *Pleurotus eryngii* was intermediate between these two. Lin (1999) identified *Flamulina velutipes, Lentinula edodes, Pleurotus cystidiosus and Pleurotus ostreatus* as having moderate to high activity against

lipid peroxidation using the 1,3-diethyl-2-thiobarbituric acid (DETBA) method which measured the secondary breakdown products of lipid hydroperoxides such as malondiadehyde.

Butylated hydrotoluene (BHT) a synthetic antioxidant was compared to aqueous and ethanolic extracts of oyster mushrooms. Filipek (1992) reported that BHT and the oyster mushroom extracts inhibited lipid peroxidation and malondialdehyde formation in phosphatidylcholine liposome systems. Lin (1999) showed that *P. cystidiosus* and *P. ostreatus,* had a moderate to high antioxidant activity (24.71- 62.30 % inhibition of lipid peroxidation) using the 1,3-diethyl-2-thiobarbituric acid (DETBA) method. Lu and Chang (1985) showed that *P. eryngii*, and *P. ostreatus* to had moderate to high levels of ascorbic acid (36-58 mg/100g) a finding that could explain the antioxidant effect of *Pleurotus* species.

Confirmation of Species Designation by Sequencing of the Ribosomal ITS Region.

Genes within the ribosomal repeat have been widely used in fungal systematics. Each repeat consists of an 18S gene (Small Subunit or SSU), an internally transcribed spacer (ITS1), the 5.8S gene, a second internally transcribed spacer (ITS2) and the large subunit gene (LSU). These genes participate, together with proteins, in the formation of the ribosomes. In some basidiomycetes, a 5S gene is found after the LSU gene flanked by two spacers (intergene spacers ISG1 and ISG2). While the 18S and LSU genes are evolutionarily conserved, the ITS1-5.8S-ITS2 region is sufficiently variable that it can be used for species

identifications in population and species level studies in fungi (see for example Bruns and Gardes, 1993). The ITS region contains two variable non-coding regions that are stationed within the rDNA repeat between the highly conserved small subunit and large subunit rRNA genes (Bruns and Gardes, 1993). The ITS primers make use of the conserved regions of the 18S, 5.8S, and 28S rRNA genes to amplify the non-coding regions between them (White *et al*., 1990). Several features make it a convenient target region for molecular identification of fungi: 1) In fungi, the entire ITS region is often between 600 to 800bp and can be readily amplified with universal primers that are complementary to sequences within the rRNA genes (White *et al*., 1990), 2) the tandem repeats of the rDNA makes the ITS region easy to amplify from small, dilute, or highly degraded DNA samples, and 3) several studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species (Gardes *et al*., 1991).

Pleurotus spp. are difficult to identify based on morphology alone. ITS sequences from the collections used in this study were matched with the existing database to determine correct species designations. Correct species identifications are important to avoid attributing medicinal attributes and results to the wrong species. Valid publication will require identifications based on more than morphology (i.e. ITS sequence). Sequences for all known *Pleurotus* species that are based on accurate morphological identifications (Ronald Peterson, Pers. Comm) and on mating studies were used in this study to confirm identifications.

Chapter II

Methods and Materials

Establishment and Maintenance of Cultures.

Collections of *Pleurotus spp*. used in this study represent the major species within the genus and are listed in Table 1.

Growth and Sacrifice of Cultures

Cultures were grown in Petri dishes containing malt extract agar (MEA = Difco malt extract, 15g; Difco Bacto Agar, 20g; distilled water, 1L). Cultures were incubated at room temperature until they covered most of the medium in the Petri dish. For antioxidant activity, lipid oxidation, protein, phenolic, and carbohydrate determinations, a plug of mycelium from MEA plates was grown in potato dextrose broth (PDA; 24 g/L Difco potato dextrose) until the mycelium covered the surface of the medium. Mycelia were filtered through mesh to remove the medium, blotted dry, dried at 41°C overnight (Fu *et al*., 2002) and stored in a desiccator until used for analysis. Mycelia were ground in liquid nitrogen to a fine powder the same day that experiments were performed.

Fruitbody and Cultures of Lentinula edodes

Fruitbodies were grown from a mushroom patch obtained from Fungi Perfecti. The patch was made up of sterilized, enriched sawdust fully colonized with a select Chinese strain of Shiitake (*Lentinula edodes*). The mushroom patch was watered and covered, and placed in a incubator at 50°C for 2 weeks.

Table 1. Fungal Collections.

ACCESSION NO.[1]	SPECIES NAME	COUNTRY	REGION
6551	*P. abieticola*	Russia	Primorsk
EA772	*P. albidus*	Argentina	
9065	*P. calyptratus*	Austria	Vienna
8966	*P. cornucopiae*	Russia	Caucasia
7167	*P. djamor*	New Zealand	North Island
6695	*P. dryinus*	Austria	Bravaria
5826	*P. dryinus*	USA	WA, Whatcomb Co.
9983	*P. dryinus*	Russia	St. Petersburg
6690	*P. eryngii*	Austria	
6689	*P. ostreatus*	Austria	Vienna
8077	*P. ostreatus*	USA	CA, Humboldt
10994	*P. ostreatus*	USA	TN, Knoxville
9936	*P. populinus*	USA	Utah
5405	*P. pulmonarius*	USA	TN, Blount
9059	*P. pulmonarius*	USA	CA, Humboldt
9891	*P. pulmonarius*	Russia	Ural Mts.
2669	*P. purpureo-olivascens*	New Zealand	
DSH-92-155 David Hibbett	*P. tuber-regium*	New Guinea	
Fruit Body[2]	*Agaricus bisporus*	Commercial	
Fruit Body[3]	*Lentinula edodes*	USA	Asia
Culture from fruit body	*Lentinula edodes*	USA	Asia

[1] Tennessee Culture Collection Accession Number
[2] Fruit bodies from Monterey Mushroom Farms, Tennessee
[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

Mushrooms were sprayed with a water bottle three to four times daily to maintain humidity. *Lentinula* fruitbodies were harvested and dried at 41°C overnight and stored in a desiccator until used for analysis. Cultures of *Lentinula* were grown and sacrificed the same way as mention above for *Pleurotus* cultures.

Fruitbody and Cultures of Agaricus bisporus

Fruit bodies of *A. bisporus* were obtained from the local supermarket in Knoxville, Tennessee (Commerical). Mushrooms were dried at 41°C overnight and stored in a desiccator until used for analysis. Cultures for *Agaricus* did not grow well enough to use in this study.

DNA Extractions.

Fungal cultures in PDA (see above) were filtered through 1mm nylon mesh to remove the medium and blotted with paper towels. Approximately 300 mg of fungal tissue was placed in the mortar together with 750µL Carlson lysis buffer (Carlson *et al*., 1991) and a small amount (pinch) of grinding sand. Fungal tissues were ground thoroughly, transferred to a 1.5 mL microfuge tube and incubated for 30 minutes at 74°C with inversion every 10 minutes. After incubation, each sample was centrifuged at 10,000 RPM for 10 minutes at room temperature to sediment cell debris. The supernatant was poured into a new tube and all samples were cooled to room temperature.

Chloroform: isoamyl alcohol (24:1;750 µl/tube) was added to each tube and mixed gently by inverting the tube several times for a few minutes. Samples were centrifuged for 10 minutes at 10,000 RPM. The upper phase of each sample was transferred to a clean microfuge tube and an equal volume of isopropanol was added to each tube. Tubes were inverted several times to mix the solution and precipitate DNA, then centrifuged for 10 minutes at 10,000 RPM to sediment DNA. The DNA pellet that formed at the bottom of each tube was rinsed with 250 µl of ice cold 70% ethanol (ETOH), to wash DNA from the sides of the tube and to replace isopropanol with ethanol. Tubes were re-centrifuged at 10,000 rpm for 10 mins to collect the DNA. Ethanol was poured out of each tube and tubes were turned upside down to dry. After a few minutes, any remaining liquid was blotted off with a kimiwipe from the sides of the microfuge tube. The nucleic acid pellet was re-suspended by adding 100µl of TE buffer (Sambrook *et al*., 1989;see Appendix).

PCR of the Ribosomal ITS Region.

The internally transcribe spacer region of the ribosomal genes (ITS1-5.8S-ITS2) was amplified by adding 1 µl of fungal DNA to 24µl of PCR reaction mix (see Appendix). The primers used were ITS1F and ITS4 (Bruns and Gardes, 1993; White et al., 1990).

The PCR program was as follows:

Cycle 1 4 mins at 94 C 1 repetition

Cycle 2 1 min at 94 C 35 repetitions

1 min at 52 C

1 min at 72 C

Cycle 3 3 mins at 72 C

Cycle 4 24 hrs at 4 C (or however long was needed)

To evaluate if ITS regions amplified, 5 µl of the product was examined by gel electrophoresis in a 1.5 % agarose gel (LE agarose, FMC corp). The buffer was 1X TBE (Sambrook *et al*., 1989;see Appendix).

DNA Sequencing.

To remove unused dNTPs and primers, 5 µl of the PCR product was incubated with 1 µl "Exosap" (U S Biochemicals, Cleveland, OH) in a 0.2 ml reaction tube. All transfers were carried out by using a plugged pipette tip. Tubes were incubated at 37 °C for 15 mins, then 80 °C for 15 mins. When the Exosap reaction was completed, 3 µl of Big Dye terminator mix (Applied Biosystem, Foster City CA) and 1 µl of diluted (3.3 µM) primer was added using a plugged pipette tip. The sequencing reaction was 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Centri-Sep columns (Princeton Separations, Adelphia, NJ) containing Sephadex G50 were used to remove primers and unincorporated nucleotides from the sequence reaction. Columns were centrifuged at 3000 RPM for 2 minutes, paying attention to the position of the column using the orientation mark molded into the tube. The columns were

then placed in 1.5mL microfuge tubes and 10μL of sequencing reaction was added to middle of the column. Columns were centrifuged at 3000 RPM for 2 minutes, to collect the purified sequencing reaction in the bottom of the tubes. DNA was dried in a spinvac at 45 °C for 20 minutes. The sequencing reaction was electrophoresed at the Molecular Biology Resource Facility and the sequence file was returned.

Sequence Alignment Procedures.

Sequences were aligned using the GCG program (Accelrys, 2001) on a Unix work station. Data were imported into the sequence alignment file and aligned by comparing imported sequences to a database of ITS sequences representing the collections. To see if the sequences were correctly aligned, files were viewed using the Seqlab program in GCG and alignment manually adjusted.

Phylogenetic Analyses.

A bootstrap 50% majority-rule consensus tree was obtained using the program Paup* 4b version 10 (Swofford, 2001). Gaps were few, were informative, and were treated as a fifth base. Names assigned to collections were deemed correct if collections used in this study fell within clades of similarly named collections which had been identified morphologically, by Dr. Ronald H. Peterson.

Determination of Protein Content.

Bradford Protein Assay

Total proteins were determined by the Bradford assay (Bradford, 1976). The Bradford assay depends on a change in absorbance of the dye Coomassie Brilliant Blue G-250 when proteins are present (Bradford, 1976). This dye specifically binds to amino acid residues in proteins including arginine, tryptophan, tyrosine, histidine and phenylalanine (Bradford, 1976). When phenolic compounds are present, the most reliable method of protein determination is the Bradford method (Robinson, 1979).

Bradford Stock Solution

Comassie Brilliant Blue G-250 (100mg) was dissolved in 50 ml of 95% methanol and mixed well. To this solution was added 100ml of 85% phosphoric acid. The solution was diluted to 1 liter.

Bovine Serum Albumin Protein Standards

Bovine Serum Albumin (SIGMA) was used as a protein standard to prepare a chart representing the relationship between protein concentration and absorbance at 595nm. Stock solutions were prepared in microfuge tubes with 1ml of ddH_2O as follows in Table 2.

Table 2. Protein Stock Solution

Initial concentration	Final concentration
25mg/ml	.125mg/ml
30mg/ml	.150mg/ml
35mg/ml	.175 mg/ml
40mg/ml	.200 mg/ml
45mg/ml	.225mgml
55mg/ml	.275mg/ml
60mg/ml	.300mg/ml
65mg/ml	.325mg/ml
70mg/ml	.350mg/ml
75mg/ml	.375mg/ml
80mg/ml	.400mg/ml
85mg/ml	.425mg/ml
95mg/ml	.475mg/ml
100mg/ml	.500mg/ml
150mg/ml	.750mg/ml

Each stock solution was diluted twice: 1)10µl of BSA stock plus 990µl of ddH_2O [1/100]; 2) 500µl of BSA stock plus 500µl of ddH_2O for a total dilution of 1/200. Twenty microliters of each diluted BSA sample were added to 1 ml of Bradford solution. The solution was mixed well and incubated at room temperature for 5-20 minutes. Absorbance was measured at 595 nm against a control without protein (Bradford reagent plus 20 µl distilled H_2O) using a Hitachi 2000 duel beam spectrophotometer. A standard linear curve (absorbance against concentration of BSA) was plotted [See Figure 1 for example] and the regression line slope (b_{xy}) and intercept (a) was calculated.

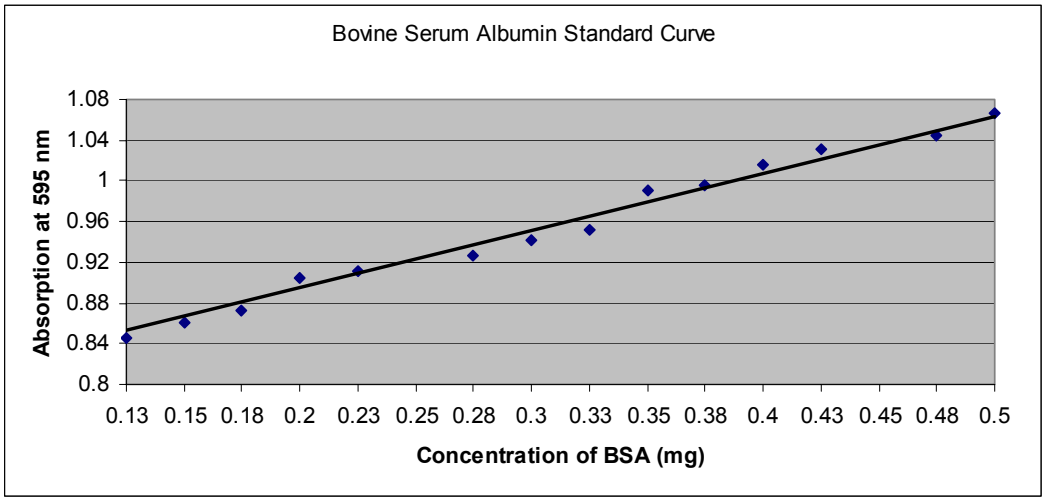

Figure 1. Protein Standard Curve.

Fungal Protein Determination

For fungal protein determinations, 0.02g of dried mycelia was extracted with 1ml of double distilled water (ddH_2O) and centrifuged for 10 minutes at 10,000 RPM. The supernatants were used for the assay the same day. Fungal protein was determined by adding 20µl of each aqueous fungal extract to 1 ml of Bradford solution, mixing well and incubating for 5-20 minutes as described above. Absorbance was measured at 595 nm against a control without protein [Bradford solution and ddH_2O only] as described above. Standard curve was repeated for each experiment. Protein in the fungal extracts was estimated from the linear portion of the standard curve using the formula $y = a + b_{xy} X$. This was adjusted for dilutions to give protein per gram dry weight of fungal tissue by multiplying the amount of protein in 20 µl extract by 50 to give total protein in one gram dry weight of fungal tissue.

Tests for Polysaccharides.

Polysaccharides levels were determined by the anthrone procedure. Dreywood (1946) initially demonstrated the use of anthrone as a specific qualitative test for carbohydrates and suggested its possible quantitative use. The test is made by rapidly adding a solution of anthrone (0.05 to 0.20%) in concentrated sulfuric acid to an aqueous solution or suspension of the carbohydrate and mixing immediately (Viles and Silverman, 1949). Under controlled conditions the amount of green color produced is proportional to the carbohydrate content (Viles and Silverman, 1949).

Anthrone Reagent

Anthrone (0.2%) was dissolved in 100 ml of concentrated sulfuric acid. The solution was mixed and stored in the refrigerator. The solution was made fresh weekly.

Glucose Standard Curve

Dextrose (Matheson Coleman & Bell) was used as a carbohydrate standard to prepare a curve representing the relationship between carbohydrate concentration and absorbance at 625 nm. Stock solutions were prepared in microfuge tubes with 1ml of ddH_2O as follows in Table 3:

Table 3. Glucose Stock Solution

Initial concentration	Final concentration
10 mg/ml	0.1 mg/ml
20 mg/ml	0.2 mg/ml
30 mg/ml	0.3 mg/ml
40 mg/ml	0.4 mg/ml
50 mg/ml	0.5 mg/ml
60 mg/ml	0.6 mg/ml
70 mg/ml	0.7 mg/ml
80 mg/ml	0.8 mg/ml
90 mg/ml	0.9 mg/ml
200 mg/ml	2 mg/ml

Each stock solution was diluted once; 10 µl of dextrose stock plus 990 µl ddH_2O [1/100]. Stock solutions 333 µl were added to 667 µl of anthrone reagent. Each solution was mixed well and boiled in a hot water bath for 20 minutes. Samples were allowed to cool and absorbance was read at 625 nm. A standard curve (absorbance against concentration of dextrose) was plotted [See Figure 2 for example] and the slope of the line (b_{xy}) and intercept (a) was calculated.

Fungal Polysaccharide Determination

To release soluble polysaccharides, 0.15g of dried fungal tissue was added to 1.5 mL of 80 % ethanol, vortexed and incubated at 95 ° C for 1 hour. Samples were cooled then centrifuged at 10,000 rpm for 10 minutes to precipitate cell debris. The supernatant was transferred to new tubes and stored in the refrigerator overnight to precipitate polysaccharides. Samples were centrifuged to collect the polysaccharides. The supernatants were poured off and polysaccharides were redissolved in 1 ml of ddH_2O.

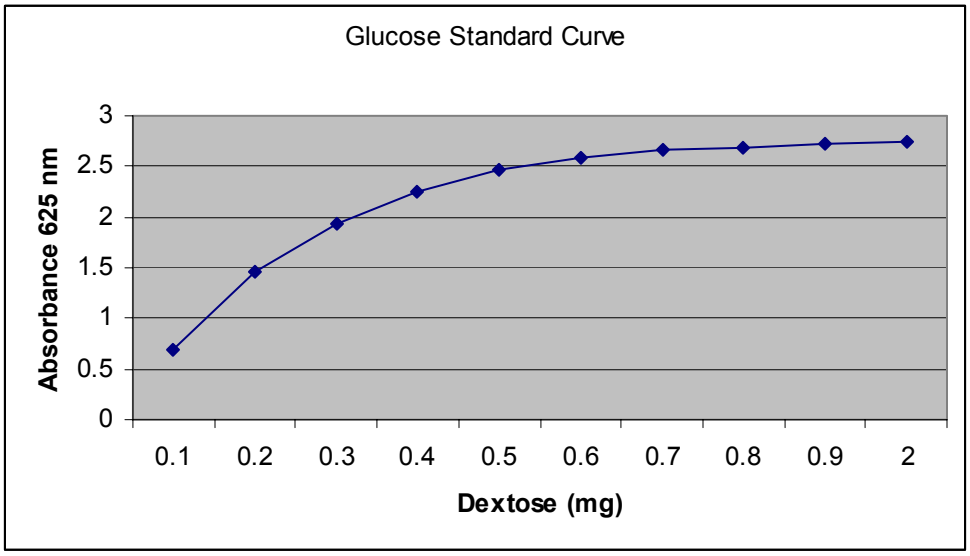

Figure 2. Glucose Standard Curve.

To test for total carbohydrate, 333 µl of polysaccharide solution from fungal samples was mixed with 667 µl anthrone reagent. Samples were boiled in a water bath for 20 minutes and cooled. Absorbance was read at 625 nm against a control without dextrose (Anthrone reagent and ddH_2O). Standard curve was run for each experiment. Polysaccharides in the fungal extracts were estimated from the linear portion of the standard curve using the formula $y = a + b_{xy} X$. This was adjusted for dilutions to give polysaccharide per gram dry weight of fungal tissue by multiplying the amount of polysaccharide in 333 µl extract by 3 to give total polysaccharide in 1 ml ethanolic fungal extract, and by 6.67 to give total polysaccharide in one gram dry weight of fungal tissue (total X 20).

Test for Fungal Phenolics.

Fungal phenolics were determined by the Folin-Denis procedure. The test is based on the reduction of phosphomolybdic acid by phenols (Folin and Denis, 1912). Phosphomolybdic in the phenol reagent is reduced by phenol derivates in acid solution and the reduced compound gives blue salts on adding alkali. The blue color obtained is not very stable in an excess of alkali; it gradually fades and the stronger the alkali the more quickly it fades (Folin and Denis, 1912). Choosing the right alkali is very important, especially in quantitative work where the maximum as well as the most stable color is needed. The best alkali is sodium carbonate (Folin and Denis, 1912).

Phenol Standard Curve

2-Phenylethanol (Sigma) was used as the phenol standard to represent the relationship between the concentration of phenolics and absorbance read at 725 nm. A stock solution was prepared in flasks. Nine amounts of phenylethanol (50 µl, 100 µl, 200 µl, 300 µl, 400 µl, 500 µl, 600 µl, 700 µl, & 800 µl) were added to 25 mL of glass distilled water and 1.6 mL of Folin-Denis reagent (Fluka) and mixed thoroughly on a shaker for 3 minutes. To each flask, 3.3 mL of sodium carbonate (Lab Chem Inc.) was added and brought to a final volume of 33 mL with glass distilled water. Flasks were shaken continuously for 30 minutes and absorbance read at 725 nm. A standard curve (absorbance against concentration of phenolic) was plotted [See Figure 3 for example] and the regression line slope (b_{xy}) and intercept (a) was calculated.

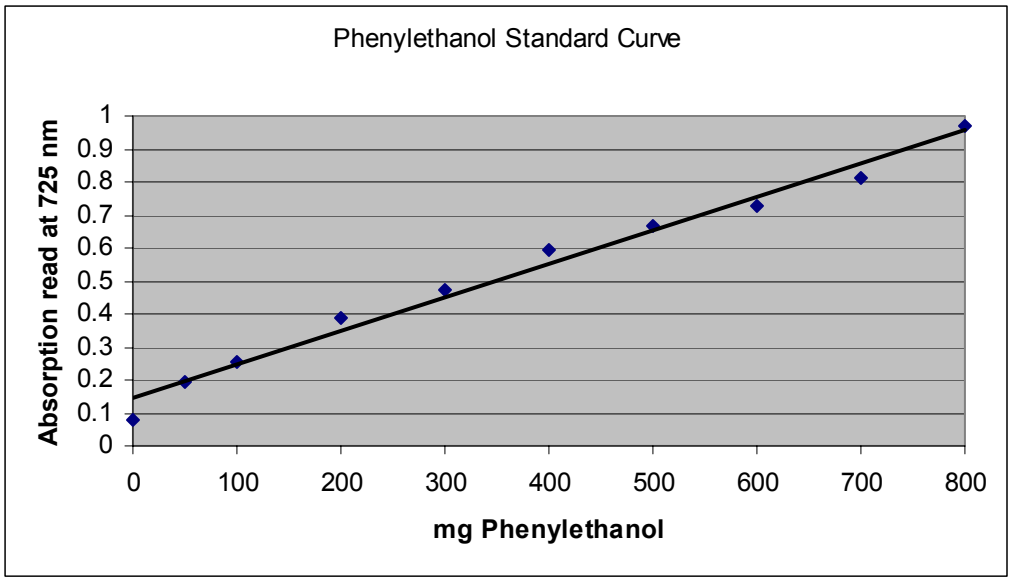

Figure 3. Phenolic Standard Curve

Determination of Phenolics

To release phenolics from fungal tissue, 0.30 g of dried fungal mycelia was added to 6 mL of 80 % ethanol and incubated for 1 hour at 60 °C. After the incubation period, fungal extracts were filtered and used the same day. Five-hundred micoliters of fungal extract was added to 25 mL of double distilled water and 1.6 mL of Folin-Denis reagent to a 125 mL flask. Flasks were mixed thoroughly for 3 minutes, then 3.3 mL of sodium carbonate was added and each flask was brought to a final volume of 33 mL with glass distilled water. The standard curve was prepared at the same time the fungal samples were measured. Samples were shaken for 30 minutes at room temperature. Absorbance was read against a control without phenolics at 725 nm. Standard control, 2-phenylethanol, was prepared at the same time the fungal samples were measured. Phenolics present in the fungal extracts were estimated from the linear portion of the

standard curve using the formula $y = a + b_{xy}X$. This was adjusted for dilutions to give phenolics per gram weight of fungal tissue by multiplying the amount of phenolics in 500 µl extract by 12 to give total phenolics in 6 ml ethanolic fungal extract, and by 3.3 to give total phenolics in one gram dry weight of fungal tissue (total X 19.8).

DPPH Assay for Free-Radical Scavenging Activity.

Scavenging Effect on the DPPH Radicals

The 2,2-diphenyl-1-picryhydrazl radical (DPPH) has been used to evaluate antioxidant activity of plant extracts (Espin *et al*. 2000;Yamaguchi *et al*., 1998; Yen and Chen 1995). Because of its odd electron, 2,2-diphenyl-1-picryl-hydrazl shows a strong absorption band, 517 nm (in ethanol), its solution appearing a deep violet color (Blois, 1958). The violet color of DPPH is modified to yellow in the presence of scavengers by appearance of reduced 2,2-diphenyl-1-picrylhydrazine. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen electron, this gives rise to the reduced form with the loss of the violet color (Molyneux, 2004). Fungi with stronger scavenging ability will quickly show a reduction in absorbance at 517 nm

DPPH Stock Solution

Free radical scavenging activity of fungal extracts followed procedures outlined in Fu *et al* (2002) using DPPH (see Appendix also). DPPH (Sigma: 0.012g) was dissolved in 100ml of 95% ethanol for a concentration of 3×10^{-4}M.

The solution was mixed and stored in the dark for 30 minutes before use. The solution was made fresh prior to each use.

Determination of Free-Radical Scavenging Activity

Dried mycelia were extracted with 95% ethanol so that the proportion of mycelia tissue to alcohol was 0.1g tissue: 20ml ETOH, and stored at 5 ± 1° C overnight. The extract was then centrifuged for 10 minutes at 10,000 RPM to pellet cell debris. Supernatants were stored at –80°C until used for further experiments. Different amounts (0µl, 33µl, 66µl, 99µl, 132µl, 165µl, & 198µl) of each ethanolic fungal extract were added to 333µl of a 3X solution DPPH in ethanol and made up with ethanol to a final volume of 1mL (final concentration of DPPH 1.0 X 10^{-4}) listed in Table 4.

The reaction was shaken vigorously and incubated in the dark for 60 minutes. Absorbance was measured at 517nm against a control without DPPH [ETOH only] using a HITACHI 2000 spectrophotometer. DPPH assays were carried out in triplicate. The scavenging activity (% SA) of fungal extract was calculated by an equation used from Cheung (2003):

Table 4. Preparation of Samples

Tube	1	2	3	4	5	6	7	8
DPPH	0	333	333	333	333	333	333	333
Extract	0	0	33	66	99	132	165	198
ETOH	1.0 ml	667	634	601	568	535	502	469

SA = (1 – Abs in the presence of fungal extract/Abs in the absence of fungal extract) X 100

From this equation, the percent scavenging ability of ethanolic fungal extract was calculated from the seven concentrations. Three concentrations 66µl, 132µl, and 198µl were selected to note the percentage increase in scavenging activity in relation to the increase in concentration of fungal extract. For example a sample that has 198µl of fungal extract would have a higher scavenging ability than the same sample using only 66µl of fungal extract. So, with an increase in fungal extract concentration there should be an increase in scavenging ability.

Oil-in-Water Emulsion for Determination of Lipid Peroxidation.

Extraction and Incubation of Fungal Extracts

Dried mycelium (0.33g) was extracted with 6.6 mL of 95% ethanol stored overnight and centrifuged the next day for 10 mins at 10,000 RPM. The supernatants were stored –80°C until the day of the experiment. For each reaction, 33µl of fungal extract was added to 1 mL of oil-in-water emulsion [30µl of stripped corn oil (USB Corporation), 3µl of Triton X-100 (Sigma), and 967µl of ddH_2O, vortexed together for 1 min]. For the control, 33µl butylated hydroxyanisole (BHA) a synthetic antioxidant for comparison with fungal extracts and 33µl ddH_2O were used. Each tube was incubated at 60°C and shaken at 250 rpm for 12 days (Duh and Yen, 1995; Fu *et al*., 2002).

Lipid oxidation in stripped corn oil emulsion was monitored by the ferric thiocyante method for 12 days (Fu *et al.,* 2002). In the oil-in-water emulsion systems hydrogen peroxides are a primary product of lipid oxidation. The ferric thiocyanate method consists of ammonium thiocyanate and ferrous iron in an acid solution. Hydrogen peroxide oxidizes ferrous iron to the ferric state, resulting in the formation of a red thiocyanate. For each sample, 783 µl of ethanol (75%), 16.7 µl of ammonium thiocyanate (30%), 16.7 µl of sample, and 16.7 µl ferrous chloride (20 mM in 3.5 % hydrochloric acid) were added to a 1.5 mL microfuge tube, vortexed and incubated for 3 minutes. Absorbance of each sample was measured at 500 nm over the course of twelve days. Daily absorbance for fungal emulsion samples was measured in triplicate against a water control. Percent inhibition of hydroperoxide formation in stripped corn oil was calculated by an equation used from Frankel et al. (1996):

%Inhibition = [(control (ddH_2O) – fungal extract)/ control (ddH_2O)] x 100

Percent inhibition of lipid oxidation for days 1, 3, 6, 9, and 12 were selected to note change in hydroperoxide formation over time. Also, a graph of lipid oxidation for the twelve days sampled was plotted as absorbance against time (days) to give a visual representation and comparison of species sampled time.

TBARS Method for Measuring Lipid Peroxidation

The thiobarbituric acid (TBA) test is one of the most widely used assay for the measurement of lipid peroxidation. The sample under test is treated with TBA at low pH, and a pink chromogen is measured (Punchard and Kelly, 1996). In the

Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid

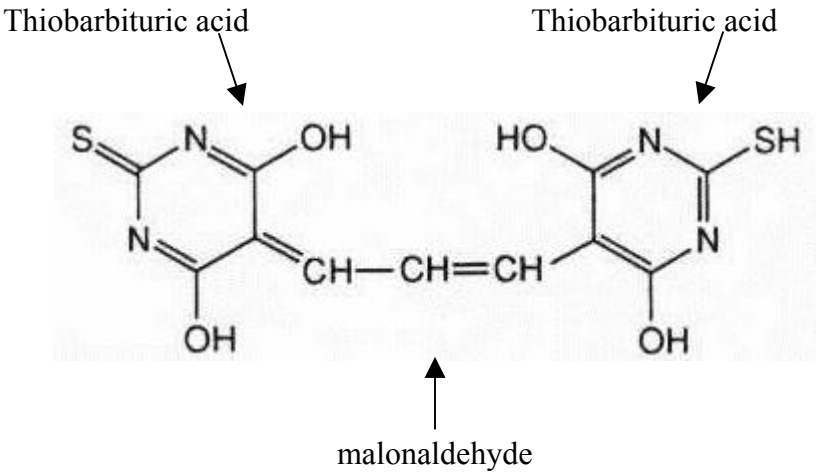

Figure 4. Structure of Malondialdehyde

TBA reaction, one molecule of malondialdehyde (MDA), a secondary breakdown product of lipid hydroperoxides, reacts with two molecules of TBA with the production of a pink pigment with an absorption maximum at 532-535 nm (See figure 4).

Fungal Extraction

Dried mycelium 0.33g was extracted with 6.67mL of 95% ethanol stored overnight and centrifuged the next day for 10 mins at 10,000 RPM. The supernatants were stored –80°C until the day of the experiment.

Oxidation of Stripped Corn Oil

Stripped Corn Oil was used as the lipid standard to test antioxidant abilities of *Pleurotus* species. To each 1.5mL microfuge tube, 100µl of fungal extract or ddH_2O (as a control) was added to 100µl stripped corn oil in the presence of 100µl of ferrous chloride used as a catalyst to induce lipid peroxidation. Samples were shaken for 30 minutes at 37°C.

Antioxidant Screening

In 1 X 7.5 cm glass test tubes, 100µl of each oxidized sample (see above) was added. To this mixture 100µl of 8.1% SDS was added and mixed together thoroughly. To each tube 2.5 mL TBA/Buffer reagent (ZeptoMetrix; see Appendix) was added making sure to pipette down the side of the test tube. Test tubes were covered with marbles and placed in a water bath at 95°C for 60 minutes. After incubation, test tubes were cooled to room temperature for 15 minutes in a ice bath and then samples were transfer to 15 mL centrifuge tubes and centrifuged for 15 minutes at 3000 rpm. The supernatant was transferred to a smaller test tube and the absorbance of each sample was read at 532 nm. From the absorbance values obtained, the percent antioxidant index (AI%) formula was used to calculate fungal extracts antioxidant stability:

$AI\% = (1 - T/C) * 100$

Where *C* is the absorbance value of the fully oxidized control (sample with no extract) and *T* is the absorbance of the test sample (Ruberto and Baratta, 2000). The antioxidant index formula value was used as a measure for retardation of lipid oxidation.

Statistical Methods.

Using SPSS version 13.0, (University of Tennessee, OIT), bivariate (Pearson) correlation coefficients were calculated to test is there was any significant relationship between levels of protein, polysaccharide, or phenolics with respect to free radical scavenging and TBARS antioxidant assays. The values were considered to be significantly different when the P value was less then 0.05. Repeated Measures ANOVA was performed on results from the oil-in-water emulsion. Since repeated samples (three samples) were taken daily from the same flask, ANOVA was performed comparing day 1 vs. day 3; day 3 vs. day 6; day 6 vs. day 9; and day 9 vs. day 12. Mauchly's Test of Sphericity ($P<0.05$) was used to accept or reject null hypothesis which was that the days were not significantly different.

Chapter III

Results

Phylogeny of *Pleurotus.*

A phylogenetic reconstruction based on the ribosomal RNA ITS1-5.8S-ITS2 region is given in Figure 5. All collections except one were correctly named and appear with other exemplars in clades representative of that species. A culture of one collection, putative *P. levis*, was apparently contaminated and was excluded from further analysis. Several attempts to sequence this collection produced two overlapping sequences. A blast search for a portion of the sequence that could be read gave the closest match as an unknown leaf-litter ascomycete.

Protein Levels in *Pleurotus* Species.

Protein levels in fungal tissues grown in liquid medium are given in Table 5. Protein levels varied from a low of 1.6 mg of protein per gram tissue dry weight to a high of 17.25 mg/g. There were large differences in the quantity of cultured mycelial protein within some species. Protein in *P. dryinus* cultures, for example, varied from 1.6 mg/g to 11.85 mg/g. Within *P. pulmonarius*, protein varied from 3.6 mg/g to 12.95 mg/g and within *P. ostreatus* from 6.45 mg/g to 10.43 mg/g. There were differences between protein levels in the fruit body and mycelium of *P. ostreatus*. *Pleurotus ostreatus* fruit body protein was 14.2 mg/gm and mycelial protein varied within species (6.45, 9.9, 10.45 mg/g).

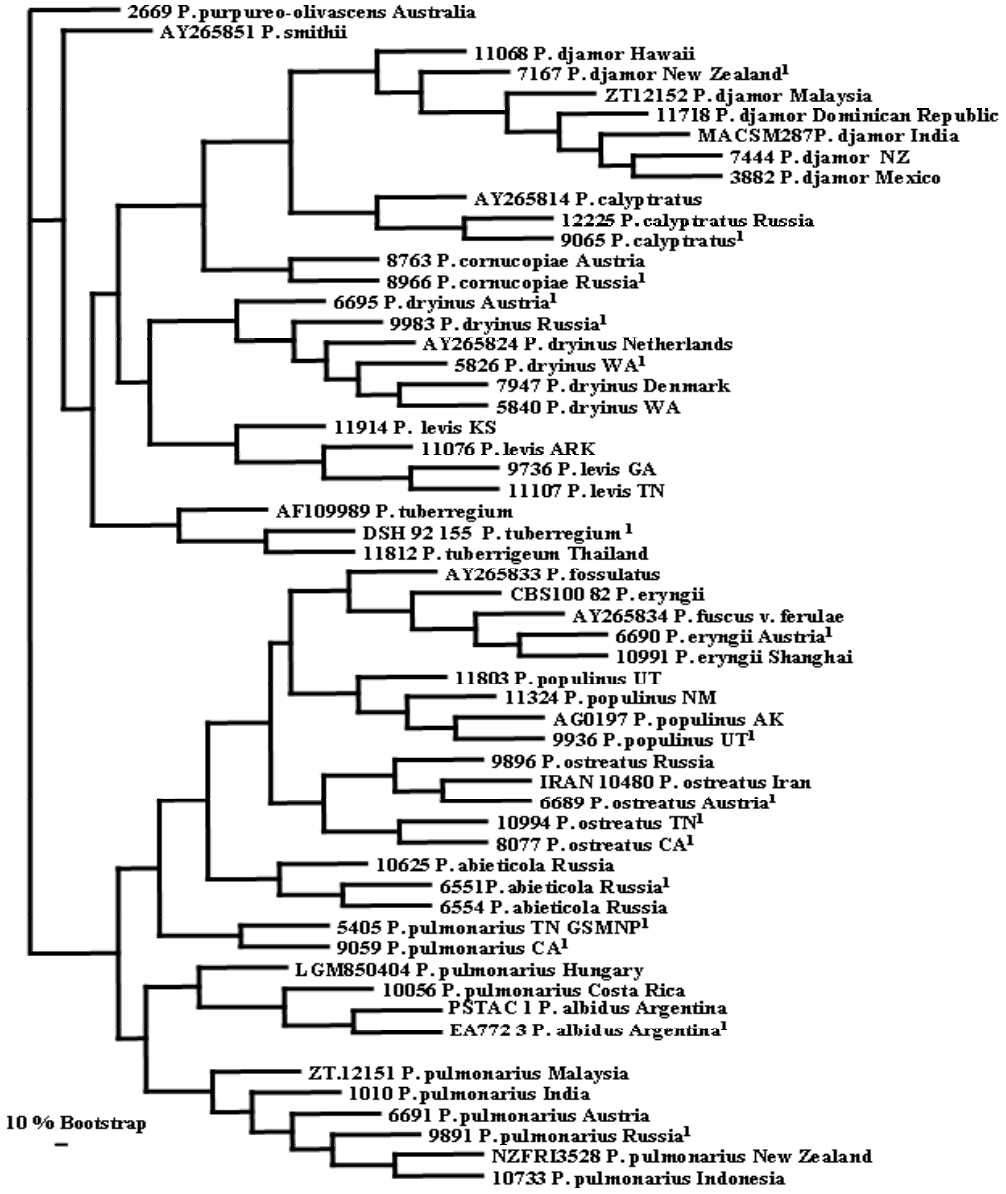

Figure 5. Phylogenetic Tree

A phylogenetic reconstruction of selected *Pleurotus* ribosomal RNA ITS1-5.8S-ITS2 sequences. Of 790 total characters, all characters were considered unordered and equally weighted. Three-hundred one characters were constant, 96 were parsimony uninformative and 393 were parsimony-informative. Gaps were treated as a fifth base. Tree length = 1327; Consistency index (CI) = 0.6745; Homoplasy index (HI) = 0.3255.

Table 5. Protein levels in *Pleurotus*

		Protein Determination				
Species	No.[1]	Protein sample 1	Protein sample 2	Protein sample 3	Mean mg Protein sampled	mg protein per 1g tissue dry weight
P. abieticola	6551	0.08	0.119	0.111	0.103 ± 0.021	5.15
P. albidus	EA772	0.024	0.033	0.053	0.036 ± 0.015	1.8
P. eryngii	6690	0.293	0.266	0.264	0.274 ± 0.016	13.7
P. populinus	9936	0.048	0.033	0.039	0.04 ± 0.007	2.0
P. pulmonarius	5405	0.246	0.283	0.246	0.259 ± 0.023	12.95
P. pulmonarius	9059	0.109	0.043	0.064	0.072 ± 0.034	3.6
P. pulmonarius	9891	0.246	0.239	0.22	0.235 ± 0.014	11.75
P. ostreatus	6689	0.184	0.175	0.237	0.198 ± 0.034	9.9
P. ostreatus	8077	0.109	0.155	0.125	0.129 ± 0.023	6.45
P.ostreatus	10994	0.203	0.226	0.235	0.221 ± 0.016	11.05
P. ostreatus Fruit body[1]	10994	0.289	0.278	0.285	0.284 ± 0.005	14.2
P. calyptratus	9065	0.043	0.009	0.014	0.073± 0.018	3.65
P. cornucopiae	8966	0.152	0.055	0.05	0.085 ± 0.057	4.25
P. djamor	7167	0.03	0.077	0.082	0.063 ± 0.029	3.15
P. dryinus	6695	0.19	0.184	0.178	0.184 ± 0.006	9.2
P. drynius	5826	0.018	0.048	0.03	0.032 ± 0.015	1.6
P. dryinus	9983	0.225	0.24	0.246	0.237± 0.011	11.83
P. purpureo-olivascens	2669	0.332	0.364	0.339	0.345 ± 0.017	17.25
P. tuberregium	92-155	0.043	0.062	0.03	0.045 ± 0.016	2.25
A. bisporus Fruit body[2]		0.344	0.395	0.329	0.356 ± 0.035	17.8
L. edodes Fruit body[3]		0.303	0.285	0.225	0.271 ± 0.041	13.55

[1] Tennessee Culture Collection Accession Number
[2] Fruit bodies from Monterey Mushroom Farms, Tennessee
[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

Polysacchride Levels in *Pleurotus* Species.

Carbohydrate levels in fungal tissue grown in liquid medium are given in Table 6. *Pleurotus* species were approximately equal with respect to carbohydrate level, ranging from 16.8 mg to 25.6 mg among species. There was a considerable difference between carbohydrate levels in the *P. ostreatus* fruit body versus mycelium. The carbohydrate level of the fruit body was 1.6 mg; mycelial carbohydrate varied between the three within-species collections (23.8, 24.8, and 24.8 mg). Clearly carbohydrate levels are higher in the mycelia than in fruit body.

Phenolic Levels in Pleurotus Species.

Phenolic levels in fungal tissue grown in liquid culture are given in Table 7. Phenolic compounds ranged from a low of 0.229 phenolics per gram tissue dry weight to a high of 12.2 g. Phenolics differed within species for *P. ostreatus* and *P. dryinus*. Within *P. ostreatus*, cultures varied from 1.75 g to 2.78 g. Within *P. dryinus* there was a considerable difference in phenolic compounds, with cultures varying from 0.229 g to 12.2 g. There were differences in the level of phenolics of the fruit body compared to mycelium for *P. ostreatus*. The fruit body phenolic content was much lower, 0.542 g, compared to within-species mycelium which was 1.75, 2.78, and 2.87 grams.

Table 6. Polysaccharide level in *Pleurotus*

Species	No[1]	Poly 1	Poly 2	Poly 3	Mean mg	mg poly per 1 g tissue dry weight
P. abieticola	6551	1.17	1.25	1.13	1.17 ± 0.04	23.4
P. albidus	EA772	0.98	1.05	1.11	1.05 ± 0.07	21
P. eryngii	6690	1.29	1.26	1.25	1.27 ± 0.02	25.4
P. populinus	9936	1.20	1.22	1.22	1.21 ± 0.01	24.2
P. pulmonarius	5405	1.19	1.26	1.20	1.22 ± 0.04	24.4
P. pulmonarius	9059	0.83	0.82	0.85	0.84 ± 0.01	16.8
P. pulmonarius	9891	1.20	1.18	1.25	1.21 ± 0.04	24.2
P. ostreatus	6689	1.23	1.25	1.25	1.24 ± 0.01	24.8
P. ostreatus	8077	1.19	1.22	1.16	1.19 ± 0.03	23.8
P. ostreatus	10994	1.26	1.23	1.21	1.24 + 0.02	24.8
P. ostreatus Fruit body[1]	10994	0.097	0.086	0.06	0.08 ± 0.02	1.6
P. calyptratus	9065	1.27	1.26	1.22	1.25 ± 0.03	25
P. cornucopiae	8966	1.23	1.24	.125	1.24 ± 0.01	24.8
P. djamor	7167	1.15	1.21	1.22	1.19 ± 0.04	23.8
P. dryinus	6695	1.26	1.28	1.30	1.28 ± 0.02	25.6
P. drynius	5826	1.26	1.28	1.30	1.28 ± 0.02	25.6
P. dryinus	9983	1.26	1.28	1.30	1.28 ± 0.02	25.6
P. purpureo-olivascens	2669	1.26	1.26	1.26	1.26 ± 0.001	25.2
P. tuberregium	92-155	1.27	1.28	1.26	1.27 ± 0.01	25.4
L. edodes	mycelium	1.20	1.21	1.20	1.20 ± 0.004	24

[1] Tennessee Culture Collection Accession Number

[2] Fruit bodies from Fungi Perfecti, Olympia, Washington

Table 7. Phenolics levels in *Pleurotus*

Species	No[1]	Phenolic 1	Phenolic 2	Phenolic 3	Mean mg	phenolics per 1 g
P. abieticola	6551	21.2	22	24	22 ± 1.5	0.4356
P. albidus	EA772	136.4	137.4	139.3	137.7 ± 1.5	2.726
P. eryngii	6690	133.4	137.4	137.4	136.1 ± 2.2	2.694
P. populinus	9936	70.4	75.3	74.3	73.4 ± 2.6	1.453
P. pulmonarius	5405	210.3	210.3	210.3	210.3	4.163
P. pulmonarius	9059	51.7	54.6	54.6	53.6 ± 1.7	1.061
P. pulmonarius	9891	20.2	22.1	21.2	21.2 ± 0.9	0.419
P. ostreatus	6689	147.3	144.3	144.3	145.3 ± 1.7	2.876
P. ostreatus	8077	86.2	86.2	94.1	88.8 ± 4.5	1.758
P. ostreatus	10994	141.3	139.4	141.3	140.7 ± 1.1	2.785
P. ostreatus Fruit body[1]	10994	27.1	27.1	28.0	27.4 ± 0.5	0.542
P. calyptratus	9065	223.1	214.3	218.2	218.5 ± 4.4	4.326
P. cornucopiae	8966	162.0	163.0	167.9	164.3 ± 3.1	3.253
P. djamor	7167	62.5	62.5	63.5	62.8 ± 0.5	1.243
P. dryinus	6695	609.33	614.3	619.2	614.3 ± 4.9	12.16
P. drynius	5826	140.4	139.3	142.3	140.7 ± 1.5	2.785
P. dryinus	9983	11.3	12.3	11.3	11.6 ± 0.5	0.229
P. purpureo-olivascens	2669	176.8	178.7	177.8	17+7.8 ± 0.9	0.336
P. tuberregium	92-155	77.3	78.3	76.3	77.3 ± 0.9	1.530
A. bisporus	Fruitbody[2]	69.4	71.4	70.4	70.4 ± 0.9	1.393
L. edodes	Fruitbody[3]	5.4	4.4	3.4	4.4 ± 0.9	0.087

[1] Tennessee Culture Collection Accession Number
[2] Fruit bodies from Monterey Mushroom Farms, Tennessee
[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

Free-Radical Scavenging Activity of *Pleurotus* Extracts.

Results of tests for free-radical scavenging activity using DPPH are given in Table 8. Free-radical scavenging activity was highest in *A. bisporus* fruit body (85.67%) followed by *Pleurotus dryinus* (71-75%). Moderate activity occurred in the fruitbodies of *P. ostreatus* and *L. edodes* and in cultures of *P. populinus, P. cornucopiae, P. pulmonarius, P. calyptratus, P. tuberregium, P. erygnii, P. djamor, P. purpureo-olivascens, P. albidus, P. abieticola, P. ostreatus*.

Antioxidant Activity of *Pleurotus* extracts in Oil-in-Water Emulsion.

The ability of *Pleurotus* extracts to retard corn oil-in-water oxidation is given in Figure 6 and Table 9. Retardation of emulsion oxidation of *Pleurotus* extracts was highest in *P. dryinus*, *P. ostreatus*, *P. calyptratus*, and *P. tuberregium*. The additional *Pleurotus* species were approximately equal with respect to retardation of oxidation in the corn oil-in-water system but clearly did retard oxidation.

TBARS Assay

Results from the TBARS assay are given in Table 10. Antioxidant activity was highest in *P. albidus* followed by *P. dryinus*, *P. ostreatus, P. purpureo-olivascens, P. erygnii,* and *P. populinus*, *P. tuberregium, P. cornucopiae, P. calyptratus,* and *P. pulmonarius.* The antioxidant activity of *P. ostreatus* fruitbody was 34.7% which differed from the mycelium ranging in averages of within species 59.7% and 56.0% respectively.

Table 8. Free-radical Scavenging Activity

Species	No.[1]	Sample Concentration (µl/mL) 66 µl	132 µl	198 µl
P. abieticola	6551	4.6 ± 0.91	6.9 ± 4.1	14.4 ± 4.0
P. albidus	EA772	7.6 ± 3.9	14.8 ± 8.4	20.6 ± 8.2
P. erygnii	6690	6.6 ± 4.0	11.5 ± 5.2	16.7 ± 5.7
P. populinus	9936	4.5 ± 1.9	18.3 ± 2.6	36.8 ± 7.4
P. pulmonarius	5405	7.4 ± 3.4	9.9 ± 0.8	13.5 ± 2.9
P. pulmonarius	9059	11.5 ± 4.7	17.6 ± 2.6	27.5 ± 3.6
P. pulmonarius	9891	7.4 ± 0.28	15 ± 3.6	18.5 ± 2.5
P. ostreatus	6689	10.2 ± 3.8	14.3 ± 5.8	25.2 ± 3.1
P. ostreatus	8077	8.1 + 4.1	14.6 ± 4.5	19.1 ± 6.1
P. ostreatus	10994	5.3 ± 8.4	14.8 ± 3.5	20.8 ± 1.3
P. ostreatus Fruit body[1]	10994	19.1 ± 5.0	33.7 ± 3.8	47.5 ± 2.3
P. calyptratus	9065	12.5 ± 0.8	18.1 ± 1.4	23.8 ±1.3
P. cornucopiae	8966	12.1 ± 3.4	20.6 ± 2.3	29.2 ± 5.1
P. djamor	7167	8.7 ± 2.8	15.4 ± 3.9	22.5 ± 4.1
P. dryinus	6695	23.8 ± 3.5	52.2 ± 1.9	71.1 ± 7.5
P. dryinus	5826	26.8 ± 3.1	50.3 ± 3.4	74.2 ± 5.5
P. dryinus	9983	30.5 ± 3.7	53.1 ± 9.0	75.5 ± 2.8
P. purpureo-olivascens	2669	10.9 ± 3.0	21.4 ± 3.4	29.6 ± 1.9
P.turberregium	DSH92-155	8.9 ± 6.2	19.5 ± 4.6	27.8 ± 4.4
A. bisporus Fruit body[2]		51.4 ± 1.6	85.4 ± 0.5	85.6 ± 0.5
L. edodes Fruit body[3]		10.8 ± 2.6	20.5 ± 4.2	29.2 ± 2.7

[1] Tennessee Culture Collection Accession Number
[2] Fruit bodies from Monterey Mushroom Farms, Tennessee
[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

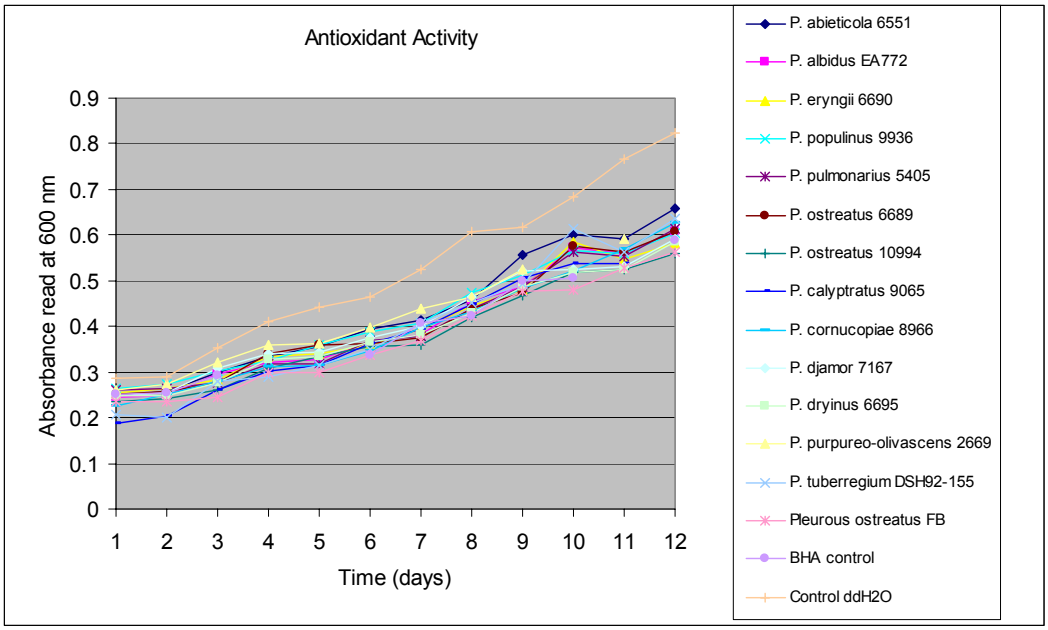

Figure 6. Antioxidant Activity in Oil-in-water Emulsion

Table 9. Antioxidant Activity in Oil-in-water Emulsion

Species	No.[1]	Percent Inhibition of Fungal extracts in Oil-in-water Emulsion %Inhibition = [(control – sample)/control] x 100				
		Day 1	Day 3	Day 6	Day 9	Day 12
P. abieticola	6551	13.1 ± 6	15.4 ± 2	14.5 ± 6	12.3 ± 3	20.2 ± 2
P. albidus	EA772	13.9 ± 7	17.0 ± 5	20.9 ± 8	19.8 ± 11	24.5 ± 5
P. erygnii	6690	10.9 ± 3	20.2 ± 2	22.4 ± 10	21.0 ± 5	25.9 ± 3
P. populinus	9936	7.3 ± 8	14.8 ± 6	16.1 ± 12	15.6 ± 2	24.3 ± 4
P.pulmonarius	5405	11.3 ± 3	22.1 ± 7	19.8 ± 10	20.1 ± 1	25.2 ± 2
P.pulmonarius	9059	17.8 ± 5	19.6 ± 8	20.2 ± 11	19.2 ± 4	27.7 ± 2
P.pulmonarius	9891	13.1 ± 4	20.6 ± 3	21.1 ± 11	19.6 ± 2	23.5 ± 2
P. ostreatus	6689	7.7 ± 4	22.0 ± 8	20.5 ± 12	21.6 ± 6	26.5 ± 1
P. ostreatus	8077	26.8 ± 16	22.7 ± 1	23.1 ± 4	16.4 ± 3	30.7 ± 6
P. ostreatus	10994	13.3 ± 7	26.1 ± 2	23.1 ± 5	23.1 ± 10	26.9 ± 5
P. ostreatus Fruit body[1]	10994	17.1 ± 10	25.8 ± 10	26.8 ± 5	21.2 ± 10	28.5 ± 3
P. calyptratus	9065	31.9 ± 10	26.8 ± 9	20.5 ± 4	16.8 ± 4	29.5 ± 4
P. cornucopiae	8966	17.2 ± 17	20.9 ± 5	25.0 ± 10	20.3 ± 5	23.1 ± 1
P. djamor	7167	13.1 ± 4	12.7 ± 5	18.6 ± 6	14.1 ± 3	28.4 ± 2
P. dryinus	6695	11.5 ± 1	21.9 ± 6	21.1 ± 5	19.9 ± 5	29.4 ± 1
P. dryinus	5826	19.4 ± 7	22.8 ± 7	22.2 ± 11	21.4 ± 3	25.5 ± 5
P. dryinus	9983	37.8 ± 0.5	24.9 ± 7	28.6 ± 1	28.5 ± 5	30.1 ± 2
P. purpureo-olivascens	2669	8.65 ± 4	9.02 ± 5	13.9 ± 8	13.5 ± 4	20.4 ± 3
P. tuberregium	DSH92-155	25.3 ± 3	29.5 ± 5	24.0 ± 13	18.7 ± 8	24.2 ± 3
BHA	Commerical	12.1 ± .2	23 ± 4	27 ± 8	17 ± 9	28.4 ± 7
A. bisporus	Fruit body[2]	21.7 ± 7	17.4 ± 3	23.1 ± 8	22.1 ± 3	27.5 ± 3
L. edodes	Fruit body[3]	18.7 ± 3	27.5 ± 3	29.0 ± 5	23.9 ± 4	26.1 ± 5

[1] Tennessee Culture Collection Accession Number

[2] Fruit bodies from Monterey Mushroom Farms, Tennessee

[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

[4] Butylated hydroxyanisole, Sigma

Table 10. Thiobarbituric Acid Assay

Species	No.[1]	Percent Inhibition of lipid peroxidation			Mean avg.
		Trial 1	Trial 2	Trial 3	
P. abieticola	6551	47.1	45.8	46.9	46.6 ± 0.7
P. albidus	EA772	64.9	63.3	64.5	64.3 ± 0.8
P. erygnii	6690	61.4	48.7	60.5	56.8 ± 7.1
P. populinus	9936	59.3	53.8	54.3	55.9 ± 2.9
P.pulmonarius	5405	28.2	37.9	13.6	26.6 + 12.2
P.pulmonarius	9059	49.8	49.1	48.9	49.3 + 0.4
P.pulmonarius	9891	57.1	58.2	56.5	57.3 + 0.8
P. ostreatus	6689	61.2	63.7	54.4	59.7 ± 4.8
P. ostreatus	10994	59.7	62.8	45.5	56.0 ± 9.2
P. ostreatus Fruit body[1]	10994	45.0	38.3	20.8	34.7 ± 12.4
P. calyptratus	9065	58.8	57.6	56.8	57.8 ± 0.9
P. cornucopiae	8966	67.2	50.3	49.6	55.7 ± 11.7
P. djamor	7167	47.1	47.5	50.6	48.4 ± 1.9
P. dryinus	6695	57.5	54.1	54.9	55.5 ± 1.7
P. dryinus	5826	54.5	62.7	62.7	60.0 ± 4.7
P. dryinus	9983	56.7	56.1	55.9	56.3 + 0.4
P. purpureo-olivascens	2669	67.2	57.5	46.0	56.9 ± 10.6
P. tuberregium	DSH92-155	61.4	60.6	40.7	54.3 + 11.7
A. bisporus	Fruit body[2]	21.0	25.6	14.2	20.3 ± 5.7
L. edodes	Fruit body[3]	53.8	50.3	40.6	48.3 ± 6.8

[1] Tennessee Culture Collection Accession Number
[2] Fruit bodies from Monterey Mushroom Farms, Tennessee
[3] Fruit bodies from Fungi Perfecti, Olympia, Washington

Statistical Analyses.

Using Pearson correlation coefficients, there was a significant correlation ($P<0.04$) between protein and polysaccharide but not phenol and protein or phenol and polysaccharide in *Pleurotus* species sampled. There was a significant correlations ($P<0.04$) between protein and polysaccharide content in relation to TBARS assay. There was not a significant relationship between free radical scavenging in conjunction with protein, polysaccharide or phenol content respectively. Correlation data is listed in Table 11.

Table 11. Correlations

		Protein	Poly	Phenol	Radical	TBARS
Protein	Pearson Correlation	1	-.453(*)	-.011	.198	-.480(*)
	Sig. (2-tailed)		**.039**	.961	.391	.032
	N	21	21	21	21	20
Poly	Pearson Correlation	-.453(*)	1	.335	-.282	.595(**)
	Sig. (2-tailed)	.039		.138	.216	.006
	N	21	21	21	21	20
Phenol	Pearson Correlation	-.011	.335	1	.210	.158
	Sig. (2-tailed)	.961	.138		.361	.505
	N	21	21	21	21	20
Radical	Pearson Correlation	.198	-.282	.210	1	-.217
	Sig. (2-tailed)	.391	.216	.361		.358
	N	21	21	21	21	20
TBARS	Pearson Correlation	-.480(*)	.595(**)	.158	-.217	1
	Sig. (2-tailed)	**.032**	**.006**	.505	.358	
	N	20	20	20	20	20

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Using Mauchly's test of Sphericity there was a significant (P<0.01) difference between days sampled for the oil-in-water assay. Tests of within subjects contrasts were made for day 1 vs. day 3, day 6 vs. day 9 and day 9 vs. day 11 and demonstrated significant differences between days (P<0.008). For day 3 vs. day 6, there was no significant difference (P>0.2) between days which is listed in Table 12.

Table 12. Mauchly's test of Sphericity
Measure: MEASURE_1 Mauchly's Test of Sphericity(b)

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon(a)		
					Greenhouse-Geisser	Huynh-Feldt	Lower-bound
factor1	.214	29.929	9	.000	.551	.619	.250

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.
a May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.
b Design: Intercept
Within Subjects Design: factor1

Tests of Within-Subjects Contrasts

Measure: MEASURE_1

Source	factor1	Type III Sum of Squares	df	Mean Square	F	Sig.
factor1	Level 1 vs. Level 2	540.836	1	540.836	17.593	.001
	Level 2 vs. Level 3	9.850	1	9.850	.781	.388
	Level 3 vs. Level 4	97.316	1	97.316	13.593	.002
	Level 4 vs. Level 5	957.743	1	957.743	82.053	.000
Error(factor1)	Level 1 vs. Level 2	553.361	18	30.742		
	Level 2 vs. Level 3	226.965	18	12.609		
	Level 3 vs. Level 4	128.864	18	7.159		
	Level 4 vs. Level 5	210.100	18	11.672		

Chapter IV

Discussion and Conclusions

Accurate species identification is important for research to ensure that medicinal attributes are assigned appropriately. Sequencing fungal genes, specifically the internal transcribe spacer (ITS) region, has helped identify and group related species (White *et al*., 1990). This study employed the use of the internal transcribe spacer (ITS) region to identify *Pleurotus* species that could not be clearly identified morphologically. In this study twelve species of *Pleurotus* were used. In a few cases, collections representing geographical variants within species were also assayed. All of the species were correctly identified based on an already established collection of ITS sequences (Peterson, R. H. pers. comm.), however one culture of *P. levis* was shown to be contaminated with an unknown ascomycete and was excluded from further analyses.

Following molecular confirmation of *Pleurotus* cultures, antioxidant activity was evaluated. Three methods were used to evaluate antioxidant activity; 1) free-radical scavenging; 2) oil-in-water emulsion; and 3) TBARS assay. These assays were used by pervious researchers (Fu *et al*., 2002; Mau *et al*., 2002, Ruberto and Baratta, 2000). Total protein, carbohydrate, and phenolics in fungal extracts were also determined. Correlation coefficients were used to determine if there was a significant correlation between these compounds and antioxidant activity.

Free radical scavenging is a well-known mechanism by which antioxidants inhibit lipid oxidation (Cheung *et al*., 2003). In general, *Pleurotus* species showed some ability to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH), which increased as the concentration of fungal extract increased. The order of scavenging activity from highest to lowest was as follows: *P. dryinus, P. populinus, P. cornucopiae, P. pulmonarius, P. calyptratus, P. tuberregium, P. erygnii, P. djamor, P. purpureo-olivascens, P. albidus, P. abieticola, P. ostreatus.* Fu *et al*., (2002), Yang *et al*., (2002) also found that scavenging of the free radical DPPH was concentration dependent. Of the various mushrooms sampled by Fu *et al* (2002), *P. ostreatus* and *P. eryngii* had low scavenging ability, ranging from 14.9% to 29.1%, which is in agreement with results of this research. Yang *et al*., (2002), in contrast, observed that *P. ostreatus* scavenging ability was high, about 80%, and *P. cystidiosus* had a moderate to high scavenging ability of 65% (*P. cystidiosus* was not included in this study). The two outgroups used, *A. bisporus* and *L. edodes* varied in scavenging ability, with the former having a high scavenging ability 85%, and the latter low scavenging ability 29%. These findings also agree with findings by Fu *et al.,* (2002).

Determination of metabolic/developmental differences between mycelial and fruit body stages were compared. *Pleurotus ostreatus* fruit body scavenging ability was 47.5% which was considerably better then mycelia (19.4%, 22%, and 25%). Unfortunately the culture of *Agaricus bisporus* grew poorly under our standard growing conditions so it was not possible to compare the mycelial and fruit body stages. Comparing only one sample does not give an accurate

comparison between mycelia and fruit body stage since all species were not compared but does suggest that fruitbodies and undifferentiated mycelia differ in their ability to quench free radicals.

Two lipid systems were used to compare antioxidant activity in *Pleurotus* species. The oil-in-water emulsion protocol used stripped corn oil because regular corn oil contains tocopherols which would interfere with antioxidant activity assays. The ability of *Pleurotus* species to inhibit lipid oxidation in the oil-in-water system fluctuated over the 12 days sampled. For example, for *P. ostreatus* at day 1, the percent inhibition was 9.6% compared to day 3 with a percent inhibition 22 %. The repeated measures ANOVA table, using Mauchly's Test of Sphericity was run to evaluate if there was significant ($P<0.05$) difference between days sampled. Two-percent butylated hydroxyanisole (BHA), a known antioxidant, was compared with *Pleurotus* extracts for ability to inhibit lipid oxidation in the oil-in-water. Inhibition for BHA was similar to *Pleurtous* extracts sampled. Fu *et al*., (2002) stated that *P. ostreatus* and *P. eryngii* had low antioxidant activity in retarding lipid oxidation with a peroxide value (absorbance 500 nm) more than 0.8 after eight days. However, this study showed that all of the *Pleurotous* species sampled on the eighth day had peroxide values below 0.5 A_{500} indicating that the fungal extracts from *Pleurotus* were retarding lipid oxidation, a process which continued to up until the tenth day for most samples. Differences in the results of the two studies may reflect differences in the substrate used or other experimental factors.

In the oil-in-water assay (Fu et al., 2002) the amount of hydrogen peroxide generated through this system was a direct indication of lipid degradation. In this research, the oil-in-water assay did not show a clear indication of hydrogen peroxide production because of fluctuation over the 12 days sampled. This may indicate that there were other components in this system initiating lipid oxidation which interfered with the assay. After critically examining the oil-in-water system, there could be several explanations for the fluxes in hydrogen peroxide results. One possibly involves oxygen. Oxygen exists in air as a molecule (O_2) known as dioxygen or molecular oxygen. When molecules are oxidized with oxygen, the oxygen molecule itself becomes reduced and forms intermediates, two of which are free radicals ($HO_2^{\bullet}$, $^{\bullet}OH$) and are formed together with H_2O_2 (Gutteridge, 1995). Iron ions are themselves free radicals, and ferrous ions can take part in electron transfer reactions with molecule oxygen:

$Fe^{2+} + O_2 \rightarrow Fe^{2+}O_2 \leftrightarrow Fe^{3+}O_2^- \rightarrow Fe^{3+} + O_2^-$

Generation of superoxide, in the presence of iron can lead to the formation of hydroxyl radicals by Fenton chemistry.

Another thing to consider is the purity of the lipid preparation. All commercial and biological samples of unsaturated fatty acids contain trace to large amounts of peroxidized material (Guteridge and Kerry, 1982). The addition of a iron complex to such a preparation will stimulate peroxidation by peroxide decomposition, generating $LO^{\bullet}$ and $LOO^{\bullet}$ radicals. Lastly iron is a well known catalyst of lipid oxidation, and it is most effective in initiating biological oxidations when Fe^{3+} can be recycled to Fe^{2+} by various reducing agents (i.e

glutathione) (Schaich and Borg, 1988), and from this iron can be used to catalytically produce damaging fluxes of OH•.

The thiobarbituric acid (TBA) assay, concerns the spectrophotometric detection of thiobarbituric acid reactive substances (TBARS), primarily malonaldehyde (MDA), one of the secondary lipid peroxidation products, whose quantification gives a measure of the extent of lipid degradation (Ruberto and Baratta, 1999). *Pleurotus* species showed moderate to high ability to inhibit lipid degradation as follows: *P. albidus P. dryinus, P. ostreatus, P. calyptratus, P. pulmonarius, P. purpureo-olivascens, P. erygnii, P. cornucopiae, P. populinus, P. tuberregium, and P. abieticola*. Yang *et al* (2002) used a similar method (1,3-diethyl-2-thiobarbituric acid; DETBA), and concluded that *P. cystidiosus* and *P. ostreatus* had moderate to high activity in inhibiting lipid degradation.

The thiobarbituric acid assay has been shown to overestimate results concerning protection against lipid peroxidation (Frankel, 1985; Janero, 1990) and results should be interpreted with caution. Besides aldehydes, specifically MDA, other substances such as ketones, ketosteroids, acids, ester, sugars, and oxidized proteins, can react with TBA (Guillen-Sans and Guzman-Chozas, 1998). Using the TBA assay for this study, the majority of *Pleurotus* species sampled prevented lipid oxidation at a level of 50% or greater. There maybe other substances reacting with TBA to give elevated results. However, other researcher's studies have shown that the formation of thiobarbituric acid reactive substances is correlated with and similar to the actual malondialdehyde (MDA)

formation regardless of overestimated results (Kikugawa *et al*., 1992; Wong *et al*., 1995).

It had been reported that the antioxidant activity of plant material is correlated with presence of plant phenolic compounds (Velioglu *et al*., 1998). Thus it was important to consider the effect of phenolic content regarding antioxidant activity of mushroom extracts and also address other compounds such as proteins and carbohydrates in association with phenolic content. *Pleurotus* species varied in their phenolic content which was not significantly ($P>0.2$) correlated with scavenging ability, and prevention of lipid degradation. Some species, in particular *P. purpureo-olivascens, P. calyptratus, P. cornucopiae and P. .pulmonarius* had relatively high phenolic content but scavenging ability was low 29%, 23.8%, 29.2%, and 13.4% respectively.

There was a problem with the phenol levels calculation regarding *Pleurotus* species sampled. Using 2-phenylethanol as a standard control produced results that over estimated the total amount of phenol level in fungal samples. To evaluate whether the standard used for the assay, 2-phenlyethanol, was causing the overestimate of phenolics, a standard curve was calculated using catechin hydrate as the phenol standard. The standard curve for catechin, was approximately three times higher than that for phenol ethanol. Catechin, however, differs in the number of phenolics groups present in the compound. Phenylethanol has a single phenolic group; catechin has three phenolic groups. Solubility of the phenol standards in the assay mixture could also be a problem. If phenol standards were not completely dissolved, phenol levels would be

overestimated; however, no evidence of solubility problems was observed in the assay system. From these results, it appears unlikely that the standard used to estimate the amount of phenolics is the problem. Folin and Dennis (1912) reported 26 substances that reacted with their reagent, several of which (salicylic acid, tannic acid, vanillin) are common plant secondary compounds. Possibly, there are reactive substances in *Pleurotus* extracts which produce a blue color and increase estimates of phenolics.

Nakamura et al., 1992, covalently linked polysaccharides (dextran) to ovalbumin and evaluated the antioxidative effect of ovalbumin-polysaccharide conjugates in food system using commercial salad oil as the oxidizable substrate. They found that the ovalbumin-polysaccharide conjugates inhibited salad oil oxidation with a peroxide value of 40 meg/kg compared to a control (no extract) with a peroxide value of 80 meg/kg with heating time of 4 hours. Xue et al., 1998, and Rupérez et al., 2002 stated that antioxidative activities of several marine polysaccharides and their derivatives might be related to sulfate content in the molecules and molecular weight. Liu *et al*., 1997, stated that polysaccharides are usually associated with protein as complexes. He concluded that scavenging activity of polysaccharides extracts appeared to depend on the amount of protein present as polysaccharide-proteins complexes. In this study, polysaccharide extracts were not used to test directly for scavenging ability. Antioxidant activity in fungal extracts, were used in conjunction with phenol, and protein content to deduce if there was a significance relationship. Polysaccharide content in *Pleurotus* species ranged from 16.8 mg to 25.6 mg. When comparing scavenging

ability of fungal extracts to polysaccharide content, there was not a significant correlation (P>0.2), but *P. drynius* had the highest polysaccharide level (25.6mg) and was the best scavenger. However, there were other *Pleurotus* species that had polysaccharide contents of 20 mg or greater, but had a low scavenging ability. When comparing protein content with scavenging ability, there was not an apparent correlation (P>0.3). There was a significant correlation between polysaccharide (P< 0.007) and protein (P>0.04) levels and TBAR assay.

In conclusion, this research posed the question "is there a correlation between the phylogenetic distributions of *Pleurotus* species in relation to their antioxidant activity?" The moderate to high antioxidant activities together with varying levels of phenolics, proteins, and polysaccharides, were distributed throughout the Genus *Pleurotus*. Therefore, the antioxidant activity of the species sampled from the Genus *Pleurotus* was not related to any phylogenetic distribution. Overall, this research expanded on previous studies (Breene 1989; Gunden-Cimerman, 1999;Ikekawa 2001)concerning the abilities of *Pleurotus* species to scavenge for radicals and inhibit lipid oxidation. *Pleurotus* along with many other types of mushrooms have been investigated as a source of natural antioxidants for nutritional, therapeutic, and medicinal purposes. Findings from this research showed that fungal mycelia have moderate to high antioxidant activity which is significantly correlated with protein and polysaccharide content using the TBARS assay. One species sampled in particular, *P. dryinus,* constantly proved to be a better scavenger, inhibitor of lipid oxidation, and was found to have moderate to high levels of levels of protein, polysaccharide and phenol

levels. The use of *P. dryinus* as a source for a natural antioxidant needs to be further investigated. *Pleurotus dryinus* is found in the Pacific Northwestern regions of Canada and the USA and is not commercially cultivated as an edible or medicinal fungus.

Different vegetative states (fruitbody vs. mycelia) were also compared with respect to their antioxidant activity. There were significant differences in protein, polysaccharide, and phenol, scavenging activity and inhibition of lipid degradation. This research did not compare both vegetative states for all species sampled and further research to see is this difference is significant in the search for natural antioxidants for medicinal purposes.

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Appendix

PCR REACTION MIXTURE

Reaction Mix:	
dd water	30.75
10X PCR Buffer (Promega)	5μL
MgCl, 25mM	6 μL
dNTP stock 10mM	4 μL
Primer ITS 1F (10mM)	1 μL
Primer ITS 4 (10mM)	1 μL
BSA 400 mg/ml	1 μL
Taq polymerase (Promega)	0.25 μL

SOLUTIONS USED IN DNA EXTRACTIONS OF FUNGAL MYCELIA

Tris/EDTA (TE)
Reference: Sambrook, Fritsch, and Maniatis (1989). "Molecular Cloning: A Laboratory Manual"

10mM Tris Cl (pH 7.4)
1 mM EDTA (pH 8.0)

ELECTROPHORESIS BUFFER

Tris-borate/EDTA (TBE)
Reference: Sambrook, Fritsch, and Maniatis (1989). "Molecular Cloning: A Laboratory Manual"

Concentrated 5X stock solution (per liter):

54g Tris base
27.5g boric acid
20 mL 0.5 EDTA (pH 8.0)

Working solution: 1X

3X DPPH STOCK SOLUTION (3 X 10^{-4}M) AND PROTOCOL

Reference: Fu et al., 2002

Prepare fresh solution before each use (at least weekly). A total of 2.5 mls will be needed for 1 assay. The final concentration in the assay is 1 X 10^{-4} M DPPH FW = 394.3 (Sigma Chemicals)

0.012 g/L DPPH (3 X 10^{-4}M)
100 mls 95% ethanol

Mix solution and store in the dark for 30 mins before use.

For each assay, add 333µL DPPH 3X stock to 666 µL fungal extract +95% ETOH in a 1 ml cuvette (Add DPPH last). Cover cuvette with parafilm and shake vigorously. Store in the dark for 20 min then read absorbance at 517 nm.

OIL-IN-WATER EMULSION PROTOCOL

Reference: Fu *et al.,* 2002

Solutions needed

C.A. 75% ETOH for extraction

23% Ammonium thiocyanate: Dissolve 30 g ammonium thiocyanate in 100 ml of ddH_2O, and store in the refrigerator until used in the experiment. The percentage of ammonium thiocyanate should have been 30 % w/v.

Ferrous chloride:
Add 10ml of concentrated hydrochloric acid (37%) to 105.7 ml of ddH_2O to make a 3.2% sulfuric acid solution.
Add 3.97 g of ferrous chloride to the 3.2% (should be 3.5 %) hydrochloric acid solution and dissolve.
Add acid to 100 ml of ddH_2O. Store in a glass container in the refrigerator until used for assays.

Oil-in-Water Emulsion

For each reaction; Add 30 µl of stripped (tocopherol-free) corn oil (USB Corporation) to 3 µl of Triton X-100 and bring to a final volume of 1 mL with ddH_2O. Vortex for 1 min.

Mushroom Extract

Extract 0.33 g of dried mycelium in 6.67 mL of 95% ethanol. Centrifuge for 10 mins at 10,000 RPM. Remove and keep supernatant.

Protocol for Antioxidant Activity in Oil-in-Water Emulsion (Fu et al., 2002)

Add 33 µl of mushroom extract to 1mL of the oil-in-water emulsion (above). For controls, use 33 µl BHA and 33 µl ddH_2O in place of the mushroom extract. Place the mixed emulsion in a 15 mL centrifuge tube.

Incubate tubes at 60 °C w 250 rpm for 24 hours.

Measurement of oxidation by Ferric thiocyanate (Duh and Yen, 1995)
In a microfuge tube add
783 µl of 75 % ethanol
16.7 µl of ammonium thiocyanate
16.7 µl of the fungal mixture (oil-water-emulsion).
Wait 3 minutes, than add 16.7 µl ferrous chloride. Read absorbance at 500 nm.
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TBA/BUFFER REAGENT
Reference: ZeptoMetrix Thiobarbituric Acid Assay kit

In a glass tube bottle add the contents of one thiobarbituric acid (TBA) vial to 45 mL of TBA Diluent 1. Rinse the vial with the remaining 5 ml of TBA diluent 1 and add to the bottle. Mix on a stir plate until fully dissolved (adding low heat will speed dissolution). When TBA is completely dissolved add 50 mL of TBA diluent 2. Continue mixing
for additional 10 minutes.

Vita

Sharon Rose Sparks Jean-Philippe was born the 8th of thirteen children in Atlanta, Georgia on August 15, 1980. She attended Beecher Hills Elementary and Benjamin Elijah Mays High School, in Atlanta, GA graduating in 1998 as a Hope Scholar. After graduation from high school she attended Tennessee State University in Nashville, TN where she majored in biology. During her undergraduate career she attended Roland McNair Research Program at the University of Tennessee, Knoxville where she was introduced to research in the field of Botany, sparking an interest in medical plants. After graduating from Tennessee State University Magnum Cum Laud, she was accepted in to the Botany Department at the University of Tennessee, Knoxville where she studied Antioxidant Properties of Some Edible Fungi in the Genus *Pleurotus*.