STUDIES ON THE THERAPEUTIC POTENTIAL OF FRUITING BODIES AND CULTURED MYCELIA DERIVED COMPONENTS OF AN EDIBLE MUSHROOM, *HYPSIZYGUS ULMARIUS* (BULL.:FR.)

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CERTIFICATE

This is to certify that thesis entitled "**STUDIES ON THE THERAPEUTIC POTENTIAL OF FRUITING BODIES AND CULTURED MYCELIA DERIVED COMPONENTS OF AN EDIBLE MUSHROOM, *HYPSIZYGUS ULMARIUS* (BULL.:FR.)"** is a bonafied research work done by **Mrs GREESHMA PV**, under my guidance and co-guidance of Dr. Fathimathu Zuhara K, at Amala Cancer Research Centre, Thrissur - 680555. No part of this work has formed the basis for the award of any degree, diploma or other similar titles of any other university.

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DECLARATION

I, hereby declare that thesis entitled "**STUDIES ON THE THERAPEUTIC POTENTIAL OF FRUITING BODIES AND CULTURED MYCELIA DERIVED COMPONENTS OF AN EDIBLE MUSHROOM, *HYPSIZYGUS ULMARIUS* (BULL.:FR.)"** is a based on the original research work done by me, under the guidance of Dr. K.K.Janardhanan, PhD, FNABS, Professor at Amala Cancer Research Centre, Thrissur – 680555 and co-guidance of Dr.Fathimathu Zuhara K, PhD, Professor, University of Calicut, Thenjippalam for the partial fulfilment for the award of Doctor of Philosophy in Microbiology. No part of this work has formed the basis for the award of any degree, diploma or other similar titles of any other university.

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Abbreviations

$ABTS^{+}$	2, 2'-azinobis (3-ethyl benzothiazolin -6- sulphonic acid)
AIDS	Acquired Immuno Defficiency Syndrome
ALDH	Alcohol Dehydrogenase
ALDS	Alcoholic Liver Diseases
ALP	Alakaline Phosphatase
ALT	Alanine amino transfrease
AST	Aspartate amino transferase
CAT	Catalase
CCl_4	Carbon tetra chloride
CETP	Cholesteryl Ester Transfer Protein
COX	Cyclooxygenase
CVD	Cardiovascular diseases
DLA	Dalton's Lymphoma Ascites
DNA	Deoxy nucleic acid
DPPH	1, 1-diphenyl -2- picryl hydrazyl
FB	Fruiting bodies
FRAP	Ferric Reducing Antioxidant Power
GSH	Reduced glutathione
GST	Glutathione S transferase
HCD	High Cholesterol Diet
HDL	High Density Lipoprotein
HMG-CoA	3-Hydroxy-3-methylglutaryl CoA
HMW	High Molecular Weight
HPTLC	High Performance Thin Layer Chromatoghraphy
IL	Inter Leukins
LDL	Low Density Lipoprotein
LMW	Low Molecular Weight
LPL	Lipo Protein Lipase
MDA	Malondialdehyde
MMs	Medicinal Mushrooms
MY	Mycelium
NAD	Nicotinamide Adenine Dinucleotide
NF	Nuclear Factor

$NO^{\cdot}$	Nitric oxide
NSAID	Nonsteroidal anti-inflammatory drugs
$O_2^{\cdot}$	Superoxide anion
OD	Optical Density
PAF	Platelet Activating Factor
PG	Prostaglandins
RA	Rheumatoid Arthritis
RBC	Red Blood Cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruvate Transaminase
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TC	Total Cholesterol
TCA	Trichloro acetic acid
TG	Triglycerides
TLC	Thin Layer Chromatography
TNF	Tumor Necrosis Factor
UV	Ultra Violet
VLDL	Very Low Density Lipoprotein

Abstract

Despite the advances we made in the field of synthetic chemistry, natural products and their derivatives still play major role in the development of new drugs. Plants have been the most important natural source for a large number of drugs since ancient times. Later the importance of microorganisms as a source of therapeutical agents was realised. Among the microbes, fungi enjoy a unique position as an important source for the production of many bioactive compounds. Mushrooms the most visible form of some fungi are valuable natural products having medicinal value. Though medicinal properties of mushrooms were known to humankind from very ancient period onwards, only a few have been studied adequately. They include *Lentinus edodes, Schizophyllum commune, Cordeceps sinensis, Ganoderma lucidum, Flammulina velutipes, Pleurotus ostreatus, Trametes versicolor, Grifola frondosa, Agaricus blazei, Phellinus linteus* etc. These mushrooms possess a wide range of biological activities such as antioxidant, anticancer, antifungal, antidiabetic, anti-inflammatory, cardiovascular, hepatoprotecive and immunomodulatory activities.

Some of the medicinal mushrooms are also edible. *Agaricus, Pleurotus and Volvariella* spp. are notable examples and *Pleurotus* clearly stands apart from other edible mushrooms. Many aspects of these mushrooms such as appropriate cultivation conditions, methods to increase yield, nutritional value and medicinal properties have been studied. Still there are certain areas which are either not studied or the available data are inadequate to bring about the best use of them. So it is necessary to carry forward such studies. *Hypsizygus ulmarius* earlier known as *Pleurotus ulmarius* is one such mushroom which has not been studied for its various properties. It is relatively a rare mushroom, distributed throughout North America, Europe and Japan. It found as a saprophyte on elms, cottonwoods, beech, maple and oak. It is excellently edible. To our knowledge, no significant investigation of the medicinal properties of *H. ulmarius* has been carried out to date. Hence, we examined the antioxidant, anti-inflammatory, antitumor, hepatoprotective and cholesterol lowering properties of the fruiting bodies and cultured mycelia of this mushroom.

Generation of free radicals are often associated with cellular metabolic process. These by-products are usually reactive oxygen species (ROS) such as super oxide anion, hydroxyl radicals lipid peroxides etc. However, an overwhelming production of ROS generates oxidative stress that can damage cell structures including lipids, proteins and DNA (Pham-Huy *et al*., 2008). Synthetic antioxidants are not widely preferred

due to their potential toxic side effects. However, natural antioxidants are considered promising. Mushrooms are increasingly being used as sources of antioxidants because of their bioavailability and lesser side effects. *In vitro* antioxidant activity of the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* was evaluated using various assays such as DPPH radical, ABT^{+} radical, Hydroxyl radical, FRAP assay, nitric oxide radical scavenging assays. Here the extracts were found to scavenge DPPH radicals, hydroxyl radicals and ABTS radicals by donating electrons to corresponding oxidizing agents. Similarly the extracts reduced ferric ions in the FRAP assay. Also the extracts prevented lipid peroxidation indicating the antioxidant activity. Enhancement of antioxidant defences through dietary supplementation would be able to provide a reasonable approach to reduce the level of oxidative stress.

The IC_{50} values of fruiting body and mycelium extract were found to be 225µg and 300µg respectively in DPPH assay. In the hydroxyl radical scavenging assay, the extracts exhibited significant hydroxyl radical scavenging activity. The IC_{50} values of fruiting body and mycelium extract were found to be 300 and 430µg/ml respectively. FRAP assay also denote electron transfer reaction. The extract successfully transferred electrons to the Fe (III) $(TPTZ)_2$ complex thereby reduced it in to Fe (II) $(TPTZ)_2$ complex. The results of the current study showed that extracts significantly eliminated $ABTS^{+}$ radicals either by scavenging or inhibiting $ABTS^{+}$ radicals. IC_{50} values of fruiting body and mycelium extract were found to be 120 and 180µg/ml respectively. The extracts exhibited nitric oxide scavenging activity and IC_{50} values of fruiting body and mycelium extract were found to be 830 and 460 µg/ml respectively and lipid peroxidation inhibiting activity was in a dose dependent manner and IC_{50} values of fruiting body and mycelia extract were found to be 850 and 600 µg/ml respectively. Enhancement of antioxidant defences through dietary supplementation would be able to prevent the level of oxidative stress.

Inflammation is a natural response of the body to many foreign agents characterised with edema formation, leukocyte infiltration and granuloma formation (Mitchell and Cortran, 2000). The present investigations reveal that aqueous ethanolic extracts of *H.ulmarius* possessed profound anti-inflammatory activities in the anti-inflammatory response induced by carrageenan and formalin in mice. The fruiting bodies and

mycelia extracts of *H. ulmarius* significantly inhibited the chronic inflammation induced by formalin. The fruiting bodies and mycelia extracts at concentrations of 1000, 500, 250 mg/kg given orally, reduced the paw thickness 42.7, 38.3, 27.7% and, 55.2, 27.6, 22.3% respectively as compared to that of control. Acute inflammation induced by carrageenan at same concentrations of fruiting bodies and mycelia extract reduced the edema by 52, 41.4, 15%, and 63.2, 41.1, 19.1% respectively. Mycelia extract showed higher activity than fruiting bodies extract. The standard drug, Diclofenac at a dose of 10mg/Kg concentration showed 52% inhibition as compared to control.

In the *in vitro* COX-2 inhibition enzyme activity analysis, the extracts significantly inhibited COX-2 activity as compared to control which reveals the anti-inflammatory activity of *H.ulmarius*. At concentrations 25, 50, and 100 µg/ mL, fruiting bodies and mycelia extract showed 40.35, 50.62, 60.89% and 35.12, 47.87, 55.07 % inhibition respectively. Also standard anti- inflammatory drug Diclofenac at same concentrations exhibited 72.02, 80.19 and 84% inhibition. The results suggest the significant anti-inflammatory effect of the mushroom. Since *H. ulmarius* is an excellent edible mushroom, the anti-inflammatory properties of this mushroom have important practical application. Because, consumption of this mushroom might be useful to provide relief to patients suffering from inflammation and arthritis.

Over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and microorganisms. The macrofungi especially mushroom are well known for the production of anti-tumor drugs. Mushroom metabolites are known to complement chemotherapy and radiation therapy by lowering the side-effects of cancer. Extracts of *H.ulmarius* possessed significant antitumor activity. The ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* significantly reduced the DLA–induced solid tumor. The extracts showed preventive antitumour effect in a dose dependent manner.The mycelia extract showed higher antitumour effect than fruiting body extract. The effect of the standard anticancer drug, Cyclophosphamide used in the experiment prevented the tumor growth by 97%, when given orally at concentration 40mg/Kg in mice, while fruiting body and mycelia extract showed 66.7 and 78.9% tumor growth inhibition at a dose of 1000 mg/Kg, respectively. Ethanolic extracts of fruiting bodies and cultured mycelia

exhibited significant cytotoxicity towards Dlaton's lymphoma ascites cancer cells and non-cytotoxic to normal cells. The IC_{50} of fruiting bodies and mycelia extracts were found to be 30µg/ml and 35µg/ml respectively.

Water soluble polysaccharides were isolated from the fruiting bodies and mycelia of *Hypsizygus ulmarius* and it significantly reduced the tumour volume induced with DLA cells in mice. Polysaccharides isolated from fruiting bodies and mycelia of *H. ulmarius* showed remarkable antitumor activity against solid tumor induced by DLA cell line. At concentrations 25, 50 and 100 mg/kg body weight of polysaccharides from fruiting bodies prevented 37.9, 41.44 and 44.8% tumor volume after 5 weeks compared to control whereas at same concentrations polysaccharides from mycelia reduced 12.4, 24.4 and 36 % of tumor volume. The standard reference drug cyclophosphamide decreased 85 % tumor volume as compared to control.The results of the current studies thus suggest the potential therapeutic use of *H. ulmarius* as an adjuvant in the treatment of cancer**.**

Liver plays a major role in regulation of physiological processes, metabolism, synthesis, secretion and storage of vitamins. It has the capacity to detoxify toxic substances such as a variety of drugs, xenobiotics, environmental pollutants. Results of experimental studies showed that aqueous ethanolic extracts of *H.ulmarius* effectively protected the liver from hepatotoxicity induced by CCl_4 and ethanol in rats. Extracts efficiently decreased the elevated serum GOT, GPT and ALP activities in a dose dependent manner. The treatment with the extracts also restored the depleted levels of antioxidants in liver. The histological observations of liver tissues also supported the protective effect. Histopathological observation of the hepatic tissue of CCl_4 and ethanol challenged group showed severe areas of necrosis and plenty of inflammatory cells. About 80% of the hepatocytes showed cytoplasm vacuolation. The necrosis and vacuolation in hepatocytes were significantly reduced by Silymarin and *H.ulmarius* extracts treatments. The results indicated that aqueous-ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* possessed significant hepatoprotective activity. Being an excellently edible mushroom, the findings suggest potential therapeutic use of elm oyster mushroom for preventing liver disorders.

Hypercholesterolemia increases the risk of cardiovascular diseases, fatty liver and stroke (Woo *et al*., 2009). The hypolipidemic activity of *H.ulmarius* was studied using

triton WR-1339 and high cholesterol diet induced models. Triton Wr-1339 has been widely used to block clearance of triglyceride-rich lipoproteins to induce acute hyperlipidemia in rats for screening natural or chemical hypolipidemic drugs. The results of the present study revealed that at 250, 500 and 1000mg/Kg concentration, the extracts of *Hypsizygus ulmarius* were effective in significant reduction in cholesterol level. The hypolipidemic activity of extracts showed dose dependent activity compared to control as well as atorvastatin treated groups. Similarly administration of *H.ulmarius* along with high cholesterol diet significantly reduced serum TG, TC, LDL with elevating HDL. TBARS in heart and liver significantly decreased and antioxidants level were increased. SGOTand SGPT levels were significantly reduced in extracts treated groups as compared to control. In both models, extracts of *Hypsizygus ulmarius* showed a significant ameliorative effect on the elevated atherogenic index as well as LDL/HDL ratio. Also the hypolipidemic activity of *H.ulmarius* can be ascribed to its inhibitory effect on the liver HMG-CoA reductase activity.

Toxicity studies are carried out for the identification of the hazard of drugs. Acute and chronic toxicity were evaluated using animal models in mice (20 ± 5g). In subacute toxicity studies, animals were administered with extracts at various doses for a period of one month. Animals were observed for toxic symptoms and mortality. Current investigation revealed that the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* did not show any toxicity at a dose of 5000 mg/Kg body weight in acute toxicity study. There was no significant difference in hematological Parameters such as total leucocyte count, differential count, RBC and Hb as compared to normal group were observed in chronic toxicity assay. After 30 days, a slight increase in serum SGPT and SGOT in the extracts treated groups were observed as compared to normal value. But these changes were statistically non-significant. This finding reveals the therapeutic significance of *Hypsizygus ulmarius* as a potent nontoxic drug.

Phytochemical analysis using standard methods indicates the presence of polysaccharides, phenols, steroids, tannins and alkaloids in the aqueous ethanolic extracts of fruiting bodies and cultured mycelia. Detectable amounts of polysaccharides, phenolic compounds and alkaloids were observed as the minor compounds in the extracts. The HPTLC analysis also confirmed this conclusion.

Quantitative analysis using anthrone reagent and Bradford's reagent showed that total polysaccharide and protein present in the fruiting body extract of *H. ulmarius* was 20% and 28.5% respectively whereas in the mycelia extract the content was 18.9% and 25.6% respectively. Presence of these phytochemicals might be responsible for the medicinal properties of *Hypsizygus ulmarius.*

In conclusion the results of the current investigation reveal that elm Oyster mushroom *Hypsizygus ulmarius* possessed profound antioxidant, anti-inflammatory, anti-tumour, hepatoprotective and cholesterol lowering activities. The finding thus suggests the therapeutic use of fruiting bodies and cultured mycelia of this mushroom in alternative medicine and as an adjuvant. The findings also reveal the therapeutic significance of this mushroom as a dietary supplement or nutraceutical. However further investigations are needed to find out the mechanism of action of bioactives at molecular level.

Chapter:1

Introduction

Bioactive compounds of natural origin have been the most consistent and successful source for new drugs. Exploration of natural sources for novel bioactive compounds for medicine has been an emerging area of research over the past decades. Among natural sources, microbes especially fungi enjoy a unique position as an important source for the production of many bioactive compounds. In this mushrooms have continued to generate a lot of interest particularly in its consumption as food, in nutrition and cure of diseases and in bioremediation. They are important products of commerce all over the world due to their nutritional, antioxidant and therapeutic values. The antioxidant properties of mushrooms have been extensively studied and many antioxidant compounds extracted from them and isolated active compounds includes phenolic compounds, polysaccharides, terpenoides, proteins, tocopherols, ascorbic acid, carotenoids, minerals etc. They are becoming important in our nutrition due to their high content of proteins and low fat levels, making them well suited for the prevention and treatment of many chronic diseases such as atherosclerosis, diabetes, cancer, cirrhosis etc. (Suabjakyong *et al*., 2015)**.**

Mushrooms have been used in medicine since the Neolithic and Paleolithic eras (Samorini, 2001). Although mushrooms as medicine have been used in China since 100 A.D. (Gunde-Cimmerman, 1999), it was only in 1960 scientists begun to investigate the basic active principles of mushrooms which are health promoting. Mushrooms have been used in health care for treating simple and age old common diseases like skin diseases and pandemic disease like acquired immunodeficiency syndrome (AIDS). Mushrooms in the twentieth century are well known to people all over Asian countries as an important bio- source of novel secondary metabolites. The secondary metabolites of mushrooms are chemically diverse and possess a wide spectrum of biological activities, which are explored in traditional medicines (Rai *et al.,* 2005). In India, several mushrooms have been reported as medicinal having antioxidant, antimicrobial, anti-inflammatory activity with antitumor and other properties.

Mushrooms are mini pharmaceutical factories which produces several bioactive compounds. It has been used traditionally for the maintenance of health and for the prevention of diseases (Patel and Goyal, 2012). Mushroom contains all essential amino acids required for humans. In addition they are a relatively good source of the nutrients like phosphorus, iron and vitamins including thiamine, riboflavin, ascorbic

acid, ergosterol, and niacin (Barros *et al.,* 2008). The mechanism of action of various secondary metabolites isolated from medicinal and wild edible mushroom is yet to be discovered. New proteins with biological activities which can be used in biotechnological processes and for the development of new drugs, lignocellulose-degrading enzymes, lectins, proteases and protease inhibitors, ribosome inactivating proteins have been found (Erjavec *et al*., 2012). High nutritional and therapeutic potential of mushrooms can find different applications, namely as functional foods or as a source of nutraceuticals for maintenance and promotion of health and life quality. Also it act as metabolic activators, prevent or control intoxication, microbial infections, help in immune balancing, and immunomodulation, as antioxidants with rejuvenating and energy boosting properties (Wasser, 2002).

A diet rich in natural antioxidants can effectively influence antioxidant potential of the organism, and the risk of some diseases caused by free radicals. Adequate level of antioxidants supplied through diet induces immunological processes and increases cells abilities to fight against foreign molecules (Galek and Targonski, 2003). An antioxidant is a molecule which donates an electron to a free radical, thus reducing its capacity of damaging other cellular molecules. They delay or inhibit cellular damage by their free radical scavenging property (Halliwell, 1995). Although oxidation reactions are crucial for life, they can also be damaging; hence, plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Antioxidants interact with free radicals and terminate the chain reaction before vital molecules are damaged. Insufficient level of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and thus damaging the cells. Therefore, there is growing interest in the use of natural additives as potential antioxidants. Mushrooms are potential source of antioxidants and are capable of inhibiting lipid peroxidation (Acharya *et al*., 2004; Pal *et al*., 2010; Ajith and Janardhanan, 2007).

Some of the inhaled oxygen is converted to free radicals such as hydroxyl, superoxide radical, peroxyl radicals etc. by univalent reduction of O_2 (Maxwell, 1995). The results of epidemiological and scientific studies show that nutritional factor plays important role in prevention of the consequences of free radicals activity in the organism. The imbalance between reactive oxygen species (ROS) and antioxidants

may increase damage of DNA, proteins or carbohydrates etc. Such processes are thought to play a role in pathological process of many chronic diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, diabetes etc. In nature there are wide varieties of antioxidants including mushrooms which contain compounds that act as free radical scavengers by inhibiting their formation, by binding to transition metal ions, decomposition of lipid peroxides etc. (Niwa *et al*., 2001). Edible mushrooms are used as delicacy and for its health benefits. Numerous synthetic antioxidants can effectively improve defense mechanisms, but because of their adverse toxic effects under certain conditions, preference is given to natural compounds.

The material for the production of pharmacologically interesting compounds from mushrooms could be obtained from the wild, from cultivated mushroom fruiting bodies or from mycelia grown in solid substrates and in fermenters. Pure compounds could be obtained by isolation from the natural or cultivated material or by chemical synthesis. Very often, the natural products serve as leading compound for the preparation of a variety of derivatives, because most mushrooms can be cultivated economically. Mushroom derived commercial products, dietary supplements, and nutritional foods with claimed biological activities have been illustrated. Most of these products are recommended for adjutant therapy or as alternative medicine but not for direct cure of any diseases. These can improve the comfort of patient's life or prevent certain diseases or support drug treatment in chronic diseases to reduce side effects. In addition, despite the claimed therapeutic potential of these products, intensive research in this area is still required to meet the growing need for better therapeutic drugs (Jasinghe *et al*., 2007).

Among the less evaluated mushrooms, various species of the genus *Hypsizygus* is gaining popularity. *Hypsizygus* is a small genus and studies on *Hypsizygus marmoreus* possessed significant antioxidant activity and exhibited higher hydroxyl radical scavenging activity and ability to inhibit lipid peroxidation (Tsai *et al*., 2006). Recent investigations reveal that the *Hypsizygus ulmarius* is rich in antioxidants and it has antidiabetic activity (Meera *et al*., 2011).

Hypsizygus ulmarius is known as blue oyster mushroom, included in family *Tricholomataceae* often grows in clusters on living elm trees or elm logs in forest and

thus also called as elm oyster mushroom. The fruiting bodies of elm oyster mushroom are larger, heavier and white to bluish grey in color (Ruchita Dixit and Shukla, 2012). *Hypsi* means "high" or "on high" and *zygus* means a "yoke" *Hypsizygus*, then, referring to position of this mushroom often high in the tree. *Ulm-* refers to "elm" indicating one of the common substrates for this fungus. Common name is Elm oyster mushroom; Shirotamogitake (Japanese for white Elm mushroom). An oyster like mushroom earlier known as *Pleurotus ulmarius*. It is relatively a rare mushroom, widely distributed throughout the temperate forests of eastern North America, Europe and Japan, probably widespread throughout similar climate zones of the world. A saprophyte on elms, cottonwoods, beech, maple and oak. It is excellently edible.

The majority of mushroom products are developed from the fruiting bodies of mushrooms, but the mycelia are an equally good source of pharmacologically active metabolites. Furthermore, the mycelium biomass can be produced by modern biotechnological methods in a short span of time. Hence, evaluation of the medicinal effects of the mycelia is of significant importance.To our knowledge, no significant investigation of the medicinal properties of *H. ulmarius* has been carried out to date. Therefore, we examined the antioxidant, anti-inflammatory, antitumor, hepatoprotective and cholesterol lowering properties of the fruiting bodies and cultured mycelia of this mushroom.

Chapter 2:

Review of literature

MUSHROOMS

Fungi are important sources of food which provide nutrients such as proteins, minerals and vitamins. Mushrooms are macrofungi valued for culinary and medicinal purposes for humans. Among the 14000 currently known mushrooms, approximately 1000 species are edible (Aida *et al*, 2009). Medicinal mushrooms (MMs) are used as dietary supplements, mushroom pharmaceuticals, dietary food, natural biocontrol agents and cosmeceuticals (Wasser, 2010; Chang and Wasser, 2012). A knowledge on the life cycle of a basidiomycete helps to understand the medicinal activities of the respective stages, since all stages are currently utilized for their medicinal properties. The mycelium "body" which uses energy to spread and ultimately produce a mushroom. "Fruiting body" is known to be mushroom and during its lifecycle it will produce spores .These spores will germinate into "hyphae." It will grow together in a bundle of thread to form the mycelium (Roberts and Evans, 2013). Now the life cycle of the fungal organism is completed. The mushroom and the mycelium are similar in composition both are made up of hyphae and are meaningfully different in structure and function.

NUTRITIONAL VALUE OF MUSHROOMS

Compositional analyses of the main cultivated varieties have revealed that on a dry weight basis, mushrooms normally contain 19 to 35% protein. It contain all essential aminoacids rich in lysine, valine, methionine and leucine, low fat content with high proportion of polyunsaturated fatty acids. Most usual minerals found in mushrooms are calcium, magnesium, potassium, iron, zinc and copper. Carbohydrates found in large proportions in edible mushrooms include chitin, glycogen, trehalose, and mannitol; besides, they contain fiber, β-glucans, hemicelluloses, and pectic substances.Glucose, mannitol, and trehalose are abundant sugars in edible mushrooms, whereas fructose and sucrose are found in low amounts. Mushrooms are also a good source of vitamins with high levels of riboflavin (vitamin B_2), niacin, folates, and traces of vitamin C, B_1, B_{12}, D and E.

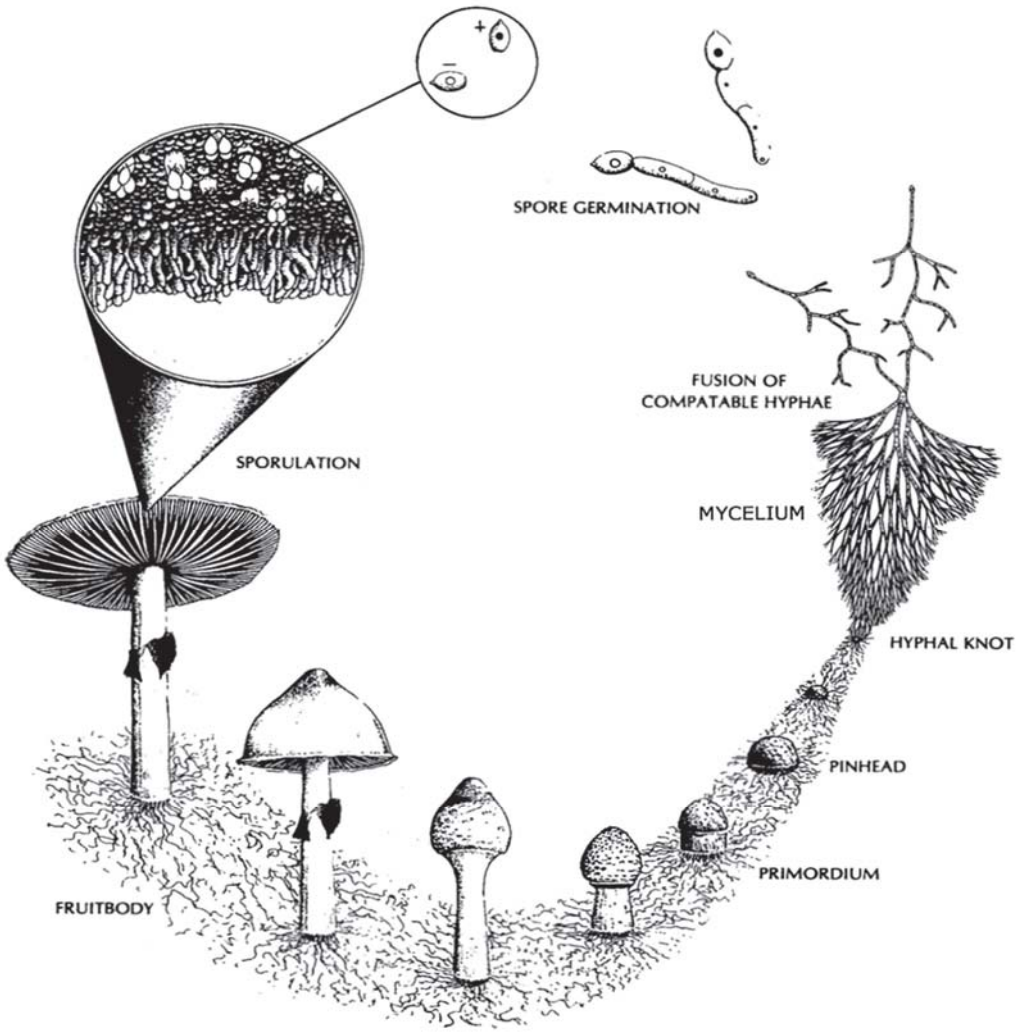

Figure.2.1. Life cycle of Basidiomycetes

More than 3000 mushrooms are said to be "the main edible species", of which only 100 are cultivated commercially, and only ten of those on an industrial scale. Their global economic value is nevertheless now staggering, and a prime reason for the rise in consumption is their medicinal and nutraceutical values. Production of mushrooms continuously increases over time, being China the biggest producer (more than 1.5 million metric tons in 2007). The most cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinula edodes, Pleurotus* spp. and *Flammulina velutipes* (Chang and Miles, 2004). These mushrooms require shorter growth time when compared to other edible mushrooms, they demand few environmental controls, and they can be cultivated in a simple and cheap way.

MEDICINAL POTENTIAL OF MUSHROOMS

Mushrooms are nutritionally functional food as well as source of physiologically beneficial and non-toxic medicines. Since ancient times they have been used in folk medicine throughout the world (Wasser *et al*., 1999). Many pharmaceutical substances with potent medicinal properties were extracted from mushrooms and make their way all around the world. In traditional Chinese medicine extracts from many medicinal mushrooms have long been used for a wide range of diseases. Modern scientific and medical studies support many of these claims. The reported medicinal properties are anticancer, antitumor antiinflamatory, cholesterol and blood pressure lowering, heaptoprotective, antifibrotic, antidiabetic and antimicrobial activity. (Wasser and Weis, 1999). In addition to the therapeutic potential hundreds of mushrooms are being explored worldwide to study their biotechnological potential.

OYSTER MUSHROOMS

Pleurotus sp. are commonly called 'oyster mushrooms'. They are the second most popular mushrooms after button mushroom all over the world (Adejoye *et al.,* 2006) and the most popular in Bangladesh. The oyster mushroom, first cultivated in Germany as a subsistence measure during World War2 is now grown commercially around the world for food. Oyster mushroom significantly reduces the hepatocellular enzyme Aspartate transaminase (AST) and Alanine transaminase (ALT) in plasma (Choudhury *et al.,* 2009). β- Glucan molecules of oyster enhance the immune system of body to fight against abnormal cells as well as boost the system against damaging effects of chemo and radiation therapies used to kill tumor cells. According to folklore*, Pleurotus* can also prevent high blood pressure, impart long life and assist people in recovering from fatigue. It can also prevent hangovers, constipation, and obesity.

Some of the bioactive substances are lectins with immunomodulatory, antiproliferative, and antitumor activities; phenolic compounds with antioxidant activities; and polysaccharides (polysaccharopeptides and polysaccharide proteins) with immunoenhancing and anticancer activities. In addition, the edible oyster mushroom may be considered a functional food due to its anti-inflammatory activity (Jedinak *et al*., 2011).Moreover, *P. ostreatus* exhibits hypocholesterolemic effect on rats with normal cholesterolemia or hypercholesterolemia and hereditary cholesterol

disorders (Bobek *et al*., 1998), *Pleurotus pulmunarius* apparently seems to be the richest source of fungal β-glucans and it demonstrated an anti-inflammatory response in rats with colitis (Manzi and Pizzoferrato, 2000) . On the other hand *P. sajor-caju* is a potential source of antioxidant and anticancer compounds (Finimundy *et al*., 2013).

Hypsizygus ulmarius (Bull:.Fr.) (Elm oyster) was earlier known as *Pleurotus ulmarius*. It is similar to oyster mushroom and known as blue oyster mushroom.It is a high yielding mushroom and is superior in texture, flavour and shelf life. No significant investigation has been done on the medicinal properties of *H. ulmarius*.

Elm Oyster mushroom (*Hypsizygus ulmarius)*

Blue oyster mushroom (*Hypsizygus ulmarius)* is one of the important edible mushrooms in the world, popularly cultivated in Japan, China, North America and other Asian countries nicknamed as tamo-motashi or shirotomogidake in Japanese. Nutritionally, this mushroom contains 23.6% protein, 2.2 % fat, 52.4% carbohydrate and 12.9 % fiber on dry weight basis (Chang, 1999). In nature it grows on elms and beech. This mushroom is superior in terms of yield, texture, flavor and shelf life. The niacin content is ten times higher than in any vegetables. The folic acid present in this mushroom is used to cure anemia. Since *Hypsizygus ulmarius* is a high yielding edible mushroom rich in proteins, vitamin C and B, a commercial cultivation technology has been released and is gaining popularity. This mushroom is rich in antioxidants and exhibited antidiabetic activity. The purification and characterization of laccase enzyme and its role in decolorization of different dyes has reported (Ravikumar *et al*., 2012)

Figure.2.2. Fruiting bodies of elm oyster mushroom (*Hypsizygus ulmarius*)

Figure.2.3. Mycelium of *Hypsizygus ulmarius*

OXIDATIVE STRESS:

Oxidative stress is defined as an imbalance between production of free radicals and reactive oxygen species such as superoxide, hydroperoxyl, hydroxyl, alkylperoxyl, alkoxyl, carbonate, carbondioxide radicals etc. and their elimination by protective mechanisms, referred to as antioxidants. This imbalance leads to damage of important biomolecules and cells, with potential impact on the whole organism (Durackova, 2010). The harmful effects of ROS are balanced by the action of antioxidants, some of which are enzymes such as glutathione peroxidase, catalase etc. present in the body (Halliwel, 1996). Despite the presence of the cell's antioxidant defense system to counteract oxidative damage from ROS, oxidative damage accumulates during the life cycle and has been implicated in diseases, aging and age dependent diseases such as cardiovascular disease, cancer, neurodegenerative disorders and other chronic conditions (Rahman,2003).

Sources of free radical

Internal sources can be enzymatic reactions, which serve as a source of free radicals. These include those reactions involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P450 system. Free radicals are generated internally mainly from mitochondria, peroxisomes and phagocytes, also from the reactions involving iron and other transition metals, arachidonate pathways and inflammation. External sources include non-enzymatic reactions of the oxygen with organic compounds. Free radicals also arise in reactions, which are initiated by ionizing radiations, UV light and ozone. Physiological factors include mental status like stress; emotion etc. and disease conditions are also responsible for formation of free radicals (Shiv Kumar, 2011).

MAJOR DISEASES ASSOCIATED WITH OXIDATIVE STRESS:

Uncontrolled production of free radicals has been related to more than 100 diseases including atherosclerosis, cardiovascular diseases, several kinds of cancer, cirrhosis, diabetes, lung diseases, liver disorders, neurological disorders, Alzheimer`s disease, mild cognitive impairment, Creutzfeldt-Jacob disease, meningoencephalitis, Parkinson`s disease and drug induced deafness etc. The brain is particularly vulnerable to oxidative damage because of its high oxygen utilization.

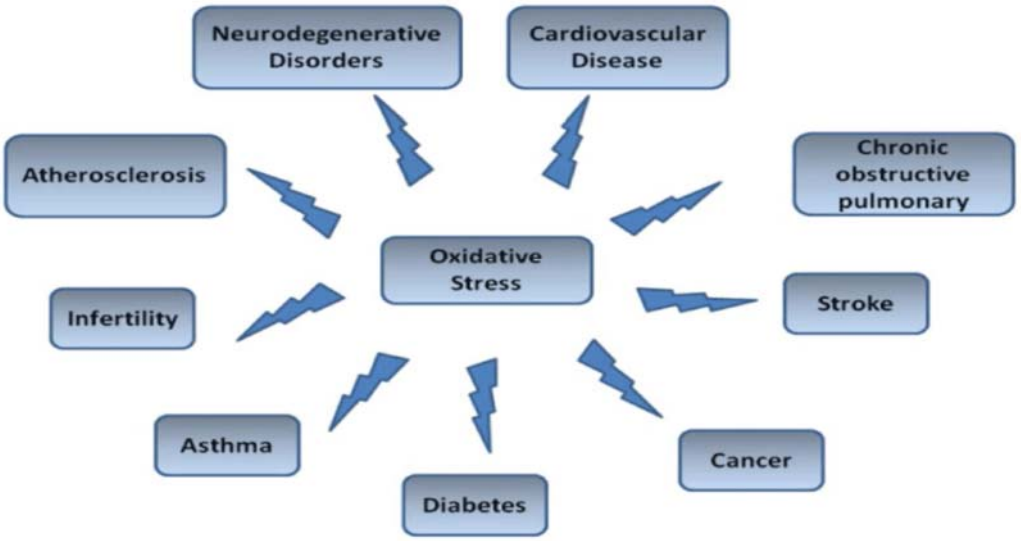

Figure.2.4. Oxidative stress-induced diseases in human

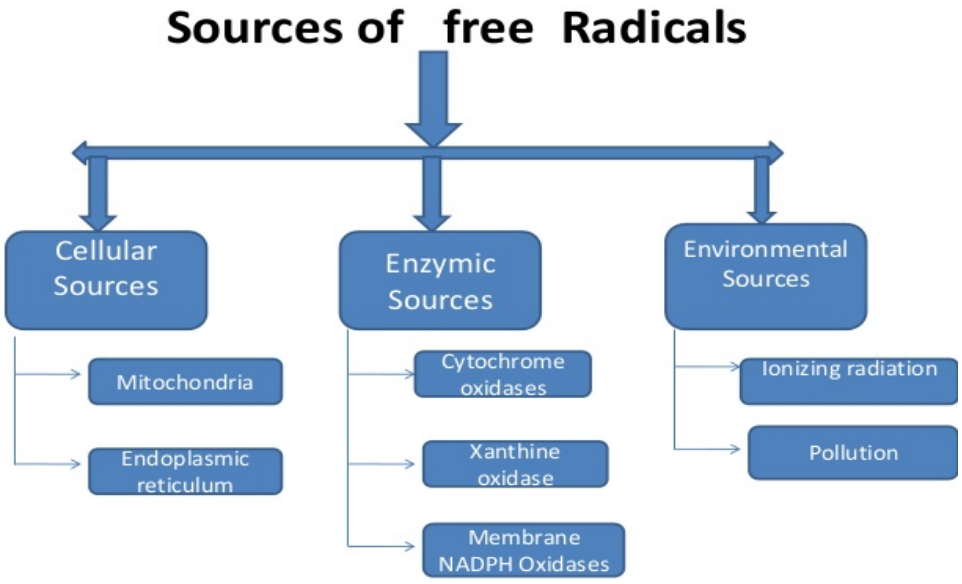

Figure.2.5. Major sources of free radicals

Oxidative stress increases with age and therefore it can be considered as an important causative factor in several neurodegenerative diseases, among older individuals. Under physiological conditions, about 1-3% of the oxygen molecules in the mitochondria are converted into superoxide. The primary site of radical oxygen

damage from superoxide radical is mitochondrial DNA (Cadenas *et al.,* 2000). The cell repairs much of the damage done to nuclear DNA, but mitochondrial DNA cannot be readily fixed.

INFLAMMATION

Inflammation is a nonspecific response to tissue malfunction and is employed by both innate and adaptive immune systems to combat pathogenic intruders **.**Inflammatory responses occur in three distinct phases, an acute, transient phase with local vasodilatation and increased capillary permeability. Second one subacute phase, characterized by leukocytes infiltration and chronic proliferative phase, in which tissue degeneration and fibrosis occur. Based on this there are many anti-inflammatory tests for measuring acute inflammation such as Paw edema in rats, vascular permeability, Pleurisy tests. Proliferative phase is measured by Cotton wool granuloma, Glass rod granuloma etc. Many irritants such as brewer‘s yeast, formaldehyde, dextran, sulfated polysaccharides like carrageenan, formalin, histamine and croton oil have been used to screen different anti-inflammatory drugs.

Molecular Signaling in inflammation

The processes leading to inflammation are usually linked to the activities of the cells involved in the restoration of tissue structure and function. When cells are exposed to immune stimulants, cells such as macrophages, monocytes, or other host cells, start to release many mediators which initiate the inflammation process. Various inflammatory mediators like interleukins (IL-1β), IL-6; IL-8; tumour necrosis factor (TNF-α); nuclear factor-κB (NF- κB), inducible type cyclooxygenase- (COX-) 2, prostaglandin E2 (PGE2); 5- lipooxygenase (5-LOX); and inducible nitric oxide synthase (iNOS) are most popular, which leads to the production of reactive nitrogen species such as nitric oxide (NO). Increased synthesis of these inflammatory mediators leads to many inflammatory diseases such as juvenile idiopathic arthritis (JIA), inflammatory bowel disease (IBD), multiple sclerosis, rheumatoid arthritis, gastritis, bronchitis, and atherosclerosis (Levine, 2012). A strong link has also been established between long-term inflammation and the development of cancers (Coussens and Werb, 2012).

Cyclooxygenases (COX) and Inflammation

Prostaglandin G/H synthases together known as COXs their main function is the production of prostaglandin. It has cyclooxygenase and peroxidase activity, exsist as isoforms COX-1 and COX-2 (Smith *et al*., 2000). COX-1 is the dominant source of prostanoids expressed in most cells whereas COX-2, induced by inflammatory stimuli, hormones and growth factors are the more important source of prostanoid formation in inflammation and in cancer (Dubois *et al*., 1998). Although COX-2 appears to be the important source of prostaglandin formation in inflammation, there is some suggestion that both isoforms may contribute to the acute inflammatory response. COX-1 is constitutively expressed in inflammatory cells and in LPS-mediated inflammatory response there is induction of COX-1 in cells (Mcadam *et al.,* 2000).In inflamed rheumatoid arthritis (RA) and in atherosclerotic plaques both COX isoforms are expressed together (Crofford *et al.,* 1994; Schonbeck *et al*., 1999).

Isoforms of cyclooxygenase:

Cyclooxygenase (COX) also known as Prostaglandin endoperoxide H synthase (PGHS, EC.1.14.99.1) and has two isoforms; PGHS-1 (COX-1) and PGHS-2 (COX-2), which catalyses the conversion of arachdonic acid to prostanoids. . The structure of COX consists of three domains; an N-terminal domain, a membrane-binding motif, and a C-terminal catalytic domain containing active sites for peroxidase and cyclooxygenase (Kurumbail *et al.,* 1996). COX-enzymes are heme proteins that are widely distributed (Morham *et al.,* 1995). These enzymes are located in two portions mainly endoplasmic reticulum membrane and the nuclear envelope (Chandrasekharan *et al*., 2002). COX-1 present in both locations equally whereas COX-2 is more abundant at the nuclear envelope than endoplasmic reticulum so there exsist two distinct prostanoid biosynthetic systems (Bonventre *et al.,* 1997).

COX-1 protein has 600-602 amino acids with 67kD molecular weight and molecular weight of COX-2 protein is 68-72kD and contains 603-604 amino acids (Tanabe and Tohnai, 2002; Reddy *et al.,* 2007). These enzymes are approximately 60% identical in terms of amino acid composition, and their catalytic regions are widely conserved. The studies on their crystal structures have revealed that COX-2 has a larger active site. Furthermore, the active sites of these two isoforms differ by only two

aminoacids, at positions 523 (Ile for COX-1 and Val for COX-2) and 513 (His for COX-1 and Arg for COX-2) (Zhang *et al.,* 1996). COX-1 and COX-2 isozymes catalyze the same reactions, but are encoded by two different genes, located on human chromosomes 9 and 1 respectively. The gene for COX-1 is approximately 22kb in length, contains 11 exons and 10 introns, whereas COX-2 gene is about 8kb, contains 10 exons and 9 introns (Bakhle, 1999).

Role COX -2 in cancer

Increased amounts of COX-2 are an important characteristic in malignant tissues (Dannenberg 2001) and this overexpression of COX-2 is due to the increased transcription and enhanced mRNA stability (Shao *et al*., 2000; Dixon *et al*., 2000). There are mainly two signaling pathways such as protein kinase C (PKC) and RA Sarcoma-mediated (RAS-mediated) signaling that stimulate COX-2 transcription. Oncogenes, cytokines, growth factors and tumor promoters induce COX-2 by increasing mRNA stability in addition to transcription stimulation. COX-2 affects many processes in different stages of carcinogenesis including xenobiotic metabolism, cell proliferation, angiogenesis, apoptosis, immune function and tumor invasiveness (Konturek *et al*., 2005; Meric *et al*., 2006). In case of carcinogens, their conversion from procarcinogen to carcinogen is carried out by the peroxidase portion of COX-2 and thus intiating tumor formation. (Eling *et al*.,1990). COX-2-derived PGs reduce the apoptosis via inhibition of the mitochondrial apoptotic pathway through depleted cytochrome c release, attenuated caspase-9 and -3 activation and upregulation of bcl-2 (Wang *et al*., 2005).

Anti-inflammatory compounds from mushroom

Edible mushrooms produce a vast diversity of bioactive compounds such as polysaccharides, proteoglucans, terpenoids, phenolic compounds, steroids, and lectins. These compounds have a wide range of therapeutic effects. Polysaccharides represent the major class of bioactive compounds among this Glucans ((1→3)-β-D-glucopyranosyl) from *Pleurotus pulmonarius* have been reported to exhibit anti-inflammatory properties in a model of acute colitis in rats (Lavi *et al*., 2012; Schwartz *et al*., 2013) Oyster mushroom containing α- and β-glucans reduced LPS- dependent

activation of TNF-α, IL-6 and 2 cells, as well as the production of prostaglandin E_2 and nitric oxide. This was mainly attributed to the downregulation of COX-2 and

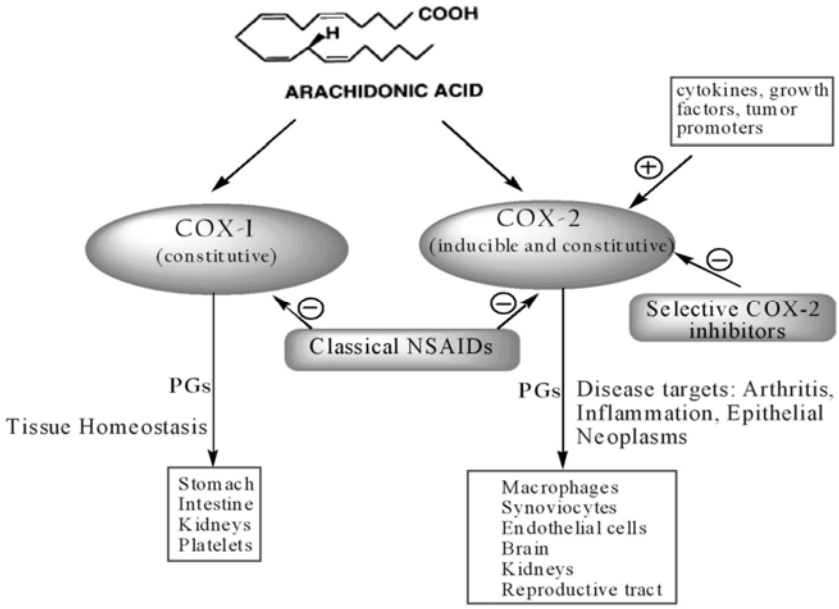

Figure.2.6.Schematic presentation of the actions of cyclooxygenases

iNOS expression, respectively (Jedinak *et al*., 2011). Mushroom polysaccharides with anti-inflammatory properties have been also reported in crude extracts of *Lentinus polychrous*, *Termitomyces albuminosus*, and *Phellinus linteus*

Terpenes are known as an important variety of naturally occurring bioactive metabolites produced by many higher fungi which exhibited anti-inflammatory properties in animal models. Diterpenoids, triterpenoids, and sesquiterpenoid are the typical representatives of terpenes with interesting biological activities. Edible mushrooms the anti-inflammatory properties are due to the presence of phenolic compounds, such as pyrogallol has been isolated from *Agaricus bisporus* (Dugler *et al*., 2004; Witkowska *et al*., 2011).

CANCER

A normal cell suddenly turns into a rogue cell and start dividing continuously without check, leading to the development of solid lumps (tumors) or an abnormal rise in the number of dispersed cells like the blood corpuscles. Cancer can occur in

any part of the body and in any organ or tissue (Uma Devi, 1989). The resultant aberrant cell behavior leads to masses of abnormal cells that destroy surrounding normal tissue and can spread to vital organs resulting in disseminated disease. The cancer-causing agents (carcinogens) can be present in food, water, air, chemicals and in sunlight (Malcolm, 2001). A cancer growth is made up of billions of cells, all originating from an initial cell which multiplies clonally, escapes to apoptosis and accumulates genetic or epigenetic alterations which converge into a neoplasic cell. The blocking of apoptosis in the phase of significant genetic damage can ease the accumulation of aberrant cells and it can become a critical point in malignant pathogenesis (Paula *et al*., 2007).

Development stages of carcinogenesis

Studies conducted using animal models, in vitro studies and epidemiologic assays enabled investigators to conclude that neoplasic pathogenesis is a complex process which can be divided into three distinct stages. These are: initiation, promotion and progression

1. INITIATION: Initiation involves direct DNA binding and damage by carcinogens, and it is rapid and irreversible (Anne *et al*., 2004).

2. PROMOTION: Promotion, which involves epigenetic mechanisms, leads to pre malignancy and is generally irreversible (Kushthar *et al*., 2013).

3. PROGRESSION: Progression is due to the genetic mechanisms and it is the period between pre-malignancy and the cancer and is also generally irreversible.

Causes of cancer

The factors responsible for cancer development are classified as exogenous and endogenous.

Table.2.1. Causes of cancer

Exogenous factors	**Endogenous factors**
Nutritional habits : food preservation and preparation	Immune system damage
Socio-economic status	Inflammation caused by uncertain aetiology (e.g. ulcerative colitis, pancreatitis, etc.)
Life style	Genetic makeup
Physical agents (Radiation-Ionising and non-ionising)	Age
Chemical compounds (Tobacco)	Endocrine balance
Biological agents (Microorganism)	Physiological condition

Anticancer substances of mushroom

Polysaccharides are the major bioactive compounds that contribute antitumor activity in which glucans with β-(1→3) bonds in the main chain and with β(1→3) bond side branches, which are essential for biological activity. Polysaccharides isolated from mushrooms prevent carcinogenesis and metastasis, also exhibits immune cell-mediated anticancer activity. This includes heteropolysaccharides, α glucans where glucose is the only monomer unit. Glucans that are isolated from mushrooms are mainly 1, 3-D-glucan or 1, 6-D-glucans. Glucans obtained from *Grifola frondosa* in which most active glucan appeared to be branched (1- 3)-glucan, known as grifolan, that exerted antitumor activity in Sarcoma 180. Lentinan the main glucan of *L. edodes* fruit bodies and Schizophyllan is also a glycan, with a side chain of glucopyranosily group at each third glucose of main chain (Rop *et al*., 2009)**.** β -glucans was also significant in mushrooms. Bioactive proteins from mushrooms, due to their pharmaceutical properties and application in protein engineering their attention have been increasing. This includes lectins, laccases etc. Terpenoids were investigated for their cytotoxic or apoptotic effects (Rios *et al*., 2012). Sesquiterpenes were isolated from the cultured mycelia of *Cordiceps ophioglossoides* and Lanostanoids are important type of tetracyclic triterpenoids derived from lanosterol that are investigated for their anticancer effects. Sterols and Phenolic compounds like Protocatechuic acid

(phenolic acid) and a related compound (cinnamic acid), also exhibited significant antitumor activities.

HEPATOTOXINS AND LIVER DAMAGE

Hepatotoxicity is the liver function damage due to a drug or non-infectious agent that reacts with cellular components results in hepatic lesions. Liver is a large, complex organ that is well designed for carbohydrate, protein and fat metabolism. It is responsible for synthesizing and secreting bile and for synthesizing lipoproteins and plasma proteins, such as clotting factors. It maintains a stable blood glucose level by taking up and storing glucose as glycogen (glycogenesis), breaking it down to glucose when needed (glycogenolysis) and forming glucose from non-carbohydrate sources such as amino acids (gluconeogenesis). It is the site where waste products of metabolism such as ammonia, are detoxified. The liver also plays an important role in drug elimination and detoxification. Liver damage may be caused by many xenobiotics, medicines, malnutrition, infection, and anaemia (Mroueh *et al*, 2004; Gowri *et al*, 2008). Liver damage is a widespread disease which involves oxidative stress and characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (Kodavanti *et al*, 1989). Tetracycline, ethanol, bromobenzene, paracetamol, sulphonamide, erythromycin, carbontetrachloride are some of the chemicals that causes hepatotoxicity (Navarro *et al*, 2006).

Carbontetrachloride (CCl_4)

Carbon tetrachloride is known as a model toxicant to be hepatotoxic as well as nephrotoxic to humans. It is considered as a standard chemical agent, whose hepatotoxictiy through various routes has been well established by various workers. Experimentally induced cirrhotic response in the rats by CCl_4 is shown to be superficially similar to human cirrhosis of the liver.

Metabolism of carbontetrachloride via CYP2E1 to highly reactive free radical metabolites plays a critical role in the postulated mode of action. The primary metabolites trichloromethyl and trichlrome-thyl peroxy free radicals, are highly reactive are capable of covalently binding

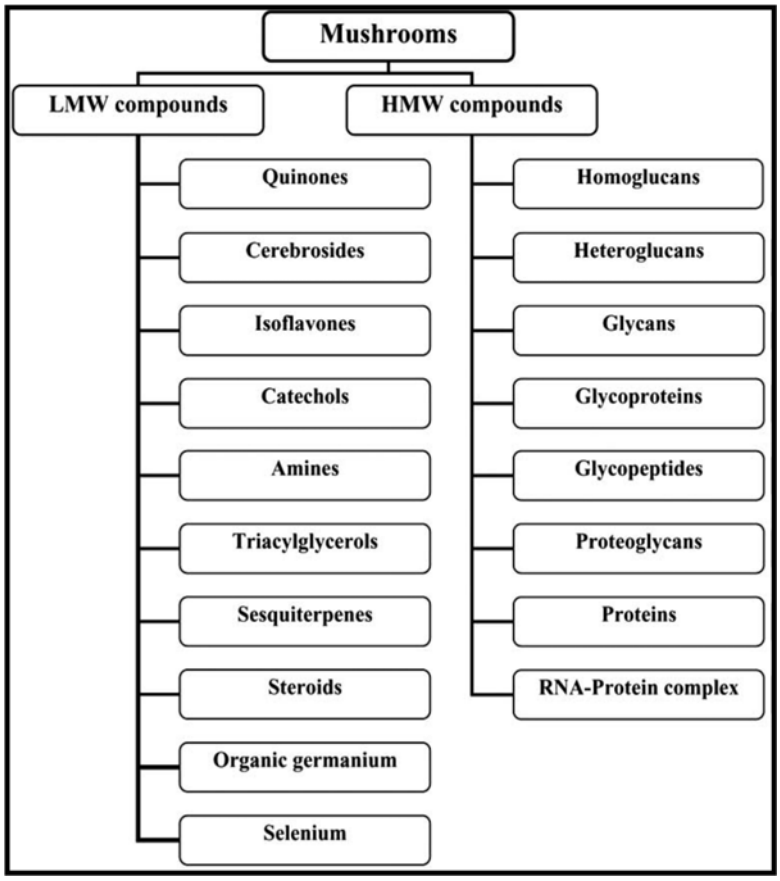

Figure.2.7. Low-molecular-weight (LMW) and high-molecular-weight (HMW) compounds with antitumor potential found in mushroom

to sufficient leakage of calcium into cytosol to disrupt the intracellular calcium homeostatis. High calcium levels in the cytosol activate calcium dependent protease and phopholipase that further increase the breakdown of membranes. Similarly the increase in intracellular calcium can activate endonuclease that can accuse chromosomal damage and also contribute to cell death. Sustained cell regeneration and prolifertation following cell death may increase mutations that can lead to cancer.

Liver damage due to CCl_4

CCl_4 damages the membranes of the hepatocyte causing leakage of the enzymes present in the cell. This results in elevation of the levels of plasma tramaminases. It leads to fat decomposition in the liver due to blockage of secretion of hepatic triglycerides into plasma. A single dose of CCl_4 leads to centrilobular necrosis and fatty liver. Within a few minutes, there is injury to the endoplasmic reticulum lending

to functional defects of the hepatocyte. Irrespective of the route of administrations it leads to centrilobular necrosis and steatosis. Biochemical changes in the blood reflect injury. Serum enzyme levels increase with cytoplasmic enzyme reaching their peak within 12 hrs. Mitochondrial enzymes reach their peak within 36 hrs. Enzymes common to both mitochondria and cytoplasm reach their peak around 24 hrs. CTC causes accumulations of fat in the liver especially by interfering with the transfer of triglycerides from the liver into the plasma. Serum glutamic pyruvic transaminase (SGPT) now called Alanine amino transferase (ALT) and serum glutamic oxaloacetic transferase (SGOT) now known as Asparatate amino transferase (AST). Asparatate and Alanine amino transferases are present in high concentration in liver. Due to hepatocyte necrosis or abdominal membrane permeability, these enzymes are released from the cells and their levels in the blood increase. ALT is a sensitive indicator to acute liver damage and elevation of this enzyme in hepatic disease is unusual. Alkaline phophatase, although is not a liver specific enzyme, the liver is major source of this enzyme. Also the levels of this enzyme increase in cholestasis, elevated serum gammaglutamyl transpeptidase levels appear to be indicative of diseases of the liver, biliary tract and pancreases. Bilirubin levels in blood also increase in liver diseases.

Alcohol liver diseases

Alcoholic liver disease represents a spectrum of clinical illness and morphological changes that range from fatty liver to hepatic inflammation and necrosis (alcoholic hepatitis) to progressive fibrosis (alcoholic cirrhosis). Furthermore, sustained excessive alcohol intake favours the progression of other liver diseases, such as virus-related chronic hepatitis, also increasing the risk of hepatocellular carcinoma Alcoholic liver disease develops in patients consuming excessive amounts of alcohol. Oxidative stress plays a pivotal role in the development of ALD. Oxidation of ethanol to water and carbon dioxide is mediated by three major hepatic enzyme systems: ADH in cytoplasm, microsomal ethanol oxidizing system in smooth endoplasmic reticulum of mitochondria (predominantly CYP2E1) and catalase in peroxisomal membrane. All these biochemical pathways produce acetaldehyde as their toxic by-product. Alcohol leads to increased liver oxidative stress via generation of highly reactive oxygen species (ROS) and adducts. ADH generates acetaldehyde, which is subsequently oxidized to acetate by ALDH.

Further oxidations in alcohol metabolism are accompanied by an excessive reduction of nicotinamide adenine dinucleotide (NAD), with a shift in the NADH/NAD ratio. Under normal circumstances, reduction of NAD (NAD / NADH) is finely regulated by the cell Krebs cycle. The shift caused by excessive alcohol consumption is thought to impair carbohydrate and lipid metabolism, finally causing impairment of gluconeogenesis and diversion of metabolism to ketogenesis and fatty acid synthesis. Finally, the NADH-induced inhibition of mitochondrial β-oxidation leads to accumulation of intracellular lipids, thus promoting steatosis.

$CH_3\ CH_2\ OH$ (Ethanol)

↓ Alcohol dehyrogenase (Cytosol)

Acetaldehyde

↓ Acetaldehyde dehydrogenase (Mitochondria)

Acetate

HYPERLIPIDEMIA

Hyperlipidemia is a condition in which blood plasma contains high levels of lipids or lipoproteins. However, elevated blood levels are harmful and have been associated with cardiovascular diseases. Abnormally elevated lipids in the blood stream allow cholesterol, particularly low density lipoprotein cholesterol (LDL-C), to be deposited within the arterial wall of large and medium sized arteries as atherosclerotic plaques. These cause obstruction of the arteries, and depending on the extent of obstruction, may contribute to hypertension, reduction in the amount of oxygenated blood that reaches the heart and in increasing the risk of coronary heart disease (CHD), myocardium infarction and cerebral arterial diseases. Despite improvement in lifestyle (diet, exercise, and weight reduction) and the use of cholesterol lowering drugs, CHD and stroke remain as major causes of death in the U.S. Once synthesized by the liver, cholesterol is transferred via the bile into the intestinal tract. About 50% of excreted cholesterol is reabsorbed by the digestive system and pumped back into circulation. This cholesterol recycling is continuous in nature. Biosynthesis and regulation of

cholesterol depends on cholesterol blood level. The higher the intake of cholesterol, the less endogenous production, and the opposite is true.

Causes of Hyperlipidemia:

Presence of diseases that tend to increase LDL blood levels such as diabetes, hypertension, hypertriglyceridemia, kidney and liver diseases. Gender is very important that men have a greater chance of developing hyperlipidemia than women. About age as a person becomes older, so does the chance for developing atherosclerosis and hyperlipidemia.. Foods, especially if consumed in relatively large quantities and frequently, can detrimentally affect cholesterol and triglyceride blood levels. Red meat, many cheeses, creamy cakes, ice cream, sausages and hot dogs have high contents of saturated fats and may affect the outcome of cholesterol blood concentration. Sedentary lifestyle has been shown that non-vigorous physical activity tends to reduce LDL and elevate HDL blood levels. Bodyweight of individuals who maintains normal bodyweight which is adequate for their frame and age are less likely to have high LDL and lower HDL levels than overweight individuals. Smoking has been reported that about 400,000 deaths annually in the US. Cigarette smoking decreases HDL level while it elevates LDL. Alcoholic persons who regularly consume large quantities of alcoholic beverages exhibit high LDL and low HDL levels.

Cholesterol

Cholesterol is a waxy, fat-like substance found in all animals including humans. It is an essential part of cells in the body and is used to make certain hormones and digest fats. A special form of cholesterol in the skin has the ability to change into vitamin D when exposed to sunlight.

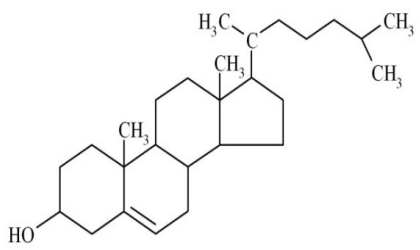

Figure.2.8.Structure of Cholesterol

Cholesterol Biosynthesis:

The cholesterol synthesis consists of five major steps:

1. Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMGCoA).
2. HMG-CoA is converted to mevalonate.
3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO_2.
4. IPP is converted to squalene.
5. Squalene is converted to cholesterol.

Regulation of Cholesterol Synthesis:

The greatest proportion of cholesterol is used in bile acid synthesis. The cellular supply of cholesterol is maintained at a steady level by three distinct mechanisms:

- Regulation of HMGR activity and levels
- Regulation of excess intracellular free cholesterol through the activity of acyl CoA:cholesterol acyltransferase (ACAT)
- Regulation of plasma cholesterol levels via LDL receptor-mediated uptake and HDL- mediated reverse transport.

Regulation of HMGR activity is the primary means for controlling the level of cholesterol biosynthesis. The enzyme is controlled by four distinct mechanisms: feed-back inhibition, control of gene expression, rate of enzyme degradation and phosphorylation- dephosphorylation. The first three control mechanisms are exerted by cholesterol itself. Cholesterol acts as a feed-back inhibitor of pre-existing HMGR as well as inducing rapid degradation of the enzyme.

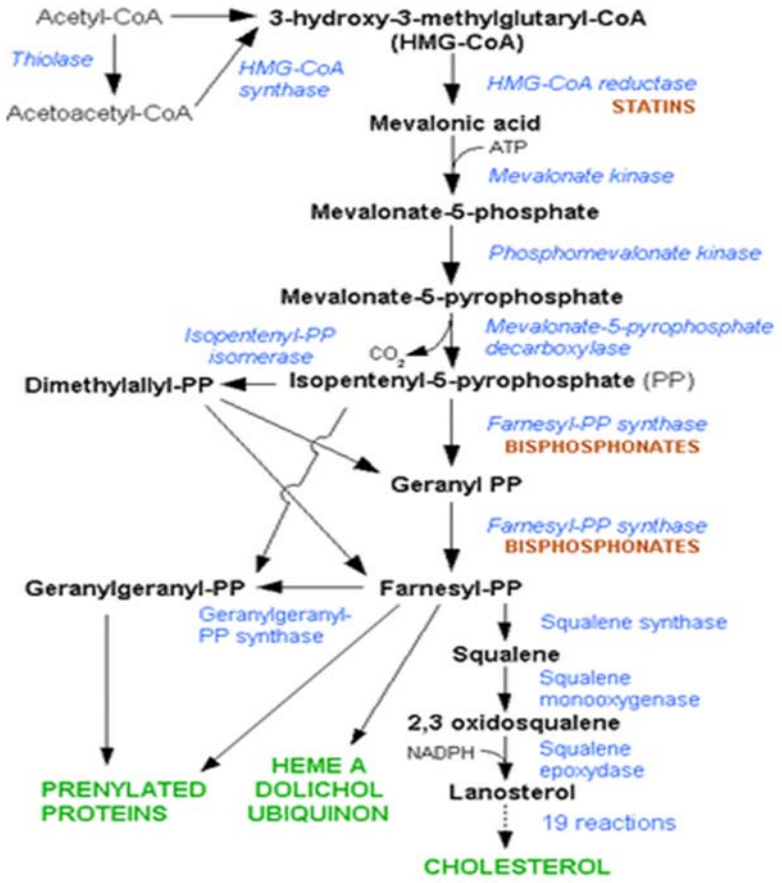

Figure.2.9.Biosynthetic pathway of Cholesterol

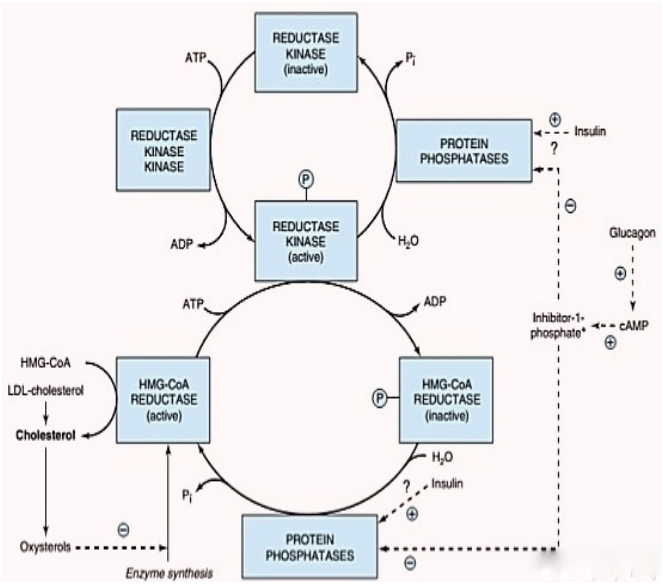

Figure.2.10. Regulation of Cholesterol Biosynthesis by HMG CoA Reductase enzyme

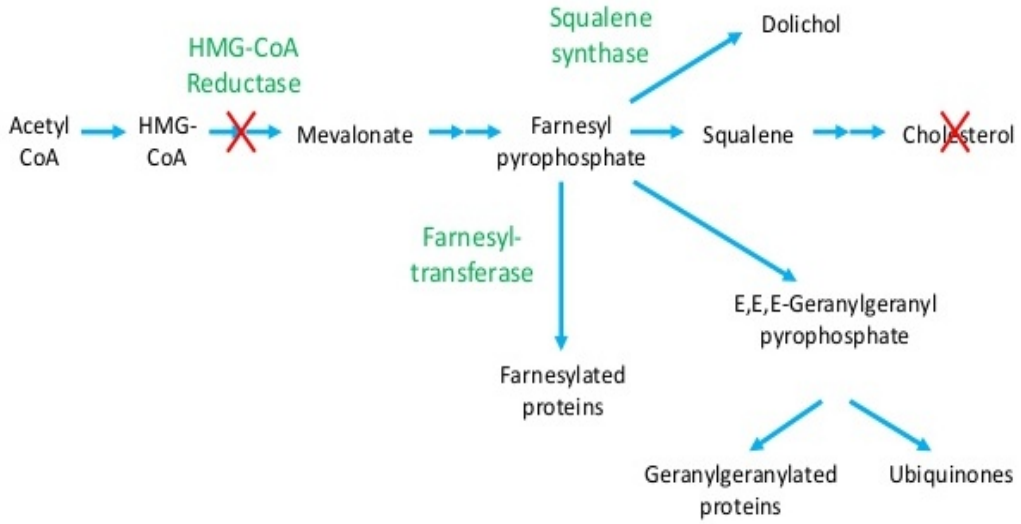

Figure.2.11. Inhibition of HMG CoA Reductase by Statins in Cholesterol biosynthetic pathway

HMG CoA Reductase-A Rate Limiting Enzyme in Mevalonate Pathway:

HMG-CoA reductase is the rate-limiting enzyme for cholesterol synthesis and is regulated via a negative feedback mechanism mediated by sterols and non-sterol metabolites derived from mevalonate, the product of the reaction catalyzed by reductase. 3-hydroxy-3- methylglutaryl coenzyme A (HMG-CoA) reductase, is a transmembrane glycoprotein that was earlier believed to be in the endoplasmic reticulum of mammalian cells (Chin *et al*., 1982).However, some report show that the enzyme in liver cells is present not only in the ER but also within peroxisomes (Keller *et al*., 1985).

Statins:

Mevastatin was the first HMG-CoA reductase inhibitor and was isolated from *Penicillum citrinum*. Other statins Simvastatin, Lovastatin and Pravastatin are also fungal derivatives, while Atorvastatin, Cerivastatin, Fluvastatin, Pitavastatin and Rosuvastatin are fully synthetic compounds. (Wierzbicki, 2003) The use of statins (Simvastatin, Pravastatin, Lovastatin, Fluvastatin, Rosuvastatin and Atorvastatin) has become the preferred method for treating elevated LDL-C levels in children and adolescents who meet the criteria for drug therapy. HMGCoA; a complex

hydrophobic ring structure that is covalently linked to the substrate analogue and is involved in binding of the statin to the reductase enzyme; side groups on the rings that define the solubility properties of the drugs.All statins are competitive inhibitors of HMG-CoA reductase with respect to the binding of the substrate, HMG-CoA, but not for that of the co-enzyme NADPH, suggesting that their HMG-CoA-like moieties bind to the HMG-CoA-binding portion of the enzyme active site.

Lovastatin:

Lovastatin is a fungal polyketide, which has a napthelin ring and a lactone ring, where the lactone ring binds to HMG-CoA reductase enzyme and there by inhibits the formation of cholesterol. Lovastatin was the first statin drug, which was approved by United States Food and Drug Administration in the year 1987. *A.terreus* is a strain that was used in the production of lovastatin, which was widely studied (Alberts *et al*., 1980; Greenspan and Yudrovitz, 1985). Many Basidomycetes species are shown to produce higher concentrations of statin drugs. Wasser *et al*., in 2002 patented the various composition and production methodology of cholesterol lowering molecule from *Pleurotus* sp (Wasser and Reshetnikov, 2002).

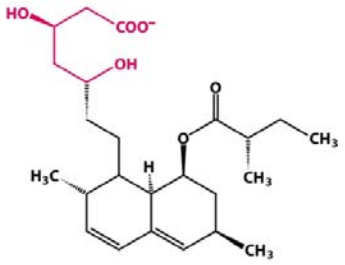

Figure.2.12. Structure of Lovastatin

The best known mushrooms are *Pleurotus* species which produces lovastatin (Gunde-Cimerman *et al*;., 1993b; Gunde-Cimerman and Cimerman, 1995).The presences of the inhibitor was determined in four species: *P. ostreatus, P. cornucopiae, P. eryngii,* and *P. sapidus.* The highest content of lovastatin was found in the fruiting bodies of the *P. ostreatus.* A screening technique was used for *Candida albicans*, which showed a greater zone of inhibition for the lovastatin producing organisms (Vilches *et al*., 2005). This method had proved to be cost effective and less time consuming.

Applications of lovastatin:

Cholesterol synthesis was reduced by lovastatin, by inhibiting the HMG-CoA enzyme which is the important enzyme for the formation of mevolanate from 3-hydroxy3-methylglutaryl coenzyme A (Alberts *et al*., 1980). The rate limiting step of cholesterol synthesis was blocked by covalent binding to the substrate analogue of reductase enzyme. Lovastatin treatment had shown to the prevalence of Alzheimer's disease in patients (Eckert *et al*., 2005). Patients, who administrated with lovastatin, had shown preventing from Multiple sclerosis. Lovastatin also helps in the prevention of kidney damage, especially in the glomerulus nephritis associated kidney disorder. But the exact role of lovastatin is yet to be elucidated (Buemi *et al*., 2002).Injection of lovastatin at particular sites heal the femoral fractures and decreased the cortical fracture gap. Lovastatin compounds had a great effect on cancer cells but the site of actions and the mechanism of actions are not known. Acute transfer of lovastatin at specific sites reduced the cancer level nearly 20%- 55% (Glynn *et al*., 2008).

Triton or Tyloxapol induced Hyperlipidemia:

The nonionic detergent, Triton WR1339 (Tyloxapol or anoxyethylated tertiary octyl phenol formaldehyde polymer), is used by several studies to induce hypercholesterolemia in animals (Harnafi *et al*., 2008). Its function is to inhibit the activity of the enzyme lipoprotein lipase, resulting in the accumulation of triglycerides and VLDL in plasma, beyond causes a significant increase in hepatic cholesterol biosynthesis by stimulating the activity of the enzyme HMG-CoA reductase (Janicki and Aron, 1962). Triton interferes with the normal rate of clearance of cholesterol from blood, not by altering any of the functions of the organs but by altering the lipoproteins of blood so that they are capable of carrying and retaining more cholesterol.

High Fat Diet Induced Hyperlipidemia

Hypercholesterolemic animals are useful models for studies on cholesterol homeostasis, and drug trials to better understand the relationship between disorders in cholesterol metabolism and atherogenesis (Jang and Wang, 2009). High saturated fat and cholesterol diets may increase triglyceride and total cholesterol blood levels, resulting in the increase of LDL synthesis by lipoprotein lipase (LPL) enzyme and

decrease of HDL synthesis. High level of cholesterol may precipitate invascular and result in cholesterol oxidation in the arterial wall leading to atherogenic level increase. Atherogenic index is a predictor of cardiovascular diseases (Kanthe *et al*., 2012). Triglyceride increase will result in the enhanced activities of Cholesteryl Ester Transfer Protein (CETP) which participates as a distribution mediator of triglyceride to HDL and ester cholesterol to LDL. CETP inhibition is necessary to develop to decrease the risk of coronary heart disease. The reduced CETP activities will be beneficial for the existence of HDL; thus triglyceride will not mostly be exchanged with HDL particle (Tjandrawinata, 2013).

LDL Cholesterol and risk of cardiovascular diseases:

LDL is made up of 51% to 58% of cholesterol and 4% to 8% of triglycerides. It makes up about 60% to 75% of all plasma cholesterol. Its main function is to deliver cholesterol from the liver cells. If large quantities of LDL are carried and no new LDL receptors are formed, the LDL absorption will be diminished and a harmful buildup of LDL will take place which may increase the risk of CHD. The process of LDL formation begins when intrahepatic cholesterol, either from gut absorption or *de novo* synthesis, is repackaged by the liver (along with proteins, triglycerides, and phospholipids) into VLDL. VLDL then enters the circulation and is converted by lipoprotein lipase and cholesteryl ester transfer protein (CETP) into more

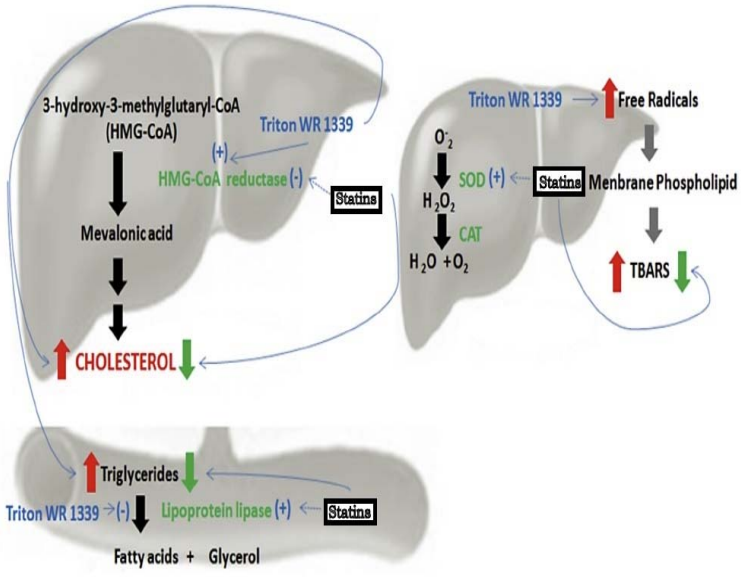

Figure.2.13. Mechanism of Triton induced Hyperlipidemia

cholesterol-enriched species, first IDL and then LDL. The liver regulates the concentration of these circulating lipoprotein species primarily by their clearance through LDL receptors on the hepatic surface. (Feingold and Grunfeld, 2000).

Circulating LDL particles are able to penetrate the endothelium of arterial walls and become oxidized, promote inflammation, and drive injury to the overlying endothelium and surrounding smooth muscle cells (Ross, 1999). The association of LDL with CV risk is also supported by data from alternative measures of atherogenic lipoproteins. Other laboratory markers of atherogenic lipoproteins correlate with LDL-C and also demonstrate similar relationships with CV risk. Both apolipoprotein (apo) B and non–high-density lipoprotein cholesterol (non-HDL-C) measure the contribution to atherogenic risk from the total number of atherogenic particles, including LDL, VLDL, IDL, chylomicrons, and lipoprotein. Statins inhibit the rate-limiting enzyme, HMG-CoA reductase, in the synthesis pathway of cholesterol. This results in lower intrahepatic cholesterol and an up-regulation of hepatic cell surface LDL receptors, resulting in enhanced receptor-mediated uptake of LDL and other Apo B-containing lipoproteins from the circulation. Evidence supports their effectiveness in lowering coronary, cerebrovascular, and peripheral vascular events.

Triglycerides and Atherogenesis:

Raised TG levels can be present in individuals at risk for CHD when the total cholesterol is normal. However, not all individuals with raised TG levels have increased risk of CHD. Factors such as: diet, age, lifestyle, and a range of medical conditions, drug therapy and metabolic disorders, can all affect the TG level. The role of TG in CHD pathogenesis is thought to involve several direct and indirect mechanisms, such as effects on the metabolism of other lipoproteins, transport proteins and enzymes. Patients with hypertriglyceridemia have been shown to respond well to dietary control and to the use of lipid lowering drugs such as 3-hydroxy-3-methylglutaryl-Coenzyme A reductase inhibitors such as statins, fibrates and nicotinic acids.

Anti-Atherogenic mechanism of HDL Cholesterol:

A low level of high-density lipoprotein cholesterol (HDL-C) is an important risk factor for cardiovascular disease. Epidemiological and clinical studies provide

evidence that HDL-C levels are linked to rates of coronary events. The association between low levels of high-density lipoprotein cholesterol (HDL-C) and an increased risk for cardiovascular disease has been well established through epidemiological and clinical studies.

Anticholesterimic activity of mushroom:

In vivo studies have shown that consumption of oyster mushrooms lowers cholesterol levels, because these mushrooms naturally contain statins such as lovastatin (Gunde-Cimerman and Cimerman, 1995) Studies have shown that the mushrooms contain up to 2.8% lovastatin on a dry weight basis. Both lovastatin and its hydroxyacid metabolite are highly bound (> 95%) to human plasma proteins. Mushroom feeding also significantly decreased hepatic cholesterol suggesting the clearance of this sterol component from liver tissue. Hypercholesterolaemia produces oxidative stress (Joseph *et al*, 1996) and for such case anti-oxidants, particularly reduced glutathione, are important factors involved in bile formation and bile flow (Ballattori and Truong, 1992). Several studies have found that a blood pressure reduction is associated with the use of statins. Oyster mushroom is very much rich in fiber. These fibers bind the cholesterols in gut; facilitate its fecal excretion from the body. Moreover, fibers forms and increase the bulk of stool, speed bowel movement, decrease the discharge time and thus reduce the risk of colon cancer and other gastrointestinal tract related disturbances.

Chapter 3:

Materials & methods

3.1. MATERIALS

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3.1.2. Chemicals

3.1.3. Cell line

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3.2.9. Determination of tissue Glutathione –S- Transferase (GST) activity

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3.2.16. Determination of HDL Cholesterol

3.2.17. Determination of hemoglobin (Hb) in blood

3.2.18. Determination of total white blood cell (WBC) count

3.2.19. Histopathology

3.2.20. Statistical analysis

3.1 MATERIALS

3.1.1 Animals

Animals were purchased from Small Animal Breeding Centre, Kerala Agriculture University, Mannuthy, Thrissur, Kerala, India. Male Swiss Albino mice weighing 20 ± 2g and Wistar rats weighing 200 ± 20g were used for the experiments. They were kept under environmentally controlled conditions with free access to standard food and water. Experiments were performed according to the guidelines and approval of Institutional Animal Ethic Committee (IAEC) under the regulation of CPCSEA (Approval No: ACRC/IAEC/15/04 -2).

3.1.2 Chemicals

Ethanol, Methanol, 2, 2-diphenyl-1-picryl hydrazyl (DPPH), Nitro blue tetrazolium (NBT) , Riboflavin, Ethylene diamine tetra acetic acid (EDTA), Potassium cyanide, Sodium dihydrogen phosphate (NaH_2PO_4), Disodium hydrogen phosphate (Na_2HPO_4), 2-azino-bis- (3-ethyl benzothiazoline-6-sulphonic acid) ($ABTS^+$), Ammonium persulphate, Sodium nitroprusside, Sodium chloride ($NaCl_2$), Potassium chloride (KCl), Potasssium dihydrogen phosphate (KH_2PO_4), Sulphanilamide, Acetic acid, Naphthyl ethylene diamene dihydro chloride, Sodium acetate, 2, 4, 6-tripyridyl-s- triazine(TPTZ), Hydrochloric acid, Ferric chloride ($FeCl_3$), Potassium hydroxide (KOH), Ascorbic acid, Deoxy ribose, Hydrogen peroxide (H_2O_2), Sodium dodecyl sulphate (SDS), Thio barbituric acid (TBA), Butanol, Pyridine, Ammonium ferrous sulphate. Formalin, Diclofenac, Cyclophosphamide, Carbon tetrachloride, 1 chloro, 2,4 dinitrobenzene (CDNB), Dextrose, 5,5'- dithiobis- (2-nitrobenzoic acid) (DTNB), Reduced glutathione (GSH), Formaldehyde, Glucose, Magnesium sulphate, Metaphosporic acid, Peptone, Riboflavin, Sodium azide, Sodium hydroxide, Sodium chloride, ,Trichloroacetic acid (TCA), 1'1'3'3' tetramethoxypropane, Yeast extract, Dichloromethane, Lovastatin (Sigma Aldrich), Iodine, Atorvastatin, Tyloxapol (Sigma Aldrich) ,Perchloric Acid, Hydroxylamine Hydrochloride.

3.1.3. Cell line

Dalton's lymphoma ascite's (DLA) cell line was obtained from cancer institute, Adayar Chennai. The cells were maintained in mice by intraperitonial inoculation.

3.1.4. Instruments

Electronic balance: Contech, Mumbai

Hot air oven: Beston, India

Hot water bath: Beston, India

Hot plate: Beston, India

PH meter: Eutech, India

Cooling centrifuge: REMI Instruments LTD, Mumbai

Tissue homogenizer: REMI Instruments LTD, Mumbai

Vortex mixer: REMI Instruments LTD, Mumbai

UV-VIS Spectrophotometer: Systronic, India

Rotary evaporator: Buchi-R-210

Incubator: Narang Scientific Works PVT LTD, Mumbai

Autoclave: Narang Scientific Works PVT LTD, Mumbai

Refrigerator: Whirlpool

Laminar Air Flow

Magnetic Stirrer - REMI Equipments Pvt LTD.

Deep freezer -20^{0}C: REMI, Cennai, India

Microscope: Olympus microscope, Japan

3.2. METHODS

3.2.1. Collection of fruiting body

Fruiting bodies of *H. ulmarius* were obtained from mushroom cultivation centre in Thrissur District. Dried fruiting bodies were used for the experiments.

3.2.2. Production of mushroom mycelium

H. ulmarius culture was grown on Glucose peptone nutrient medium (Glucose-10g, Peptone-3g, KH_2PO_4 - 1g, K_2HPO_4 -1g, $MgSO_4$-0.25g, Yeast extract-1g, Distilled water -1000ml). The medium was poured into Roux bottles and sterilized at 121^0C for 30 minutes. The medium was inoculated with 10 days old culture of *H .ulmarius* and incubated at 25-27ºC for 20 days as stationary culture. After 20 days, the medium was filtered and the mycelium mat was separated and dried at 45-50^0C for 24-48 hours.

3.2.3. Preparation of extracts

Dried fruiting bodies and mycelia biomass were powdered and extracted with 70 % (v/v) aqueous ethanol for 8-10 hours using Soxhlet apparatus. The extracts were collected and filtered through Whatman No.1 filter paper; the solvent was completely evaporated at 40^0C using a rotary vacuum evaporator and finally lyophilized. The yield of extract from fruiting bodies (11% w/w) and mycelium (8% w/w).

3.2.4. Preparation of tissue homogenates

Animals were sacrificed after the completion of experiments. Liver was excised and rinsed thoroughly in ice cold saline to remove blood. It was then blotted between the folds of the filter paper and weighed in an analytical balance. 10% of the homogenate was prepared in 0.05M phosphate buffer (pH 7) using a polytron homoginiser at 4ºC. A part of this homogenate was used for the determination of reduced glutathione. Rest of the homogenate was centrifuged at 10,000 rpm for 20 minutes for removing the cell debris, unbroken cells, nuclei, erythrocytes and mitochondria. The supernatant was used for the estimation of superoxide dismutase, catalase, glutathione peroxidase, glutathione-S- transferase and malondialdehyde.

3.2.5. Determination of protein

Protein present in the tissue was determined by Bradford's method

Principle

The assay relies on the binding of the dye Coomassie blue G250 to protein. The quantity of protein can be estimated by determining the amount of dye in the blue ionic form. This is usually achieved by measuring the absorbance of the solution at 595nm or 625nm.

Procedure

Pipette standard BSA (0.5mg/ml) and test sample as indicated in table 1. Adjust the volume to 0.2 ml with distilled water. Add 3 ml of Bradford's reagent and mix it thoroughly. Incubate all the tubes at room temperature for 10 minutes. The optical density is measured at 595 nm/ 625 nm.

3.2.6. Determination of tissue catalase (CAT) activity

Tissue catalase activity was determined according to the method of Beer and Sizer (1952) with some modification.

Principle

Catalase catalyzes the decomposition of H_2O_2. In the ultraviolet range H_2O_2 shows a continued increase in absorption with decreasing wavelength. The decomposition of H_2O_2 was followed directly by the decrease in extinction at 240nm.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

Procedure

10µl of the tissue supernatant was mixed with 1.990ml of the phosphate buffer (0.5M, pH- 7.0) and 1ml of 11Mm H_2O_2. Decrease in absorbance per minute was measured at 240 nm for the first three minutes against a blank which contained 0.01ml of tissue supernatant and 2.990ml of phosphate buffer. The activity of catalase was calculated using molar extinction coefficient of H_2O_2 (43.6) and expressed as units of catalase per milligram protein. One unit of the enzyme is equal to 1 millimole of H_2O_2 degraded per minute.

$$\text{Catalase activity (U/mg protein)} = \frac{(\Delta OD/\text{minute} \times 1000 \times 3)}{43.6 \times \text{mg protein in sample}}$$

3.2.7. Determination of tissue superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined according to the method of McCord and Fridovich (1969) with some modification.

Principle

Illumination of riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then re-oxidizes and simultaneously reduces oxygen to O_2^- which was allowed to react with a detector molecule NBT, reduced the NBT to formazan blue. The SOD in the sample inhibits the formazan production.

Procedure

20µl of the homogenate was mixed with 0.2 ml of 0.1 M EDTA (containing 0.0015% KCN), 0.1 ml of 1.5 mM NBT and phosphate buffer (67 mM, pH 7.8) in a total volume of 2.6 ml. After adding 0.05 ml of riboflavin, the absorbance of the solution was measured against distilled water at 560 nm. All the tubes were illuminated for 15 min and absorbance of the blue color formed was measured again. Percent of inhibition was calculated after comparing absorbance of sample with the absorbance of control (the tube containing no enzyme activity). The volume of the sample required to scavenge 50 % of the generated superoxide anion was considered as 1 unit of enzyme activity and expressed in U/ mg protein.

3.2.8. Determination of tissue reduced glutathione

The GSH was measured by the method of Moron et al (1979) with some modification.

Principle

The acid soluble sulfhydryl groups (non-protein thiols of which more than 93% is reduced glutathione) forms yellow colored complex with 5, 5'- dithiobis- (2-nitrobenzoic acid) (DNTB). The absorbance of the colored complex was measured at 412nm.

Procedure

0.5ml of the tissue homogenate was mixed with 0.1ml of 25% TCA and kept on ice for 10 minutes. This was then centrifuged at 2000 rpm for 10 minutes to settle down the precipitate. 0.5ml of the supernatant was mixed with 0.5ml of 0.2M sodium phosphate buffer (pH-8.0) and 2ml of 0.6mM DTNB (freshly prepared in 0.2M phospate buffer, pH-8.0). The yellow color produced was read after 10 minutes at 412 nm against a blank which contained 0.5ml of 5 % TCA in the reaction mixture instead of the supernatant. The GSH in the tissue sample was estimated by using a standard

graph plotted from different concentration (10-50 nanomoles) of GSH in 0.3ml of 5 % TCA. The values are expressed as nanomole of GSH per mg protein.

3.2.9. Determination of tissue Glutathione –S- Transferase (GST) activity

The activity GST was determined by the method of Habig et al (1974).

Principle

The activity of GST was determined by the rate of increase in conjugate formation between GSH and CDNB. The conjugate has absorption maxima at 340nm.

Procedure

The reaction mixture contained 0.05ml of 60mM GSH, 0.05ml of 60mM CDNB (in ethanol), 0.02 ml of tissue supernatant and sodium phosphate buffer (0.1M, pH-6.5) in a total volume of 3 ml. The change in absorbance per minute was measured at 340nm for the first three minutes against a blank which contained all the assay mixture except the supernatant. The activity of GST was calculated using the molar extinction coefficient of CDNB-GSH conjugate (9.6 mM-1 cm-1) and was expressed as units of GST.

One unit is defined as micromole of CDNB-GSH conjugate formed per minute/milligram of protein.

$$\text{GST (U/mg protein)} = \frac{(\Delta OD/\text{minute} \times 1000 \times 3)}{9.6 \times \text{mg protein in sample}}$$

3.2.10. Determination of tissue lipid peroxidation

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al (1979).

Principle

Malondialdehyde (MDA) was allowed to react with thiobarbituric acid (TBA). The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532nm.

Procedure

4 ml of the reaction mixture containing 0.4 ml of supernatant of tissue homogenate, 0.2 ml of 8.1% SDS, 1.5 ml of 0.8% TBA, 1.5 ml of acetic acid (20% (v/v), pH 3.5) and 0.4 ml of distilled water was kept in a boiling water bath. After 1 hour the reaction mixture was removed from the water bath, cooled and 1 ml of distilled water was added. 5 ml of butanol: pyridine (15:1(v/v)) was then added, mixed thoroughly and centrifuged at 3000 rpm for 10 minutes. Absorbance of the clear supernatant was measured at 532 nm against butanol: pyridine mixture. The MDA was estimated by using a standard graph of different concentration (1- 10 nanomole) of 1'1'3'3'-tetramethoxy propane in 1 ml distilled water. The level of lipid peroxides was expressed as nmol of MDA/mg protein.

3.2.11. Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

SGOT level in the serum was determined using Agappe diagnostic kit.

Principle

Oxaloacetate formed as a result of transamination is converted to malate using the enzyme malate dehydrogenase and NADH. The rate of consumption of NADH is measured kinetically at 340nm.

Procedure

100µl of serum was added to the working reagent containing tris buffer (70.40mM, pH – 7.8), L-aspartate (208mM), lactate dehydrogenase (1200U/L), malate dehydrogenase (720U/L),α ketoglutarate (2.4mM) and NADH (0.048mM). The reaction mixture was incubated at 37ºC for 1 minute and the decrease in absorbance per minute was read at 340nm against distilled water for the first three minutes. The activity of the enzyme was expressed as units per litre using the relation: SGOT activity = (ΔOD/minute) X 1745

3.2.12. Determination of serum glutamate pyruvate transaminase (SGPT) activity

SGPT level in the serum was determined using Agappe diagnostic kit.

Principle

Pyruvate formed as a result of transamination is converted to lactate using the enzyme lactate dehydrogenase and NADH. The rate of consumption of NADH is measured kinetically at 340nm.

Procedure

100µl of serum was mixed with 1000µl of the working reagent containing tris buffer (88mM, pH7.5), L-alanine (480mM), lactate dehydrogenase (1200U/L), α-ketoglutarate (3.2mM) and NADH (0.048mM). The reaction mixture was incubated at 37ºC for 1 minute and decrease in absorbance per minute was read at 340 nm against distilled water for the first three minutes. The activity of the enzyme was expressed as units per liter and calculated as

SGPT activity = (ΔOD/minute) X 1745

3.2.13. Determination of serum alkaline phosphatase (ALP) activity

ALP level in the serum was determined using Agappe diagnostic kit.

Principle

ALP in the serum reacts with paranitrophenyl phosphate and converts it into p-nitrophenol and the associated increase in absorbance was measured kinetically at 405nm.

Procedure

20µl of the serum was mixed with 1000µl of the working reagent containing diethanolamine buffer (100mM, pH-10.2), Magnesium chloride (0.5mM) and P-nitrophenyl phosphate (10mM). The reaction mixture was incubated at 37ºC for 1 minute and increase in absorbance per minute was read at 405nm against distilled water for the first three minutes. The activity of the enzyme was expressed as units per litre using the following relation

ALP activity (U/L) = (ΔOD/minute) X 2750

3.2.14. Determination of serum total cholesterol

Total cholesterol was determined using Agappe diagnostic kit

Principle

Enzymatic colorimetric determination of total cholesterol according to the following reactions;

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol esterase}} \text{4-Cholesten-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{Phenol} + \text{4-Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Red quinone} + 4H_2O$$

Procedure

1000µl of working reagent was added to 10 µl of cholesterol standard and 10 µl of each samples and a blank containing only 1000µl of working reagent. All tubes were mixed well using vortex mixer and incubated for 5 minutes at 37^0C. The absorbance of sample and standard against reagent blank was measured at 505 nm.

Calculation

$$\text{Cholesterol Con (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 \text{ mg/dL}$$

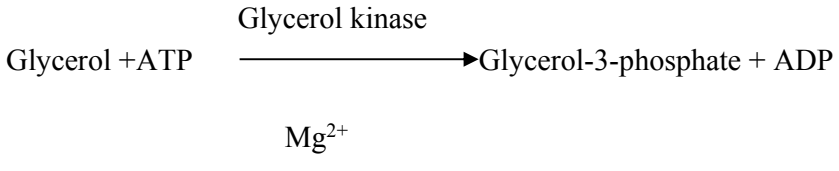
3.2.15. Determination of serum triglycerides

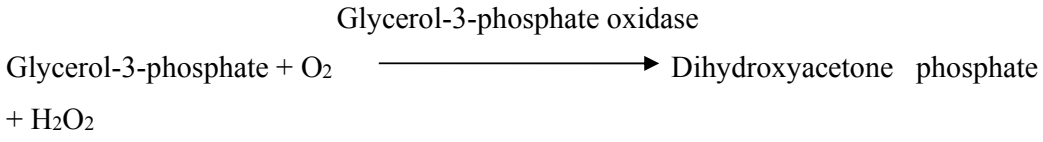
Triglycerides was determined using Agappe diagnostic kit

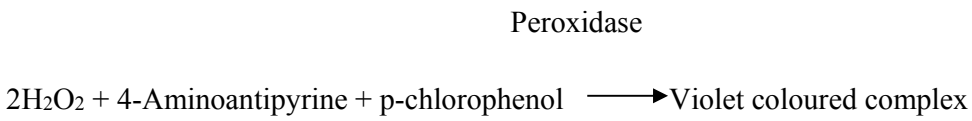
Principle

Enzymatic determination of triglyceride is based on the following reactions:

$$\text{Triglycerides} + H_2O \xrightarrow{\text{Lipoprotein lipase}} \text{Glycerol} + \text{fatty acid}$$







Procedure

1000µl of working reagent was added to 10 µl of cholesterol standard and 10 µl of each samples and a blank containing only 1000µl of working reagent. All tubes were mixed well using vortex mixer and incubated for 5 minutes at 37^0C. The absorbance of sample and standard against reagent blank was measured at 546 nm.

Calculation

$$\text{Triglycerides Con (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 200 \text{ mg/dL}$$

3.2.16. Determination of HDL Cholesterol

HDL Cholesterol was determined using Agappe diagnostic kit

Principle

The chylomicrons, Very Low Density Lipoproteins (VLDL) and Low Density Lipoproteins (LDL) of serum are precipitated by phosphotungstic acid and magnesium ions. After centrifugation, HDL content of supernatant is measured by an enzymatic method.

Procedure

300 µl of HDL reagent is added to 300 µl of serum sample. Mixed well using vortex mixer and allowed to stand for 10 minutes at room temperature. Mixed again and centrifuged at 4000 rpm for 10 minutes. After centrifugation, the clear supernatant from the precipitate was separated within 1 hour and determined the HDL Cholesterol concentration using the cholesterol reagent.

1000µl of working reagent was added to 10 µl of cholesterol standard and 10 µl of each samples and a blank containing only 1000µl of working reagent. All tubes were mixed well using vortex mixer and incubated for 5 minutes at 37^0C. The absorbance of sample and standard against reagent blank was measured at 505 nm.

$$\text{HDL cholesterol Con (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{concentration of standard}$$

Where, 2 = dilution factor, concentration of standard= 50 mg/dL

3.2.17. Determination of haemoglobin (Hb) in blood

Haemoglobin was determined according to the method of Drabkin and Austin (1932) using Agappe diagnostic kit.

Principle

Haemoglobin reagent contains potassium dihydrogen phosphate, potassium cyanide and potassium ferrricyanide. When blood was added to it, ferricyanide forms methhaemoglobin which is converted to cyanmethhaemoglobin. The optical density was read at 546 nm and OD is directly proportional to the amount of haemoglobin present in the blood.

Procedure

0.02ml of blood was mixed with 5ml of the cyanmeth reagent. It was then incubated at room temperature for 5 minutes and then the OD was read at 546 nm against reagent blank. The standard (60mg/dl) was treated in the same manner was used for calculation of concentration of haemoglobin in the blood.

Calculation

$$\text{Haemoglobin (g/dl)} = \frac{\text{Optical Density of test} \times 60 \times 0.251}{\text{Optical Density of standard}}$$

3.2.18. Determination of total white blood cell (WBC) count

Total WBC count was estimated using the method described by Chaudari, 2000

Principle

The whole blood was diluted in a dilutent which hemolyses red blood cells. The number of white blood cells in a known volume and known dilution was counted using haemocytometer.

Procedure

0.02 ml of blood was mixed with 0.38ml of diluting fluid. Well mixed fluid was then added to a haemocytometer. Total number of white blood cells in the four large corner squares of chamber was counted after 3-4 minutes.

Calculation

Total WBC Count = Number of cells counted × 50 /mm^3

3.2.19. Histopathology

A small portion of the tissue sample was taken from each sample and placed in a bottle containing 10% formalin and embedded in paraffin, cut into 4–5µm thick sections and stained with hematoxylin and eosin. The sections were examined under light microscope.

3.2.20. Statistical analysis

All the experimental data are expressed as means ± SD. Mean values were statistically analyzed using One-way analysis of variance. Dunnet's test was applied for expressing the significance. $P < 0.05$ was considered significant.

Chapter 4:

Antioxidant activity of fruiting bodies and cultured mycelia extracts of Hypsizygus ulmarius

4.1. INTRODUCTION

4.2. MATERIALS AND METHODS

4.2.1. Preparation of the extracts

4.2.2. Free radical scavenging activities

4.2.2.1. DPPH radical scavenging activity

4.2.2.2. Hydroxyl radical scavenging activity

4.2.2.3. $ABTS^{+}$ radical scavenging activity

4.2.2.4. Inhibition of lipid peroxidation

4.2.2.5. Nitric oxide scavenging activity

4.2.2.6. Ferric Reducing Antioxidant Power (FRAP) Assay

4.3. RESULTS

4.3.1. DPPH radical scavenging activity

4.3.2. Hydroxyl radical scavenging activity

4.3.3. Inhibition of lipid peroxidation

4.3.4. Nitric oxide scavenging activity

4.3.5. $ABTS^{+}$ radical scavenging activity

4.3.6. Ferric Reducing Antioxidant Power (FRAP) Assay

4.4. DISCUSSION

4.1. INTRODUCTION

The antioxidants refer to any molecule capable of stabilizing or deactivating free radicals before they attack cells. Humans have evolved with highly complex antioxidant systems (enzymatic and non-enzymatic) which work synergistically and in combination with each other to protect the cells and organ systems of the body against free radical damage. Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and normal physiological function of a living system. The antioxidant can be endogenous or obtained exogenously as a part of a diet or as dietary suppliments. The most efficient enzymatic antioxidants involve glutathione, peroxidase and superoxide dismutase. Non enzymatic antioxidants involve vitamin E and C, thiol antioxidants (thioredoxin, lipoic acid), melatonin, carotenoids, natural flavanoids and other compounds.

The most commonly used synthetic antioxidants at the present time are butylated hydroxyanisole (BHA), butylhydroxytoluene (BHT), propyl gallate and tert butylhydroxyquinone. However, BHA and BHT have restricted use in foods as they are suspected to be carcinogenic and to cause liver damage. Therefore, there is growing interest in the use of natural additives as potential antioxidants. Many edible mushrooms are good sources of carbohydrates such as glucans; phenolics such as tocopherols; B-vitamins such as niacin, flavin and pyridoxine; organic acids such as ascorbate, malate and fumarate; monoterpenoid and diterpenoid; lipids; proteins and trace elements like selenium (Aggarwal *et al*., 2012, Iwalokun *et al*., 2007). These are responsible for their medicinal properties including antioxidant, antimicrobial and antitumor potentials of mushrooms (Lindequist *et al.,* 2005)

Many bioactive compounds from mushrooms have been isolated and well-studied, in which phenolic compounds and polyphenols can act as antioxidant as they can react highly with hydrogen or electron donors, stabilize chain breaking reaction and terminate Fenton reaction (Rice evans *et al*., 1997). Phenolic compounds from mushrooms are mainly involved in hydrogen peroxide scavenging cascade. There exist a correlation between total phenolic content in the mushroom extract and their antioxidant activities. Also, polysaccharide isolated from the fruiting bodies and mycelia of mushroom exhibit a broad spectrum of therapeutic properties including immunestimulatory, anti-tumour, anti-inflammatory, antifungal, antioxidant activities

(Han *et al*., 2010) and the antioxidant properties of polysaccharides are influenced by chemical characteristics like molecular weight, degree of branching, types of monosaccharides and ratio of monosaccharides.

Organisms are well protected against free radical mediated damage. When the mechanism of this antioxidant defense system becomes unbalanced, deterioration of cellular functions may occur (Mau *et al.,* 2002).There is a growing interest to measure free radical scavenging ability of mushrooms, since the active compounds exhibiting such properties could be isolated and used for the prevention of free radicals mediated pathologies, such as cardiovascular and neurodegenerative diseases (McCord, 2000). The antioxidant capacity (radical scavenging) of *Hypsizygus ulmarius* was investigated in both fruiting bodies and cultured mycelium was examined and results are reported in this chapter.

4.2. MATERIALS AND METHODS

4.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

4.2.2. Free radical scavenging activities

4.2.2.1. DPPH radical scavenging activity

In the DPPH (1, 1-diphenyl -2- picryl hydrazyl) assay, the ability of antioxidant to scavenge stable purple colored primary radical $DPPH^+$ by depolarization is assayed spectrophotometrically at 515 nm. In this method a commercially available and stable free radical DPPH, soluble in methanol was used (Aquino *et al.,* 2001).The DPPH in its radical form has an absorption peak at 515 nm, which disappeared on reduction by an antioxidant. Different concentrations of (100-1000µg/ml) ethanolic extract of fruiting bodies or mycelia were added to freshly prepared DPPH solution. The absorbance was measured 20 minutes after reaction was started. The experiment was repeated twice with each concentration in triplicate.

4.2.2.2. Hydroxyl radical scavenging activity

Hydroxyl radicals generated from Fe^{3+}-`ascorbate/EDTA-H_2O_2system (Fenton reaction) was estimated. The assay was carried out as described by (Elizabeth and Rao,1990) The reaction mixture contained 100µl of deoxyribose (2.8 mmoles/L), 100µl of EDTA(0.1mmoles/L), 100µl of H_2O_2 (1mmoles/L), 100µl of Ascorbate (0.1mmoles/L), 100µl of KH_2PO_4-KOH buffer (20 mmoles/L), and various concentrations (100-1000µg) of the extracts of the fruiting bodies or mycelia in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37°C. 600µl reaction mixture was removed and treated with 0.2ml SDS, 1.5ml acetic acid, and 1.5 ml thiobarbituric acid. The reaction mixture was heated on a water bath at 95-100^{0}C for one hour. After cooling, 1ml of the distilled water and 5 ml of n-butanol-pyridine mixture (15:1) were added to the reaction mixture. Shaken vigorously and centrifuged at 4000 rpm for 10 minutes. The n-butanol pyridine layer was removed and its absorbance read at 532 nm against pyridine- butanol reagent. Radical scavenging activity was determined by comparing the optical density of the treatments with that of control. The experiment was repeated twice with each concentration in triplicate.

4.2.2.3. Inhibition of lipid peroxidation

The assay for the inhibition of lipid peroxidation was carried out by the method described by (Ohkawa *et al*., 1979) with slight modification. The reaction mixture contained 0.2ml rat brain homogenate (25%w/v) in Tris HCl buffer and various concentrations (100- 1000µg/ml) of the ethanolic extracts of the fruiting bodies or mycelia , KCl (150 mmoles/L), $FeSO_4$ $(NH_4)_2SO_4$.$7H_2O$ (200µl), ascorbate (200µl) and was made up to a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37^{0}C. After the incubation period, 0.1ml was removed and treated with 0.2ml of SDS, 1.5ml acetic acid, 1.5ml thiobarbituric acid. The total volume in the test tube made up to 4ml with distilled water and kept on a water bath at 95-100^{0}Cfor 1 hour. After cooling, 1ml distilled water and 5 ml of n-butanol and pyridine mixture (15:1) were added to the reaction mixture. Shaken vigorously and centrifuged at 4000 rpm for 10 minutes. The n-butanol pyridine layer was removed and its absorbance read at 532 nm against pyridine –butanol blank reagent. Inhibition of lipid peroxidation was

determined by comparing the optical density of the treatments with that of control. The experiment was repeated twice with each concentration in triplicate.

4.2.2.4. Nitric oxide scavenging activity

In this assay, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess-Illosvoy reaction (Garrat, 1964). The reaction utilizes sulfanilamide and N-1-napthylethylenediaminedihydrochloride (NED) under acidic conditions (Marcocci *et al.*, 1996). The reaction mixture (3 ml) containing sodium nitroprusside (10 mmoles/L), phosphate buffer saline (pH- 7.4), and various concentrations (100-1000µg/ml) of the extracts, were incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of Sulphanilamide solution (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed, and allowed to stand for 30 min at 25 °C. A pink colored chromophore is formed. The absorbance of these solutions was measured at 540 nm against the corresponding blank (PBS buffer) solution. The experiment was repeated twice with each concentration in triplicate.

4.2.2.5. $ABTS^{+}$ radical scavenging activity

In this assay, a model stable free radical derived from 2, 2'-azinobis (3-ethyl benzothiazolin -6- sulphonic acid) (ABTS), is allowed to interact with sample. The pre-formed radical monocation of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) is generated by oxidation of ABTS with ammonium persulfate (a blue chromogen) and is reduced in the presence of hydrogen donating antioxidants.The production of radical cation was done as described by (Halliwell *et al.*, 1999) with some modifications. A stock solution of ABTS (7 mmoles/L) was prepared in water. To this solution Ammonium persulphate (2.45mmoles/L) was added and solutions were allowed to react in dark for 16 hours at room temperature. ABTS and persulphate react with each other leading to the generation of ABTS radicals in the solution. For the evaluation of ABTS radical scavenging activity, the working solution was prepared by diluting ABTS radical solution in ethanol to obtain the

absorbance of 0.700 ± 0.02 at 734 nm. Various concentrations (50- 250µg/ml) of the extracts were mixed with the ABTS working solution (1 ml) and the reaction mixture was allowed to stand at 30 ºC for 6 min, then the absorbance was measured by using a UV-visible spectrophotometer at 734 nm against blank Ethanol (Hua *et al*., 2010).The experiment was repeated twice with each concentration in triplicate.

4.2.2.6. Ferric Reducing Antioxidant Power (FRAP) Assay

The mechanism of this method is based on the reduction of ferric 2, 4, 6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous form (Fe^{2+}-TPTZ) in the presence of antioxidants, form a blue colored complex which can be measured at 593nm.The FRAP assay was carried out as described by (Benzie and Strain, 1996). The FRAP reagent contain 2.5ml TPTZ solution, 2.5 ml Ferric chloride solution and 25 ml acetate buffer. FRAP reagent was mixed with different concentrations (50-250 µg/ml) of the extracts and final volume made up to 3 ml. A blue colored complex was formed when Fe^{3+} -TPTZ complex reduced to ferrous (Fe^{2+}) form after incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured spectrophotometrically at 593 nm against a FRAP reagent solution as blank. The results were expressed in µmole equivalent to $FeSO_4.7H_2O$ by calculating from calibration curve. For constructing the calibration curve different concentrations of $FeSO_4.7H_2O$ were used and the absorbences were measured. The experiment was repeated twice with each concentration in triplicate.

4.3. RESULTS

4.3.1. DPPH radical scavenging activity

The fruiting body and mycelia extract of *H. ulmarius* showed significant DPPH radical scavenging activity. 500, 250 and 50µg/ml fruiting body extract showed 85, 45.3 and 11.9% inhibition respectively, and the at the same concentrations the mycelia extract showed 88.3, 55.6and 20.76% inhibition respectively. The IC_{50} values of fruiting body and mycelium extract were found to be 225µg and 300µg respectively (Fig.4.1).

4.3.2. Hydroxyl radical scavenging activity

Degradation of deoxy ribose by hydroxyl radical generated from Fe^{3+}ascorbate /EDTA/H_2O_2 system was markedly decreased by the extract of fruiting body of *H. ulmarius* than mycelia extract. At concentrations of 1000, 500 ,100µg/ml fruiting body extract showed 85.6, 65.7 and 33.4% activity, and at the same concentrations mycelium extract showed 71.3, 59 and 9.4% activity respectively. The IC_{50} values of fruiting body and mycelium extract were found to be 300 and 430µg/ml respectively (Fig.4.2).

4.3.3. $ABTS^+$ radical scavenging activity

The aqueous ethanolic extract of both fruiting body and mycelia extract of *H. ulmarius* efficiently scavenged ABTS radicals generated by the reaction between 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid and ammonium persulphate. At concentrations of 250, 100 and 50µg/ml mycelia extract showed 61.95, 32.95, and 18.29% activity and at the same concentrations fruiting body extract showed 71.4, 47.8, 5.1% activity respectively. Fruiting body extract has more activity than mycelia extract. IC_{50} values of fruiting body and mycelium extract were found to be 120 and 180µg/ml respectively (Fig. 4.3).

4.3.4. Inhibition of lipid peroxidation

H. ulmarius mycelia extract was more effective to inhibit lipid peroxidation produced by Fe^{2+}ascorbate system in rat brain homogenate than the fruiting body extract. At concentrations of 1000, 500, and 100µg/ml fruiting body extract showed 41, 27 and 15% activity and at the same concentrations mycelia extract showed 56.4, 35.4 and 14.8% activity respectively. IC_{50} values of fruiting body and mycelia extract were found to be 850 and 600 µg/ml respectively (Fig. 4.4)

4.3.5. Nitric oxide scavenging activity

H. ulmarius mycelia extract showed significant nitric oxide scavenging activity. At concentrations of 1000, 500, and 100µg/ml mycelia extract showed 76, 54.6, and 2.7% inhibition and at the same concentrations fruiting body extract showed 59.42, 32.37 and 22.5% activity respectively. The IC_{50} values of fruiting body and mycelium extract were found to be 830 and 460 µg/ml respectively (Fig.4.5)

4.3.6. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay is based on the ability of an antioxidant to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2}+-TPTZ complex with an absorption maximum at 593nm. In the present study both the fruiting body and mycelia extract of *H. ulmarius* showed significant Fe^{3+} reducing power which indicated the hydrogen donating ability of the extract. Mycelia extract showed more activity than fruiting body extract. The activity was expressed as the amount of Fe^{3+} reduced. At concentrations of 150, 100, and 50µg/ml fruiting body extract showed 36.5, 24.5, and 16µmoles of Fe^{3+} reduction and at the same concentrations mycelia extract showed 42.5, 33, and 24µmoles of Fe^{3+} reduction respectively.

Figure.4.1. DPPH radical scavenging activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*

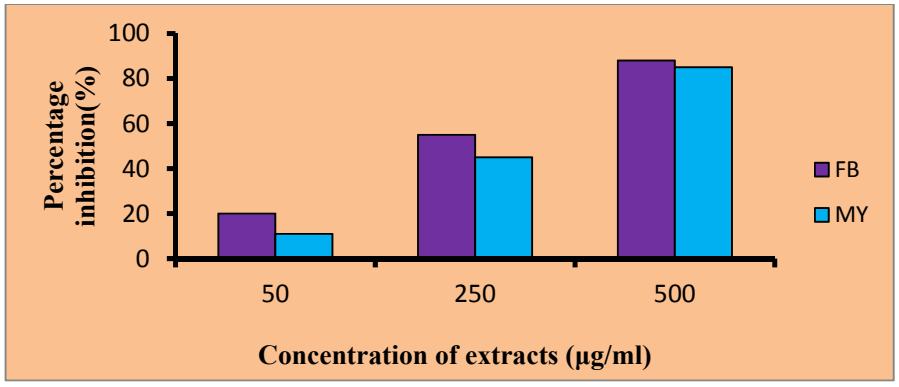


Figure.4.2. Hydroxyl radical scavenging activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*

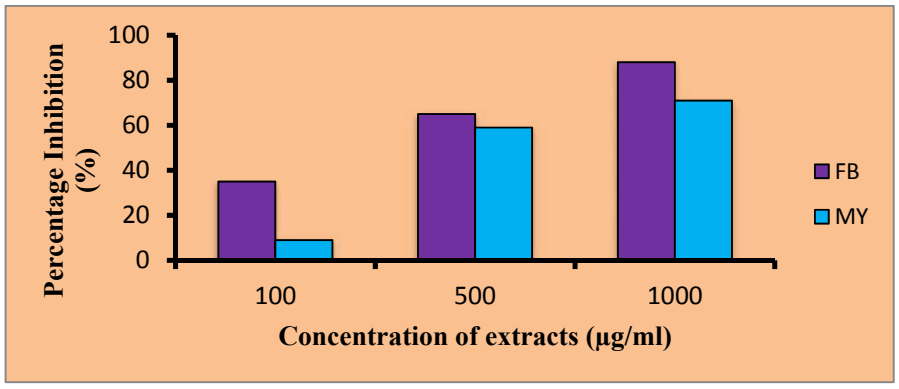


Figure.4.3 $ABTS^{+}$ radical scavenging activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*

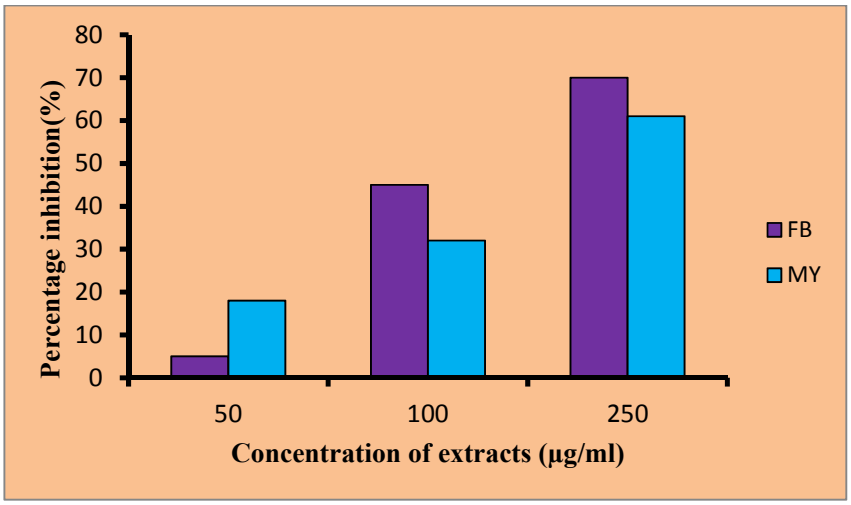


Figure.4.4 Inhibition of lipid peroxidation activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*

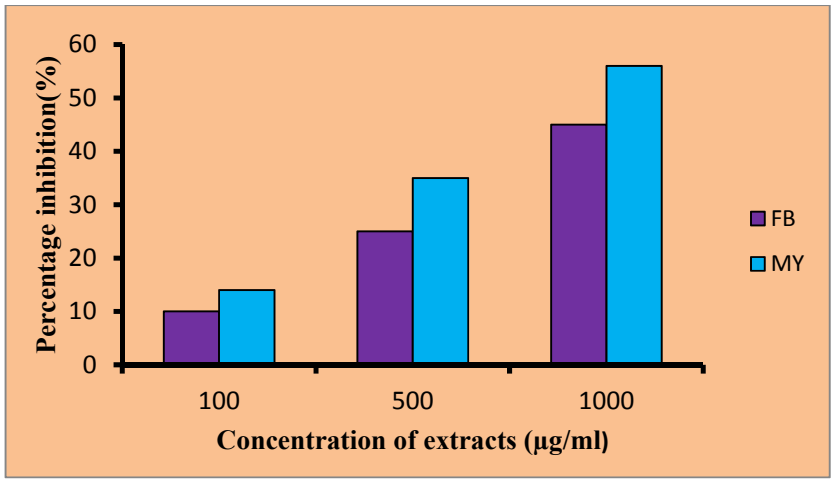
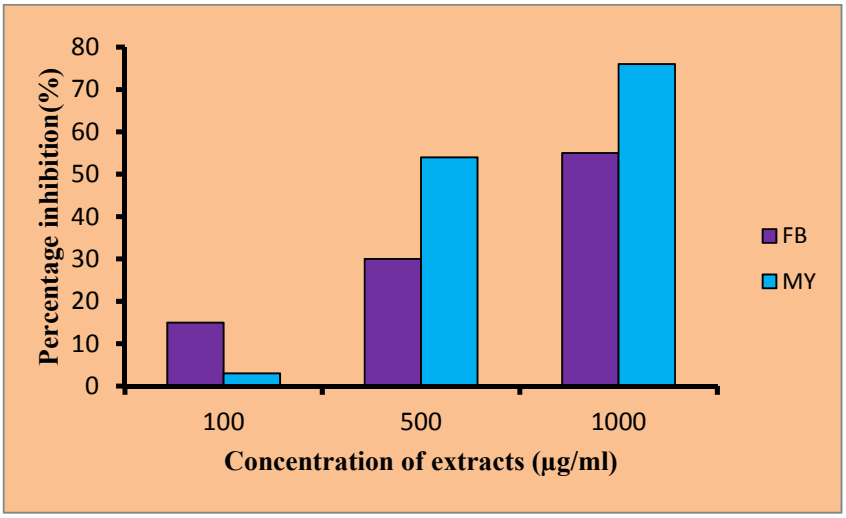


Figure.4.5 Nitric oxide scavenging activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*



4.4. DISCUSSION

Among the 14,000 known mushrooms now, *H. ulmarius* is comparatively a new genus (earlier known as *Pleurotus ulmarius)*. A number of investigations carried out on the medicinal properties of *Pleurotus* species have demonstrated that oyster mushrooms possessed remarkable antioxidant, antitumor, antimicrobial, anti-inflammatory, hypocholesterolemic, and anti-platelet aggregating effects (Nayana and Janardhanan, 2000).

Antioxidant defense systems protect human body from oxidative stress induced damage; sometimes these are not enough to repair the damages. So antioxidants in diet are of important value as possible protective agents to help human body to reduce free radical induced damage. Recently a large number of natural antioxidants have been isolated from mushrooms. Mushrooms are functional food and are traditionally used in folk medicine in several countries. Human diet containing mushrooms possessing antioxidative properties would be of great importance to prevent some human ailments.

It has been established that mushroom antioxidants have significant protective properties at different stages of the oxidation process and by different mechanisms

(Brewer, 2011; Ferreira *et al*., 2009). There are two main types of mushroom antioxidants, namely, primary (chain breaking, free radical scavengers) and secondary or preventive (Kozarski *et al.*,2015).Secondary antioxidants are the consequence of deactivation of metals, inhibition or breakdown of lipid hydroperoxides, regeneration of primary antioxidants, singlet oxygen quenching, *etc*. Some mushroom metabolites that exhibit antioxidant activity function as inducers and cell signals, leading to changes in gene expression, which result in the activation of enzymes that eliminate ROS (Chang *et al*., 2007).

The results of the present investigation reveal that the ethanolic extracts of *H. ulmarius* possessed significant capacity to inhibit free radical formation and scavenging activity. Both the extracts showed high free radical scavenging activity in a dose dependent manner. This shows the ability of the extracts to scavenge stable free radicals. Anti-oxidants may mediate their effect by directly reacting with ROS, quenching them or chelating the catalytic metal ions (Sun *et al*., 2002). In the DPPH assay, the ability of antioxidant to scavenge stable purple–colored primary radical DPPH is tested by its depolarization spectrophotometrically at 515 nm. From the result it may be postulated that the ethanolic extracts of *H.ulmarius* reduced the radical to the corresponding hydrazine when it reacts with hydrogen donors (Sanchez Moreno 2002).When the DPPH radical interacts with scavengers, the solution losses its colour depending on the concentration of the extracts (Blois, 1958). In the hydroxyl radical scavenging assay, the extracts exhibited significant hydroxyl radical scavenging activity. Whether the reduction is by scavenging of hydroxyl radicals or by some interference in the generation of the radicals has not been investigated. For the production of hydroxyl radicals Fe^{2+}must be available for H_2O_2. Many of the phytochemicals can chelate the Fe^{2+} resulting in false appearance of hydroxyl radical scavenging. At present we are unaware about chelating activity of the extracts.

$ABTS^+$ assay, another *in vitro* antioxidant screening method, applicable for both lipophilic and hydrophilic antioxidants .The assay is based on the inhibition of radical cation $ABTS^+$ which has a characteristic long wavelength absorption spectrum. The results of the current study shows that extracts significantly eliminated $ABTS^+$ radicals either by scavenging or inhibiting $ABTS^+$ radicals, since both inhibition and scavenging properties of antioxidants towards $ABTS^+$ radicals have been reported

earlier. The extracts of *H.ulmarius* showed a similar pattern of activity in donating electrons as in the case of DPPH antiradical activity. Like $ABTS^{+}$ radical assay, FRAP assay also take advantage of electron transfer reaction. The extract successfully transferred electrons to the Fe (III) $(TPTZ)_2$ complex thereby reduced it in to Fe (II) $(TPTZ)_2$ complex.

The extracts exhibited nitric oxide scavenging and lipid peroxidation inhibiting activity in a dose dependent manner. Nitric oxide generated from sodium nitro prusside prevents the peroxynitrite formation in the cell and NO scavenging activity of extracts prevents nitrosamine mediated carcinogenesis (Lawrence *et al*., 2002). NO involved in different physiological processes like inhibition of platelet aggregation, neuronal signaling etc. In the presence of nitrite ions formed by the oxidation of nitric oxide, sulfanilic acid is quantitatively converted to a diazonium salt under acidic conditions. Then the diazonium salt reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form the red pink azo dye, which can be spectrometrically measured at 540 nm. *H. ulmarius* extracts facilitated nitric oxide scavenging activity in a dose dependent manner. The extract was effective in inhibiting lipid peroxidation. There was a considerable increase in inhibition of lipid peroxidation when the extract concentration increased from 100µg/ml to 1000µg/ml. The above discussion the antioxidant reactions can be categorized into two categories. In one type of reactions, electrons are donated by the extracts thereby eliminating the unpaired electron carrying species. Examples include elimination of DPPH, $ABTS^{+}$ and FRAP radicals. In the second category, the reactive species are being captured by active components present in the extracts. Nitric oxide and hydroxyl ion scavenging are examples.

In conclusion, current investigations reveal that the extracts of fruiting body and mycelia of *H. ulmarius* possessed significant antioxidant activity indicating the therapeutic use of this mushroom.

Chapter 5:

Anti-inflammatory activity of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of Hypsizygus ulmarius

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS

5.2.1. Preparation of the extracts

5.2.2. Animals

5.2.3. Carrageenan induced acute paw edema

5.2.4. Formalin induced chronic paw edema

5.2.5. *In vitro* analysis of Cyclooxygenase enzyme activity

5.3. RESULTS

5.3.1. Anti- inflammatory activity of *H.ulmarius* against Carrageenan induced oedema

5.3.2. Anti- inflammatory activity of *H.ulmarius* against Formalin induced oedema

5.3.3. *In vitro* analysis of COX enzyme.

5.4. DISCUSSION

5.1. INTRODUCTION

When considering the natural products from different sources with potential medicinal properties, one should consider the criterias such as their traditional usage, their abundance in nature and sustainable utilisation. These criteria are best suited for the discovery of natural products from the plant kingdom. However, fungi could also achieve these criteria and most importantly, the sustainability of fungi can be achieved by artificial cultivation techniques (Baker *et al.,*1995).When searching for novel bioactive compounds, another factor is the uniqueness of the organism and its ability to produce secondary metabolites (Donadio *et al*., 2002). Among the large resources of fungi, higher basidiomycetes, especially mushrooms, represent unlimited sources of therapeutically useful biologically active agents (Mizuno, 1995). The medicinal use of mushrooms also has a very long tradition. Fruiting bodies, mycelia or the culture fluid in which the mycelia has been cultivated may all be explored for biological activity. Additionally, both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system and therefore might be useful to treat a variety of diseases including inflammatory disorders (Wasser, 1999).

Inflammation can be considered as normal protective response to a variety of cell and tissue damage and may be referred to as the innate immunity, appear due to microbial infections, physical factors (trauma, radiation, temperature), chemical substances (irritant and corrosive chemicals), as well as tissue necrosis and hypersensitivity reactions. Its role is to remove harmful cells and tissues, as well as repair them. Uncontrolled inflammatory response results in extensive cell and tissue damage, giving rise to normal cell and tissue destruction, which is associated with chronic inflammation and various human chronic diseases (Babatunji *et al*., 2015). Inflammation is a complex process and associated with oxidative stress. Inflammation has been identified in the pathogenesis and development of a great number of unrelated diseases including cancer; Alzheimer's, Parkinson's and cardiovascular diseases as atherosclerosis, rheumatoid arthritis and hepatic diseases (Aroha *et al*., 2015).

Antiinflammatory drugs or immunosuppressive drugs such as 5-amino salicyclic acid and 6- mercaptopurine, steroids and nonsteroidal anti-inflammatory drugs are

effective for temporary relief of symptoms. However drug induced side effects may occur. A number of studies reports that mushroom derived extracts or derivatives such as phenolic compounds and flavanoids show anti-inflammatory activity based on their ability to reduce the production of inflammatory mediators through downregulation of the gene expression of different inflammatory mediators such as interleukins (IL 1β, IL-6, IL-8), tumor necrosis factor (TNF-α) and prostaglandin E2 (PGE2) and COX-2. Increased attention is now being focused on efforts to discover bioactive compounds which have the ability to suppress these inflammatory mediators. In this context, the anti-inflammatory activity of several mushroom such as *Ganoderma lucidum, Pleurotus ostreatus, Agaricus bisporus* and their bioactive metabolites have been employed as potent, natural, and safe anti-inflammatory compounds. The current investigation is to evaluate the anti-inflammatory potential of fruiting bodies and cultured mycelia of elm Oyster mushroom *Hypsizygus ulmarius*.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

5.2.2. Animals

Male Swiss Albino mice weighing (20 ± 2) were used for the experiments.

5.2.3. Formalin induced chronic paw edema.

Swiss albino mice were divided into eight groups of six animals each group and experiment was performed as follows

Group I	Distilled Water
Group II	Diclofenac (10 mg / Kg)
Group III	Fruiting bodies extract (250 mg/ kg)
Group IV	Fruiting bodies extract (500 mg/ kg)
Group V	Fruiting bodies extract (1000 mg/ kg)
Group VI	Mycelia extract (250 mg/ kg)
Group VII	Mycelia extract (500 mg/ kg)
Group VIII	Mycelia extract (1000 mg/ kg)

0.2 mL of freshly prepared 2% formalin was injected to right hind paw of all animals to induce chronic inflammation. Distilled water was administered orally to group I and the standard anti-inflammatory drug diclofenac (10 mg/kg) was given orally to group II. Fruiting body extract (250, 500, and 1000 mg/kg, respectively) was administered orally using oral gavage tube to groups III, IV, and V and the same concentrations of mycelia extract was given orally to groups VI, VII, and VIII. All of these treatments were performed 1 h prior to formalin injection. The standard drug and extract administration were continued once daily for 6 consecutive days. The paw thickness was measured using vernier caliper before and 6 days after formalin injection. The increase in paw thickness and percentage inhibition were calculated as follows. Increase in paw thickness in control (P_C) or treatment (P_T) = $Pt - P_0$, % inhibition = $(P_C - P_T / P_C) \times 100$, where P_t is paw thickness at time t, P_0 is initial paw thickness, P_C is the increase in paw thickness of the control group, and P_T is the increase in paw thickness of the treatment group (Ajith and Janardhanan, 2001)

5.2.4. Carrageenan induced acute paw edema.

Swiss albino mice were divided into eight groups of six animals each and treated in the same way as described in the above experiment.

Group I	Distilled Water
Group II	Diclofenac (10 mg / Kg)
Group III	Fruiting bodies extract (250 mg/ kg)
Group IV	Fruiting bodies extract (500 mg/ kg)
Group V	Fruiting bodies extract (1000 mg/ kg)
Group VI	Mycelia extract (250 mg/ kg)
Group VII	Mycelia extract (500 mg/ kg)
Group VIII	Mycelia extract (1000 mg/ kg)

Acute inflammation was induced in all animals by subplantar injection of 20 µL of a freshly prepared 1% suspension of carrageenan in carboxymethyl cellulose on the right hind paw (Winter *et al*., 1962) The degree of edema formation was determined as the increase in paw thickness. The increase in paw thickness and percentage inhibition were calculated as described in the previous experiment.

5.2.5. *In vitro* analysis of Cyclooxygenase enzyme activity.

RAW 264.7 cells were grown and activated with 1 µL lipopolysaccharides (LPS) 1 µg/ mL. LPS stimulated RAW cells were exposed to different concentrations (25, 50, 100 µg/ mL) of fruiting bodies and mycelia extracts solutions and Diclofenac, a standard anti- inflammatory drug at varying concentrations and incubated for 24 hours. After incubation the anti- inflammatory assays were performed using the cell lysate.

The Cyclooxygenase (COX) activity was assayed by the method of (Walker and Gierse, 2010) 100 µL cell lysate was incubated in 1ml of reaction mixture containing Tris- HCl buffer (P^H 8), Glutathione 5mM / L and haemoglobin 5 mM/ L for 1 minute at 25˚C. The reaction was initiated by the addition of arachidonic acid 200mM/L and terminated after 20 minutes incubation at 37 ˚C by the addition of 200 µL of 10% trichloroacetic acid in 1 N hydrochloric acid. After the centrifugal separation and the addition of 200µl of thiobarbiturate and the tubes were boiled for 20 minutes. After cooling the tubes were centrifuged for three minutes and COX activity was determined by reading absorbance at 632 nm. The percentage inhibition of the enzyme was calculated as

$$\% \text{ inhibition} = \frac{\text{Absorbance of control- Absorbance of test}}{\text{Absorbance of control}} \times 100$$

5.3. RESULTS

5.3.1. Anti- inflammatory activity of *H. ulmarius* against Formalin induced edema

The fruiting bodies and mycelia extracts of *H. ulmarius* significantly inhibited the chronic inflammation induced by formalin. The fruiting bodies and mycelia extracts at concentrations of 1000, 500, 250 mg/kg given orally, reduced the paw thickness 42.7, 38.3, 27.7% and, 55.2, 27.6, 22.3% respectively as compared to that of control (Table 5.1)

5.3.2. Anti- inflammatory activity of *H. ulmarius* against Carrageenan induced edema

Acute inflammation induced by carrageenan at same concentrations of fruiting bodies and mycelia extract reduced the edema by 52, 41.4, 15%, and 63.2, 41.1, 19.1% respectively. Mycelia extract showed higher activity than fruiting bodies extract (Table 5.2). Diclofenac at a dose of 10mg/Kg concentration showed 52% inhibition as compared to control.

5.3.3. *In vitro* analysis of COX enzyme.

In vitro Cyclooxygenase enzyme analyses were performed using RAW 264.7 cells and both fruiting body and mycelia extracts exhibited dose dependent activity. At concentrations 25, 50, and 100 µg/ mL, fruiting bodies and mycelia extract showed 40.35, 50.62, 60.89% and 35.12, 47.87, 55.07 % inhibition respectively. Also standard anti- inflammatory drug Diclofenac at same concentrations exhibited 72.02, 80.19 and 84% inhibition. The results suggest the significant anti-inflammatory potential of the mushroom.

Figure.5.1. *In vitro* COX enzyme activity of fruiting bodies (FB) and mycelia (MY) extracts of *H.ulmarius*

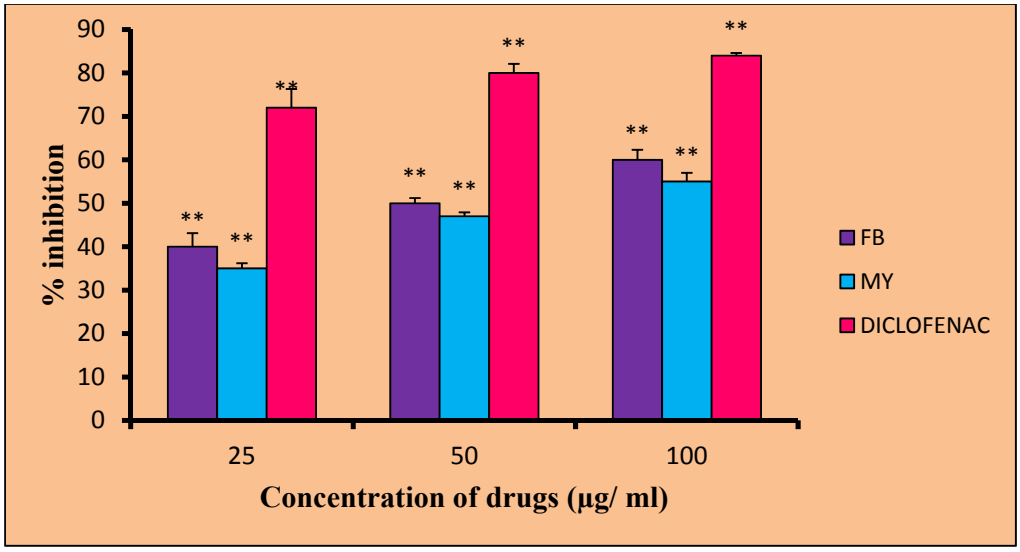


Table.5.1. Anti-inflammatory activity of aqueous-ethnolic extracts of fruiting body and mycelia of *H. ulmarius*- Formalin induced chronic paw edema.

Groups	Initial paw thickness(mm)	Final paw thickness(mm)	Increase in paw thickness(mm)	% inhibition
Control	0.2025±0.009	0.382±0.053	0.18±0.045	
Diclofenac (10mg/Kg)	0.23±0.0067	0.316±0.0057	0.083±0.0057**	53.8
Fruiting body extract (1000mg/Kg)	0.226±0.005	0.32±0.001	0.10±0.041**	42.7
Fruiting body extract (500mg/Kg)	0.232±0.005	0.34±0.015	0.11±0.014**	38.8
Fruiting body extract (250mg/Kg)	0.24±0.00	0.37±0.00	0.13±0.00*	27.7
Mycelia extract (1000mg/Kg)	0.25±0.00	0.33±0.014	0.08±0.014**	55.5
Mycelia extract (500mg/Kg)	0.24±0.014	0.37±0.041	0.13±0.028*	27.7
Mycelia extract (250mg/Kg)	0.23±0.00	0.37±0.00	0.14±0.00*	22.2

All values are expressed as Mean±SD, n=6, **p<0.01;*p<0.05 compared to control considered as significant.

Table.5.2. Anti-inflammatory activity of aqueous-ethnolic extract of fruiting body and mycelia of *H. ulmarius* - Carrageenann induced acute paw edema

Groups	Initial paw thickness(m)	Final paw thickness(mm)	Increase in paw thickness(mm)	% inhibition
Control	0.273±0.011	0.403±0.011	0.136±0.0115	
Diclofenac (10mg/Kg)	0.265 ±0.023	0.335± 0.017	0.065 ±0.019**	52.2
Fruiting body extract (1000mg/Kg)	0.232 ±0.01	0.297± 0.02	0.065± 0.019**	52.0
Fruiting body extract (500mg/Kg)	0.247± 0.009	0.337± 0.023	0.085± 0.01**	41.4
Fruiting body extract (250mg/Kg)	0.235± 0.012	0.35± 0.011	0.115± 0.017*	15.1
Mycelia extract (1000mg/Kg)	0.243±0.011	0.293±0.037	0.05±0.026**	63.2
Mycelia extract of (500mg/Kg)	0.252 ±0.026	0.332± 0.020	0.08± 0.008**	41.1
Mycelia extract (250mg/Kg)	0.245 ±0.021	0.355± 0.049	0.11± 0.028*	19.1

All values are expressed as Mean±SD, n=6, **p<0.01;*p<0.05 compared to control considered as significant

5.4. DISCUSSION

It is well known that only a small fraction of the fungal biodiversity worldwide has been investigated for bioactive compounds and their pharmacological properties. Moreover, some unique medicinal mushroom species are rare and restricted to certain small areas of the world. Therefore, identifying their ecological niches, conservation and sustainability of these precious natural resources is essential (Mortimer *et al.* 2012). *Hypsizygus ulmarius* is one among them and most of its therapeutic properties are unkown to us.

The anti-inflammatory effect of the fruiting bodies and mycelia extracts of *H.ulmarius* showed a dose dependent activity. Carrageenan induced acute inflammation in animals is used to screen anti-inflammatory agents and is one of the most suitable procedure. Carrageenan is a mixture of polysaccharides composed of sulfated galactose units and is derived from Irish sea moss, *Chondrous crispus*. The carrageenan induced edema is mediated by activation of platelet activating factor (PAF), prostaglandins, protease and other inflammatory mediators like interleukin, NO, TNF-α (Hwang *et al*., 1986). The first phase is attributed to the release of histamine, 5-HT and kinins. The second phase is related to the release of prostaglandins. Carrageenan also induces a protein rich exudate containing large number of neutrophills. Here both the extracts showed significant reduction in carrageenan induced acute edema and it was in a dose dependent manner which reveals the effect of extracts against inflammation. Formalin induced paw edema model is suitable to screen chronic anti-inflammatory agents as it closely resembled human arthritis (Greenwald, 1991). The nociceptive effect of formalin is also biphasic, an early neurogenic component followed by tissue mediated response. The result suggests the usefulness of this mushroom extracts for inflammation- associated diseases like Rheumatoid arthritis.

Inflammatory mediators according to their biochemical properties are classified as vasoactive amines, vasoactive peptides, lipid mediators, cytokines, chemokines and proteolytic enzymes (Kumar *et al*., 2003). Lipid mediators (eicosanoids and platelet-activating factors) are derived from phospholipids, such as phosphatidylcholine. After activation they generate arachidonic acid and lysophosphatidic acid, the precursors of the two classes of lipid mediator listed above, from phosphatidylcholine. Arachidonic

acid is metabolized to form eicosanoids either by cyclooxygenases (COX1 and COX2), which generate prostaglandins and thromboxanes, The prostaglandins PGE2 and PGI2, in turn, cause vasodilation, and PGE2 is also hyperalgesic and a potent inducer of fever (Terano *et al*., 1984). Prostaglandins play a key role in the generation of the inflammatory response. Their biosynthesis is significantly increased in inflamed tissue and they contribute to the development of the cardinal signs of acute inflammation. During an inflammatory response, both the level and the profile of prostaglandin production changes dramatically. Prostaglandin production is generally very low in uninflamed tissues, but increases immediately in acute inflammation prior to the recruitment of leukocytes and the infiltration of immune cells. This is very much correlating with the decreased COX enzyme inhibition activity in the control group of formalin and carragenan induced groups. Whereas there was an increase in the inhibition of COX enzyme activity in extracts treated groups in a dose dependent manner.

The anti-inflammatory potential of natural agents is studied extensively using an uncommon method by analysing COX enzymes but it has been reported in some publications (Noreen *et al*., 1998 and Zhang *et al*., 2003). COX activity is usually determined based on the conversion of arachidonic acid to PGE2 and is expressed as a percentage of the control**.** *In vitro* analysis of COX activity, both fruiting bodies and mycelia extracts of *H.ulmarius* significantly inhibited the cyclooxygenase enzyme activity which reflects the anti-inflammatory potential of this mushroom.

Current investigations reveal that the fruiting bodies and mycelia of *H. ulmarius* possessed significant anti-inflammatory activity. Since *H. ulmarius* is an excellent edible mushroom, the medicinal properties of this mushroom have important practical application. Because, consumption of this mushroom might be useful to prevent several diseases which are mediated through free radicals as evident from its significant antioxidant properties. Anti-inflammatory activity of this mushroom also will be advantageous, as long term consumption of this mushroom will be able to provide relief to patients suffering from inflammation and arthritis.

Chapter 6:

Cytotoxic and antitumor activities of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of Hypsizygus ulmarius

6.1. INTRODUCTION

6.2. MATERIALS AND METHODS

6.2.1. Preparation of the extracts

6.2.2. Cell lines

6.2.3. Animals

6.3. Antitumor activity

6.3.1. Antitumor activity of the extracts- Preventive model

6.3.2. Antitumor activity of the extracts- Curative model

6.4. *In vitro* Cytotoxicity against DLA cell lines

6.4.1. Haematological studies

6.4.1.1. Total Leucocyte Count

6.4.1.2. Differential count

6.5. Isolation of polysaccharides

6.5.1. Estimation of total carbohydrate and protein

6.5.2. Antioxidant activity of polysaccharides

6.5.3. Antitumor activity of polysaccharides - Preventive model

6.6. **RESULTS**

6.6.1. Antitumor activity of the extracts

6.6.2. *In vitro* Cytotoxicity against DLA cell lines

6.6.3. Haematological studies

6.6.4. Total carbohydrate and protein content in polysaccharides

6.6.5. Antioxidant activity of polysaccharides

6.6.5.1. $ABTS^{+}$ radical scavenging activity

6.6.5.2. Ferric Reducing Antioxidant Power (FRAP) Assay

6.6.6. Antitumor activity of polysaccharides - Preventive model

6.7. DISCUSSION

6.1. INTRODUCTION

Cancer is a leading cause of death worldwide. It is a group of diseases characterized by the unregulated proliferation of abnormal cells that invade and disrupt surrounding tissues (Cesare *et al*., 2007). Several factors of our modern life style for example excess alcohol consumption, tobacco chewing and smoking habits, exposure to toxic chemicals and radiations increase the risk of cancer. Tumor formation is a multistage process involving a series of events which include the accumulation of genetic and epigenetic alterations leading to the progressive transformation of a normal cell into a malignant one. Despite the development of new therapies, cancer is still the second leading cause of death in world (Rosa *et al*., 2012). In modern medicine chemotherapy, radiotherapy and surgery are the major therapeutic options of cancer treatment. Administration of chemopreventive agents in the early stage of carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic drugs. These agents have a narrow margin of safety and the therapy may fail due to drug resistance and dose limiting toxicities, which may severely affect the host normal cells. Hence the use of natural products has been contemplated in the control of cancer and its eradication programme (Nitha *et al*., 2006).

The current anti-cancer drugs available in market are not target specific and have been demonstrated to possess several side-effects and complications as compared with natural anticancer materials, which highlight the urgent need for novel effective and less-toxic agents from natural products (Fu-Qiang Song *et al*., 2013). Over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and microorganisms (Cragg and Newman, 2005). Compared with other microorganisms, fungi play an important role in cancer treatment. Other than micro fungi, the macrofungi especially mushroom are well known for the production of anti-tumor drugs. Mushrooms are known to complement chemotherapy and radiation therapy by lowering the side-effects of cancer. As such, medicinal mushrooms and their synthetic derivatives are expected to play an important role in developing innovative agents for prevention of human cancer. Earlier, the anticancer activity of ethanol, methanol, ethyl acetate, and hot water extracts of fruiting bodies

from different edible mushrooms such as *Ganoderma lucidum, Pleurotus ostreatus* etc. were evaluated (Ivanova *et al*., 2014).

Mushrooms have been treasured as remedies for disease and as natural health supports for thousands of years and are an incredibly popular food in most countries (Sabiha, 2014). Mushrooms have been not only used as food materials with their unique flavour and texture, but also recognized as an important source of biologically active compound of medicinal value. Both cultivated and wild edible mushrooms have been a matter of research as therapeutic agents, and several bioactive substances have been identified showing health-promoting benefits, such as phenolic compounds, polypetides, terpenes, steroids and lectins. These compounds of mushrooms have been found to have anti-cancer, antimicrobial, antiinflamatory, antidiabetic, antihypercholestrolemic, immunomodulator and antioxidant properties. Mushroom polysaccharides are the most extensively investigated among all the bioactive substances of mushrooms (Jeong *et al*., 2009). In this chapter investigations on the antitumor activity and cytotoxic activity of fruiting bodies and cultured mycelia of edible mushroom *H. ulmarius* are presented. Mushroom polysaccharides are known to possess antitumor activity. Besides isolation of polysaccharides from the fruiting bodies and mycelia of *H.ulmarius* and their antitumor activity are also presented in this chapter.

6.2. MATERIALS AND METHODS

6.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

6.2.2. Cell lines

Dalton's lymphoma ascite's (DLA) cell line was obtained from Cancer Institute, Adayar Chennai. The cells were maintained in mice by intraperitonial inoculation.

6.2.3. Animals

Male Swiss Albino mice weighing (20 ± 2) were used for the experiments.

6.3. Antitumor activity

6.3.1. Antitumor activity of the extracts- Preventive model

Swiss albino mice were divided in to 8 groups of 6 animals in each group.

Group I	Distilled Water
Group II	Cyclophosphamide (40 mg / Kg)
Group III	Fruiting bodies extract (250 mg/ kg)
Group IV	Fruiting bodies extract (500 mg/ kg)
Group V	Fruiting bodies extract (1000 mg/ kg)
Group VI	Mycelia extract (250 mg/ kg)
Group VII	Mycelia extract (500 mg/ kg)
Group VIII	Mycelia extract (1000 mg/ kg)

Viable DLA cells (1×10^6) in 0.1 ml PBS were transplanted subcutaneously in to the right groin of mice to induce tumor formation. Group I injected with DLA cells alone and group II treated with cyclophosphamide (40mg/Kg) orally were maintained as control and standard drug treatment respectively. Aqueous ethanol extract of fruiting bodies of *H. ulmarius* (250, 500 and 1000mg/Kg) was administered to group III, IV, V, and the same concentrations of mycelia extract to group VI, VII and VIII respectively, orally 24 hour after tumor implantation and the treatment continued once daily for 10 consecutive days. The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier caliper twice a week for 5 weeks. The tumor volume was calculated using the formula $V=4/3\ \pi\ r_1^2\ r_2$, where r_1 and r_2 are the radii of the tumors. At the end of the fifth week, animals were sacrificed and the tumors were extracted and weighed. The percent inhibition was calculated by the formula: (1-B/A) 100, where, A is average tumor weight of the control group and B is that of the treated group (Chihara *et al.,* 1970)

6.3.2. Antitumor activity of the extracts- Curative model

Curative antitumor activity of the ethanolic extracts was tested after tumor initiation in mice. Solid tumor was induced by DLA cell line in mice as described previously. After 15days, animals with tumor size around $1.1\pm 1\ cm^3$ were divided in to 8 groups

of 6 animals in each group and treated in the same way as mentioned above. Tumor diameter was measured using vernier caliper twice a week for a period of 5weeks and volume was calculated (Ajith and Janardhanan, 2003). At the end of fifth week, animals were sacrificed and the tumors were extracted and weighed. The percent inhibition was calculated as described earlier.

6.4. *In vitro* Cytotoxicity against DLA cell lines

Cell viability was determined by trypan blue dye exclusion method as described by Rao *et al*., 2002.

Principle: Trypan blue is not permeable in live cells due to the presence of intact plasma membrane. When the cells are dead they will take up the dye and appear as blue color. The method is an index of the dead cells in a cell population.

Procedure: The short term *in vitro* cytotoxicity of aqueous ethanolic extracts of *H. ulmarius* was studied using Dalton's ascites lymphoma (DLA) cells. Approximately 1 x 10^6 cells were suspended in 0.1 ml of PBS (0.2M, p^H 7.4) and various concentrations of extracts (10µg/ml-100µg/ml) were added to make a final volume of 1ml and tubes were then incubated for 3 hours at 37°C. After incubation viability of the cells was determined using trypan blue. The number of dead (stained) and live (unstained) cells were counted using haemocytometer.

The percentage of cytotoxicity was calculated using the formula;

$$\% \text{ cytotoxicity} = \frac{\text{No. of dead cells in test- No. of dead cells in control}}{\text{Total No. of cells in test}} \times 100$$

6.4.1. Haematological studies

6.4.1.1. Total Leucocyte Count

Total WBC count was estimated using the method described in the section 3.2.17

6.4.1.2. Differential count

The differential count was conducted using blood sample aspirated from the heart of animals (Jelalu Kemal., 2014).

A drop of blood was placed at the end of the slide and a second glass slide (spreader slide) was placed against the surface of the first slide, holding at an angle of 30-45 degrees. The spreader slide was drawn gently into the drop of blood and pushed the spreader slide forward with a steady even motion. Dry by waving rapidly in the air. The air dried blood film was flooded with Leishman's stain and kept for 5 to 7 minutes. Washed with distilled water until the film showed a pinkish tinge. Wipe the back of the slide and allow drying in upright position. Then slide was observed under low power first and then under oil immersion to count and identify the cells.

6.5. Isolation of polysaccharides

Dried fruiting bodies and cultured mycelium were extracted using hot water at 95-100˚C for 48 hours. The extract was collected and filtered through Whatmann No 1 filter paper and then concentrated. This hot water extract was used for polysaccharide isolation. Isolation of polysaccharides was carried out by the method of (Mizuno *et al*., 1992) with some modifications. Both fruiting bodies and mycelia hot water extracts were precipitated by the addition of 4X volume of chilled ethanol and kept at 4˚C for 48 hours. It was then centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded and pellet was again treated with ethanol. This procedure was repeated twice and then kept at 4˚C for 48 hours and again centrifuged at 10,000 rpm for 20 minutes. The pellet collected was dissolved in distilled water and treated with Sevag's reagent (Chloroform: n- butanol, 4:1) (Staub1965) several times to remove protein and then dialyzed against deionized water for 48 hours. The extracts were concentrated and finally lyophilized. The lyophilized extracts were used for the antitumor experiment.

6.5.1. Total carbohydrate and protein

Carbohydrate was estimated according to the method of (Yem and Wills 1994). In this method the anthrone reagent was prepared by dissolving 0.2g anthrone in 100 ml of chilled con. sulphuric acid .To all the test tubes 4ml of anthrone reagent was added. Then without disturbing the reagent in the test tubes, 0.5 ml of test solutions having unknown concentrations of carbohydrate was added. All the solution was made up to1 ml using distilled water. The blank containing 1 ml distilled water was taken as reference. Then contents of the tubes was mixed well and heated in boiling water bath for 8 minutes. It was then immediately cooled and optical density was measured at

630 nm. Protein content was estimated using Bradford's method as described in the section 3.2.4

6.5.2. Antitumor activity of polysaccharides - Preventive model

Antitumor activity of polysaccharides (Preventive model) were performed according to the procedure mentioned in the section 6.3.1

6.5.3. Antioxidant activity of polysaccharides

Antioxidant activity of the isolated polysaccharides were determined using $ABTS^{+}$ radical scavenging activity as described in the section 4.2.2.5 and FRAP assay in the section 4.2.2.6

6.6. RESULTS

6.6.1. Antitumor activity of the extracts

The ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* significantly reduced the DLA–induced solid tumor. The extracts showed preventive antitumour effect in a dose dependent manner (Table 6.1).The mycelia extract showed higher antitumour effect than fruiting body extract. Anti-tumor effect of the extracts when administered after the tumor development also reduced the tumor growth significantly in a dose dependent way (Table 6.2). The effect of the standard anticancer drug, Cyclophosphamide used in the experiment prevented the tumor growth by 97%, when given orally at concentration 40mg/Kg in mice, while fruiting body and mycelia extract showed 66.7 and 78.9% tumor growth inhibition at a dose of 1000mg/Kg, respectively.

6.6.2. *In vitro* Cytotoxicity against DLA cell lines

The aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* showed significant cytotoxic activity against DLA cancer cell line. The IC_{50} of fruiting bodies and mycelia extracts were found to be 30µg/ml and 35µg/ml respectively (Fig.6.3).

6.6.3. Haematological studies

Aqueous ethanolic extracts of *H. ulmarius* were found to maintain normal level of WBC. The total leucocyte counts were significantly higher in the DLA control mice. In standard reference, the TC was decreased significantly when compared with control. In higher dose group, it attained normal level, while other groups there were slight increase in TC level compared to control.

The differential count indicates that the percentage of neutrophiles was increased while the lymphocyte count was decreased in the extract treated mice when compared with DLA control mice. Basophils, eosinophils and monocytes showed no much variation. This showed the effect of extracts on immune system.

6.6.4. Total carbohydrate and protein content in polysaccharides

The carbohydrate content was estimated by Anthrone method and it was found to be 42.79% and 35.885% in polysaccharides isolated from fruiting bodies and mycelial of *Hypsizygus ulmarius* respectively. Also protein content in polysaccharides isolated from fruiting bodies and mycelia were 21% and 23% respectively.

6.6.5. Antitumor activity of polysaccharides - Preventive model

Polysaccharides isolated from fruiting bodies and mycelia of *H. ulmarius* showed remarkable antitumor activity against solid tumor induced by DLA cell line. The polysaccharides of fruiting bodies and mycelia were administered 24 hours after tumor implantation. At concentrations 25, 50 and 100 mg/kg body weight, fruiting bodies extract prevented 37.9, 41.44 and 44.8% tumor volume after 5 weeks compared to control whereas at same concentrations mycelia extract reduced 12.4, 24.4 and 36 % of tumor volume (Fig 6.4). The standard reference drug cyclophosphamide decreased 85 % tumor volume as compared to control. Tumor volume in the treated groups (25, 50 and 100 mg/kg body weight) at the end of fifth week decreased significantly in a dose dependent manner.

6.6.6. Antioxidant activity of polysaccharides

6.6.6.1. $ABTS^+$ radical scavenging activity

Polysaccharides of *H. ulmarius* efficiently scavenged $ABTS^+$ radicals generated by the reaction of 2, 2'-azinobis- (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and

ammonium persulphate. At 150µg, 250µg and 350 µg/ml concentrations polysaccharides of fruiting bodies and mycelia of *H.ulmarius* exhibited 26, 43 and 58 % and 41, 59 and 79 % activity respectively (Fig.6.5)

6.6.6.2. Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay is based on the ability of an antioxidant to reduce $Fe^{3+}$to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593nm. In the study the polysaccharides isolated from fruiting bodies and mycelia of *H. ulmarius* showed significant Fe^{3+} reducing power which indicated the hydrogen donating ability of the polysaccharides. Antioxidant activity expressed as concentration (µM) Fe^{3+} reduced. At 50µg, 125µg and 250µg/ml concentrations polysaccharides of fruiting bodies and cultured mycelia showed 9.18, 21.3 and 47.4 and 10.47, 24.99 and 55.6 µ moles of Fe^{3+} reduction.

Table 6.1.Antitumor activity of aqueous- ethnolic extract of fruiting bodies and mycelia of *H. ulmarius* (preventive effect)

Groups	Tumor Volume$(cm)^3$	Tumor weight(gm)	% Inhibition
Control	1.375± .0500	4.7± 0.129	_
Cyclophosphamide (20mg/Kg)	0.04± 0.008*	0.175± 0.01*	88.0
Fruiting body extract (1000mg/Kg)	0.455±0.0057*	1.15± 0.1*	66.7
Fruiting body extract (500mg/Kg)	0.7625±0.0125*	2.27± 0.09*	44.3
Fruiting body extract (250mg/Kg)	1.07± 0.095*	3.18± 0.17*	21.0
Mycelia extract (1000mg/Kg)	0.285± 0.001*	0.95± 0.1*	78.9
Mycelia extract (500mg/Kg)	0.55± 0.012*	1.85± 0.109*	59.8
Mycelia extract (250mg/Kg)	1.025± 0.020*	3.75± 0.125*	25.5

All values are expressed as Mean±SD, n=6,*$p<0.05$ compared to control considered as significant.

Table.6.2. Antitumor effect of aqueous- ethanolic extract of fruiting bodies and mycelia of *H. ulmarius* (curative effect)

Groups	Tumor Volume(cm)^3	Tumor weight(gm)	% Inhibition
Control	2.2 ±0.170	6.35±0 .34	_
Cyclophosphamide (20mg/Kg)	0.045± 0.012*	0.612 ±0.04*	85.9
Fruiting body extract (1000mg/Kg)	0.467± 0.01*	1.725 ±0.22*	78.7
Fruiting body extract (500mg/Kg)	0.705 ±0.01*	2.4± 0.18*	67.9
Fruiting body extract (250mg/Kg)	1.45± 0.20*	4.8± 0.38*	34.0
Mycelia extract (1000mg/Kg)	0.315 ±0.01*	1.17± 0.17*	85.6
Mycelia extract (500mg/Kg)	1.08± 0.08*	3.17 ±0.12 *	50.9
Mycelia extract (250mg/Kg)	1.6 ±0.40*	5.35± 0.20*	27.2

All values are expressed as Mean±SD, n=6, *p<0.05 compared to control considered as significant

Figure.6.1.The effect of fruiting bodies (FB) and mycelia (MY) extracts of *Hypsizygus ulmarius* on tumor volume (Preventive model).

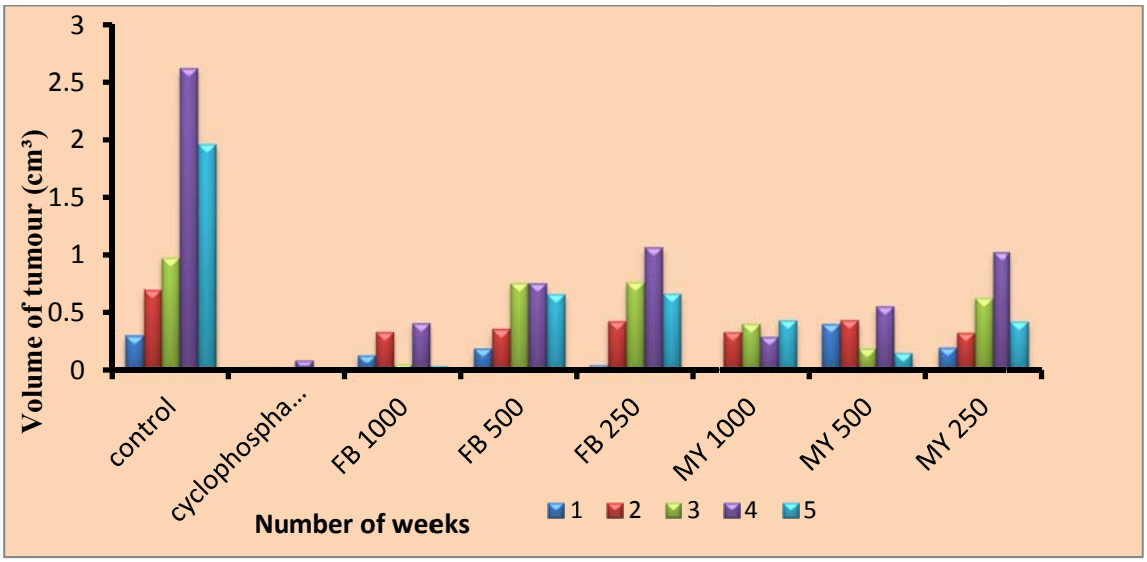


Figure.6.2.The effect of fruiting bodies (FB) and mycelia (MY) extracts of *Hypsizygus ulmarius* on tumor volume (Curative model).

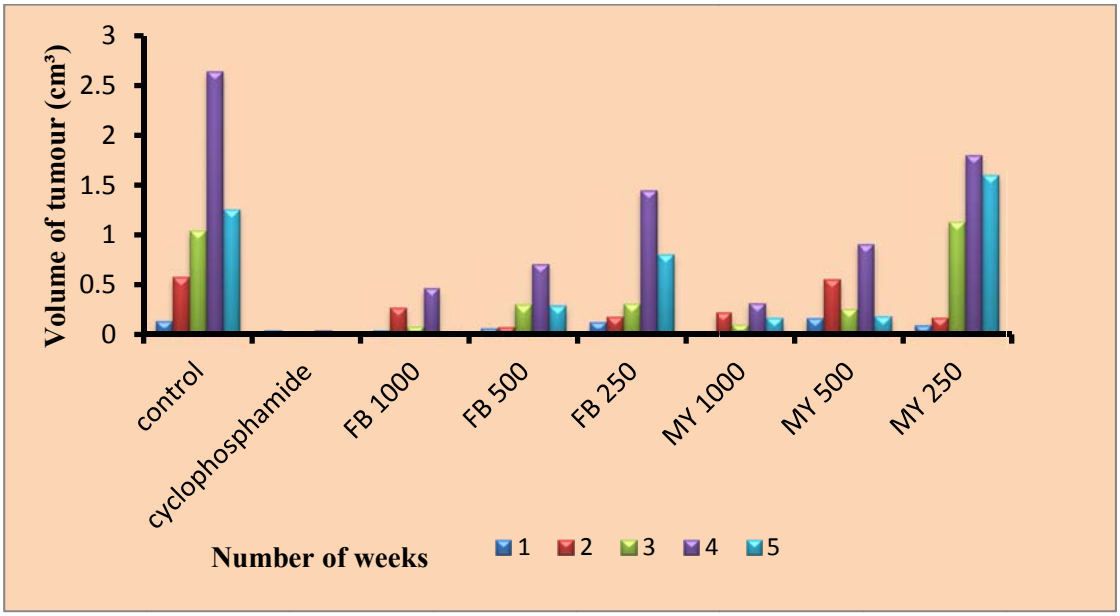


Figure.6.3. *In vitro* cytotoxicity of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* against DLA cell line.

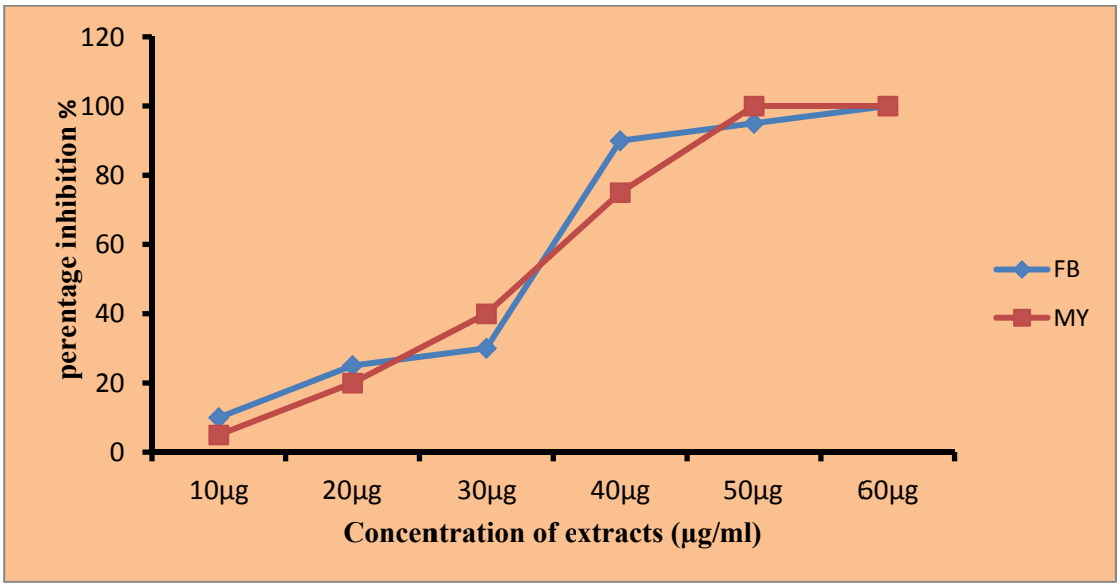


Figure.6.4. The effect of Polysaccharides isolated from fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on tumor volume

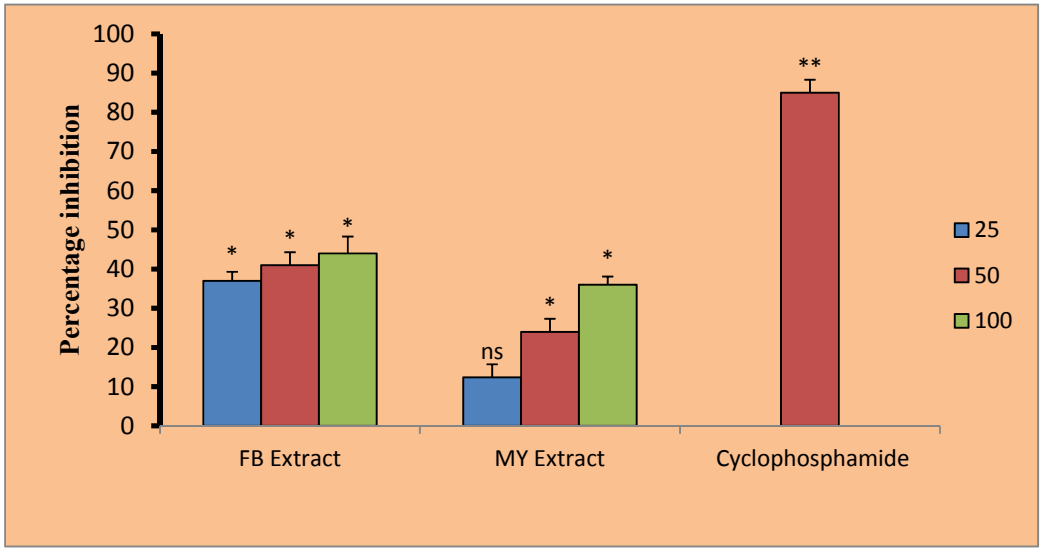
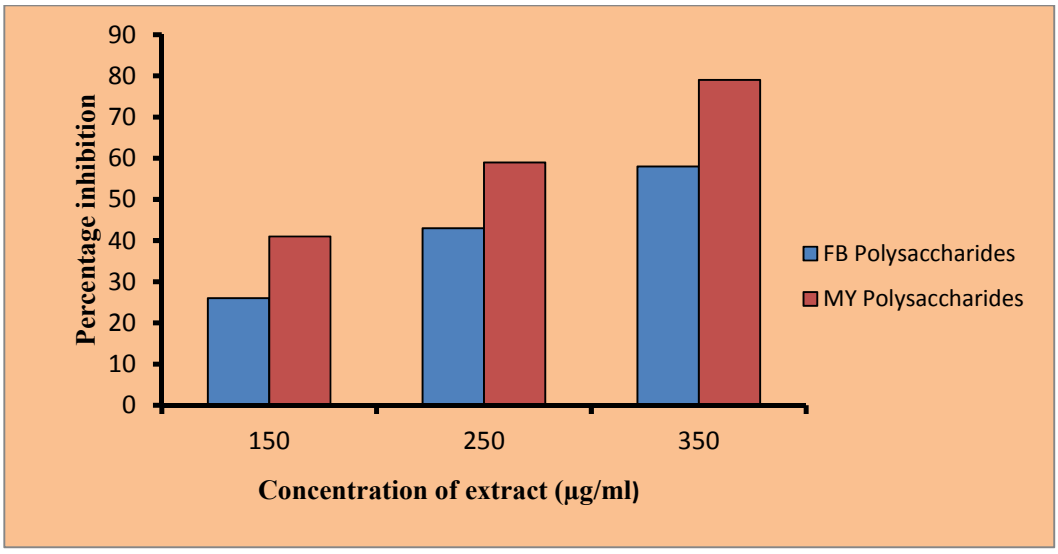


Figure.6.5. ABTS^{+} radical scavenging activity of polysaccharides isolated from fruiting bodies (FB) and mycelia (MY) of *H.ulmarius*



6.7. DISCUSSION

Cancer is a major public health problem which can affect any part of human body and is a major leading cause of death. In India, according to National Cancer Registry Program data (ICMR), the total cancer cases are likely to go up from 979,786 cases in the year 2010 to 1,148,757 cases in the year 2020. The cause of cancer can widely classified under the category include physical, chemical, biological, hormone related and due to diet and habits.

An approach to prevent cancer by the administration of natural products, especially compounds derived from mushrooms is considered as advantageous since they act through multiple cell-signaling pathways and reduce the chance of developing resistance of cancer cells (Reddy *et al*., 2003). In current experimental studies a significant reduction in tumor volume in drug treated group as compared to control group suggests the preventive effect of *Hypsizygus ulmarius* against tumor cells. The most studied potential of medicinal mushroom extracts is their use in the treatment and prevention of cancer. Approximately 200 species of mushrooms have been reported to exhibit antitumor activity. Several bioactive substances with antitumor activity have been isolated from these mushrooms. These include mainly polysaccharides; in particular β-glucans, polysaccharide-peptide complex (PSP), polysaccharide proteins, and proteins (Bao *et al*., 2001).

Carcinogenesis has three stages: initiation, promotion, and progression. It has been described that the modulation of the human immune system, attributed to mushrooms, particularly to various mushroom polysaccharides, was likely to affect primarily the promotion and progression stages, according to the referred model of carcinogenesis. Nonetheless, other substances contained in mushrooms were described as possibly being able to interfere with the referred tumor initiation process, through a variety of mechanisms such as enhancing the antioxidant capacity of cells or upregulating phase I and II enzymes involved in the metabolic transformation and detoxification of mutagenic compounds. Additionally, other mushroom constituents have been described as being able to inhibit what was considered to be the promotion or progression stages of carcinogenesis, by exerting direct cytotoxicity against tumor

cells, interfering with tumor angiogenesis, or upregulating other nonimmune tumor-suppressive mechanisms (Borchers *et al*., 2004).

Recent studies have demonstrated the extracts of *Pleurotus sajor-caju, Pleurotus florida* possessed profound antitumor activity (Nayana Jose *et al*., 2000). The results of present investigation also reveal that the aqueous ethanolic extracts of *Hypsizygus ulmarius* exihibited remarkable capacity to inhibit the tumor growth in a dose dependent manner. However, the antitumor activity of the extract at high dose is almost comparable to the recommended dose of an established anticancer drug, cyclophosphamide. The extract also possesses profound *in vitro* cytotoxic activity against DLA cell line. So, higher cytotoxicity of the extract explains its significant antitumor activity against induced solid tumor. Many previous studies have reported antitumor activity of medicinal mushrooms was due to the presence of polysaccharides, alkaloids, polysaccharide-protein complexes, dietary fiber, certain types of proteins, terpenoids, steroids, phenols, etc (Ivanova *et al*., 2014). Among these Polysaccharides are the best known and most potent bioactive compound with antitumor and immunomodulating properties in mushrooms (Wasser, 2002**).**

Clinical studies have demonstrated that mushrooms exhibit cancer-preventive and anticancer activity, which might be due to its antioxidative and radical-scavenging effects, inhibition of metabolic activation and facilitating the detoxification of carcinogens, exerting cytotoxicity against tumor cells, induction of cell-cycle arrest, apoptosis, and enhancement of host immune function (Ying *et al.,* 1987; Yang *et al.,* 1987). Use of mushroom extracts in combination with traditional chemotherapy is considered as alternative sources for adjuvant cancer therapy, as they have no adverse effects and they activate the cells of the immune system (Pauliuc *et al*., 2013). Hematological and immunological alterations are common in patients with malignant neoplasms. Previous scientific evidence indicates that dietary supplementation with medicinal fungi is capable of significantly improving the physiological condition and prognosis of patients with cancer because of their effects on red blood cells and the immune system (Soumya *et al*., 2011). The hematological studies of the extracts of *H.ulmarius* were found to maintain normal level of total leucocyte count and differential leucocyte count more or less to normal level. This clearly indicates that aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H. ulmarius* have profound immunomodulating effect**.**

Oxidative stress is a major contributor to increased cancer risk, excess accumulation of free radicals in cells disrupts the innate protective systems leading to different physiological disorders. More and more evidence suggest that cancer-inducing oxidative damage might be prevented or delimited by anti-oxidants (Dreher and Junod, 1996). The mushrooms thus have both preventive and curative anti-tumour effects**.** Recent studies have reported anti-cancer potential of exopolysaccharides isolated from *H. ulmarius* (Latha and Baskar, 2014). Hence this mushroom may represent a practical and promising approach in anti-cancer therapy based on current experimental data from *in vitro* and *in vivo* studies. The results of the current studies thus suggest the potential therapeutic use of *H. ulmarius* as an adjuvant in the treatment of cancer**.**

Chapter 7:

Hepatoprotective activity of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of Hypsizygus ulmarius

7.1. INTRODUCTION

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7.2.2. Animals

7.2.3. Determination of hepatoprotective activity

7.2.3.1. Determination of hepatoprotective activity against carbon tetrachloride induced acute toxicity.

7.2.3.2. Determination of hepatoprotective activity against ethanol induced chronic toxicity.

7.2.3.3. Biochemical analysis- Assay for liver marker enzymes

7.2.3.4. Evaluation of lipid peroxidation in liver

7.2.3.5. Determination of antioxidant status in liver

7.2.3.6. Histopathological examination

7.3. RESULTS

7.3.1 Effect of *H.ulmarius* extracts on the activities of SGOT, SGPT and ALP in carbon tetrachloride and ethanol induced toxicity.

7.3.2. Antioxidant status in liver

7.3.3. Lipid peroxidation in liver

7.3.4. Histopathological observations.

7.4. DISCUSSION

7.1. INTRODUCTION

Liver diseases such as jaundice, cirrhosis, hepatitis and liver cancer are the biggest threat to the world which is characterized by impaired metabolic and secretary function of liver (Goyal *et al*, 2012).Chemicals that cause liver injury are called hepatotoxins. In the liver these chemicals are converted to reactive metabolites which then interact with nucleic acids, lipids and proteins leading to DNA damage, protein dysfunction and lipid peroxidation results in cell death and liver failure. Some drugs when administered in overdoses and sometimes even at therapeutic range may also injure liver.

Liver plays a major role in regulation of physiological processes, metabolism, synthesis, secretion and storage of vitamins. It has the capacity to detoxify toxic substances such as a variety of drugs, xenobiotics, environmental pollutants. It helps to regulate homeostasis of the body. It is involved in almost all biochemical pathways and metabolism of carbohydrates, protein and fat (Ahsan *et al*, 2009). The role played by this organ in the removal of substances from the portal circulation makes it susceptible to persistent attack by offending foreign compounds resulting in liver dysfunction. Excessive consumption of alcohol, viral infections, parasitic infections, environmental pollutants and chemotherapeutic agents are the most common factors responsible for liver damage. Chemical agents those used in industries such as lead, arsenic, natural chemicals like microcystins, aflatoxins, some antibiotics and laboratory agents (eg: carbon tetrachloride, paracetamol) can induce hepatotoxicity (Ostapowicz *et al*, 2002).

Carbon tetrachloride is widely used in scientific research and is one of the most potent hepatotoxins. Exposure to high concentrations of carbon tetrachloride can affect the central nervous system, degenerate the liver and kidneys and may result in coma and even death. It is bio transformed to two free radical by the Cytochrome P_{450} system. The first metabolite, a trichloromethyl free radical that forms covalent adducts with lipids and proteins. It can interact with oxygen to form a second metabolite, a trichloromethylperoxy free radical. These sequence of events leads to peroxidation of lipids in membranes of liver and results in liver damage (EISisi *et al,* 1993).

Another most important common cause of liver injury is alcohol consumption. During metabolism of ethanol, enhanced lipid peroxidation result in development of hepatitis,

which ultimately leads to cirrhosis. Heavy alcohol drinkers are at high risk in developing the alcoholic liver diseases (ALD_s) (Zakhari, 2007). In the liver, alcohol is metabolized by alcohol dehydrogenase and by xenobiotic metabolism through cytochrome P-450 pathways. Alcohol consumption is known to cause changes in membrane fluidity due to increased lipid peroxidation eventually resulting in loss of membrane structure and cellular integrity (Thonda *et al*., 2012).

The efficacy of treatments of liver diseases by modern medicines is limited and associated with serious side effects. Hence, natural products have gained acceptability as effective hepatoprotective agents. Mushrooms are largely unexplored natural source for bioactive compounds for drug discovery although many of them have been used as home remedy to treat various ailments such as rheumatoid arthritis, diabetes, cardiovascular diseases, liver diseases and cancer (Aruoma, 1998). No significant investigation has been done on the hepatoprotective activity of fruiting bodies and cultured mycelia of *H. ulmarius*. Hence, the present study was undertaken to evaluate the hepatoprotective effect of cultured mycelia and fruiting bodies of the mushroom, *H. ulmarius* against carbon tetrachloride and ethanol induced hepatotoxicity.

7.2. MATERIALS AND METHODS

7.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

7.2.2. Animals

Male Wistar rats weighing (200 ± 20) were used for the experiments.

7.2.3. Determination of hepatoprotective activity

7.2.3.1. Determination of hepatoprotective activity against carbon tetrachloride induced acute toxicity.

Wistar rats were divided in to nine groups of six animals each

Group I	Distilled water
Group II	CCl_4 in paraffin oil (1:5, v/v, 3.75 mL/kg body weight, i.p)
Group III	Silymarin (100 mg/kg) + CCl_4
Group IV	Fruiting bodies extract (250 mg/ kg) + CCl_4
Group V	Fruiting bodies extract (500 mg/ kg) + CCl_4
Group VI	Fruiting bodies extract (1000 mg/ kg) + CCl_4
Group VII	Mycelia extract (250 mg/ kg) + CCl_4
Group VIII	Mycelia extract (500 mg/ kg) + CCl_4
Group IX	Mycelia extract (1000 mg/ kg) + CCl_4

Group 1 was given saline alone and kept as normal group. Group II was administered with CCl_4 in paraffin oil (1:5, v/v, 3.75 mL/kg body weight, i.p). Group III was treated with standard reference drug Silymarin (100 mg/kg p.o.). Aqueous ethanolic extract of fruiting bodies of *H. ulmarius* (250, 500, and 1000 mg/kg, respectively) was administered to groups IV, V and VI and the same concentrations of mycelia extract to groups VII, VIII and IX orally. Group III, IV, V, VI VII, VIII and IX were administered CCl_4 as in the case of group II. 72 hours after the CCl_4 injection animals were sacrificed. Blood was collected and serum was separated for the determination of liver function enzymes. Liver of each animal was removed and then stored at -40˚C (Ajith *et al*., 2006)

7.2.3.2. Determination of hepatoprotective activity against ethanol induced chronic toxicity.

Wistar rats were divided in to nine groups of six animals each

Group I	Distilled water
Group II	Ethanol (36%, 6ml/animal p.o)
Group III	Silymarin (100 mg/kg) + Ethanol
Group IV	Fruiting bodies extract (250 mg/ kg) + Ethanol
Group V	Fruiting bodies extract (500 mg/ kg) + Ethanol
Group VI	Fruiting bodies extract (1000 mg/ kg) + Ethanol
Group VII	Mycelia extract (250 mg/ kg) + Ethanol
Group VIII	Mycelia extract (500 mg/ kg) + Ethanol
Group IX	Mycelia extract (1000 mg/ kg) + Ethanol

Group 1 was given saline alone and kept as normal group. Group II was administered with ethanol (36% v/v, 6ml/animal p.o) for 30 days. Group III was treated with standard reference drug Silymarin (100 mg/kg p.o.) Aqueous ethanolic extract of fruiting bodies of *H. ulmarius* (250, 500, and 1000 mg/kg) was administered to groups IV, V and VI and the same concentrations of mycelia extract to groups VII, VIII and IX orally. Group III, IV, V, VI, VII, VIII and IX were administered with ethanol as in case of group II for 30 days. Twenty four hours after the last dose of treatments animals were sacrificed. Blood was collected from heart. Serum separated for the determination of liver function enzymes. Liver of each animal was removed and then stored at -40˚C (Nitha *et al.,* 2013)

7.2.3.3. Biochemical analysis – Assay for liver marker enzymes.

Serum was separated and the activities of SGOT (section 3.2.10) SGPT (section 3.2.11) ALP (section 3.2.12) were estimated using Agappae diagnostic kits, India

7.2.3.4. Evaluation of lipid peroxidation in liver

Lipid peroxidation was estimated as described in section 3.2.9

7.2.3.5. Determination of antioxidant status in liver

Liver was excised and rinsed thoroughly in ice cold saline to remove blood. 10% of the homogenate was prepared in 0.05M phosphate buffer (pH 7) using a polytron homoginiser at 4˚C. A part of this homogenate was used for the determination of reduced glutathione (3.2.7). Rest of the homogenate was centrifuged at 10,000 rpm for 20 minutes. The supernatant was used for the estimation of superoxide dismutase (3.2.6), catalase (3.2.5) and glutathione-S- transferase (3.2.8). The protein content was estimated by the method of Bradford (3.2.4).

7.2.3.6. Histopathological examination

A small portion of the liver was taken from each sample and placed in a bottle containing 10% formalin and embedded in paraffin, cut into 4–5µM thick sections and stained with hematoxylin-eosin. Sections were observed for the hepatocellular necrosis, fibrosis and other toxic manifestations.

7.3. RESULTS

7.3.1. Effect of *H. ulmarius* extracts on the activities of SGOT, SGPT and ALP in carbon tetrachloride and ethanol induced toxicity.

CCl_4 administration elevated the level of liver marker enzymes SGOT, SGPT, ALP to 153.12 ± 18.9, 61.65 ± 1.55, 59.18 ± 2.4 IU/L respectively. Treatment with aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* at doses of 1000, 500 and 250 mg/kg exhibited a significant reduction of SGOT, SGPT, ALP levels to 127.4 ± 5.99, 148.23 ± 4.9, 151.41 ± 14.49 IU/L and 40.60 ± 3.01, 51.16 ± 1.13, 58.19 ± 17.43 IU/L and 36.14 ± 26.444, 50.26 ± 13.85, 57.33 ± 1.37 IU/L respectively. Administration of silymarin also brought down the elevated level of marker enzymes to 111.79 ± 2.29, 35.366 ± 6.68, and 33.45 ± 8.89 IU/L.

Ethanol administration elevated the level of SGOT, SGPT, ALP to 145.02± 3.62, 56.533 ± 2.85, 234.58±4.51 IU/L respectively. Aqueous ethanolic extract of fruiting bodies of *H. ulmarius* at doses of 1000, 500 and 250 mg/kg showed a significant decrease in SGOT, SGPT, ALP levels to 94.34 ± 4.34, 107.035 ±7.77, 111.078 ± 8.644 IU/L and 41.122 ± 3.78, 41.29 ± 1.13, 47.51 ± 3.16 IU/L and 182.903 ± 3.344, 191.905 ± 1.10, 234.86 ± 3.08 IU/L respectively. At same concentrations of mycelia extract showed a reduction of SGOT, SGPT, ALP levels to 95.74 ± 3.90, 105.49 ± 4.35, 134.11 ± 3.43 IU/L and 38.02± 2.01, 42.19 ± 1.03, 55.43 ± 1.34 and 180.53 ± 3.06, 192.90 ± 3.39, 220.18 ± 9.5 IU/L respectively. Silymarin also reduced the elevated level of SGOT, SGPT and ALP levels to 111.79 ± 2.29, 35.366 ± 6.68, and 133.45 ± 8.89 IU/L.

7.3.2. Antioxidant status in liver

The activity of Catalase was observed to be decreased by CCl_4 intoxication to 38.25 ± 3.0 U/mg compared to normal 110.03 ± 9.80 U/mg. The treatment with Silymarin elevated the enzyme level to 96.44 ± 2.04 U/mg. However the administration of fruiting bodies and mycelia extracts at concentrations 1000, 500 and 250 mg/kg enhanced the activity of catalase to 94.058 ±7.41, 89.098 ± 8.390 and 84.335 ± 3.64 and 93.87 ± 17.62 U/mg, 77.60 ± 9.49 U/mg and 56.513 ± 3.0 U/mg respectively. Catalase activity was observed to be decreased by ethanol intoxication to 40.63 ± 3.25 U/mg compared to normal 69.23 ± 4.03 U/mg. Silymarin treatment elevated the

enzyme level to 65.285 ± 3.11 U/mg. Fruiting bodies and mycelia extracts of *H.ulmarius* at concentrations 1000, 500 and 250 mg/kg elevated the activity of catalase to 64.546 ±7.41, 58.76 ± 1.99 and 46.14 ± 2.25 and 66.09 ± 7.46 U/mg, 56.925 ± 2.15 U/mg and 49.066 ± 3.221 U/mg respectively.

The SOD level in the normal animals was found to be 14.09 ± 0.75 U/mg. CCl_4 intoxication depleted SOD activity to 3.08 ± 1.10 U/mg. The SOD level was raised to 13.08 ± 2.29 U/mg on administration with Silymarin. The reduced level of SOD after the CCl_4 treatment was restored by treatment with fruiting bodies and mycelia extracts at 1000, 500 and 250 mg/kg concentrations to 11.277 ± 0.41U/mg, 10.186 ± 0.31 U/mg, 7.302 ± 0.60 U/mg and 11.12 ± 0.76 U/mg, 8.23 ± 1.02 U/mg, 3.07 ± 0.91 U/mg respectively. Ethanol administration depleted SOD activity to 3.79 ± 0.347 U/mg and the normal value was found to be 14.996 ± 1.01 U/mg. The SOD level was elevated to 12.265 ± 0.496 U/mg on administration with Silymarin. The reduced level of SOD after the ethanol treatment was restored by treatment with fruiting bodies and mycelia extracts at 1000, 500 and 250 mg/kg concentrations to 11.277 ± 0.41U/mg, 10.186 ± 0.31 U/mg, 7.302 ± 0.60 U/mg and 10.77 ± 0.499 U/mg, 7.908 ± 0.337 U/mg, 6.7 ± 0.34 U/mg respectively.

GST level was also reduced in the CCl_4 treated group of animals to 493.23 ± 13.17 U/mg in comparison with the normal 1334.56 ± 189.46 U/mg. The administration of Silymarin elevated the GST level to 1259.65 ± 102.56 U/mg. The treatment with the various concentrations of fruiting bodies and mycelia extracts (1000, 500 and 250 mg/kg) increased the GST level in a dose dependent manner to 1213.53 ± 7.27 U/mg, 912.101 ± 7.311 U/mg and 548.19 ± 4.38 U/mg and 1231.80 ± 213.89 U/mg, 822.55 ± 143.04 U/mg and 645.70 ± 123.30 U/mg respectively. In the case of ethanol treated group GST level decreased to 468.241 ± 26.20 U/mg in comparison with the normal 918.89 ± 36.933 U/mg. Standard drug Silymarin elevated the GST level to 700.30 ± 6.662 U/mg. Various concentrations of fruiting bodies and mycelia extracts (1000, 500 and 250 mg/kg) also increased the GST level in a dose dependent manner to 837.87 ± 32.63 U/mg, 560.97 ± 18.02 U/mg and 442.30 ± 30.02 U/mg and 802.84 ± 20.63 U/mg, 771.92 ± 3.7 U/mg and 454.93 ± 8.39 U/mg respectively.

GSH level was decreased in CCl_4 intoxicated group to 3.75 ± 0.31 nmol/mg when compared with normal 11.24 ± 0.69 nmol/mg. The treatment with the extracts of

fruiting bodies and mycelia at different concentrations 1000, 500 and 250 mg/kg elevated the GSH level in a dose dependent manner. The increase in GSH levels was 8.45 ± 4.033, 6.88 ± 0.694 and 3.61 ± 0.475 and 8.49 ± 0.33, 6.05 ± 0.33 and 3.58 ± 0.68 nmol/mg respectively. GSH level was significantly decreased in ethanol control group to 2.085 ± 0.371 nmol/mg when compared with normal 8.56 ± 0.408 nmol/mg. The extracts of fruiting bodies and mycelia at different concentrations 1000, 500 and 250 mg/kg elevated the GSH level in a dose dependent manner. The increase in levels was 6.36 ± 0.330, 4.788 ± 0.134 and 2.165 ± 0.106 and 7.3 ± 0.142, 4.72 ± 0.074 and 2.3 ± 0.114 nmol/mg respectively.

7.3.3. Lipid peroxidation in liver

A marked increase in the level of MDA was found in CCl_4 intoxicated group (0.57 ± 0.06 n mol/mg) as compared to normal group which was found to be 0.27 ± 0.10 nmol/mg. Administration of Silymarin reduced the MDA level to 0.28 ± 0.04 nmol/mg. Treatment with fruiting body extract at different concentrations 1000, 500 and 250 mg/kg reduced MDA level to 0.234 ± 0.047, 0.315 ± 0.026 and .4321 ± 0.014 nmol/mg. Whereas mycelia extract at same concentrations decreased MDA level to 0.30 ±0.06 nmol/mg, 0.36 ± 0.021 nmol/mg and 0.43 ± 0.05 nmol/mg.

MDA level was increased significantly in ethanol administered group (0.86 ± 0.013 nmol/mg) as compared to normal group (0.319 ± 0.002 nmol/mg). Silymarin reduced the MDA level to 0.451 ± 0.006 nmol/mg. Treatment with fruiting body extract at different concentrations 1000, 500 and 250 mg/kg reduced MDA level to 0.305 ± 0.024, 0.319 ± 0.004 and 0.402 ± 0.014 nmol/mg. Whereas mycelia extract at same concentrations decreased MDA level to 0.410± 0.06 nmol/mg, 0.552 ± 0.0214 nmol/mg and 0.727 ± 0.05 nmol/mg.

7.3.4. Histopathological observations.

Histopathological observation of the hepatic tissue of CCl_4 challenged group showed severe areas of necrosis and plenty of inflammatory cells. About 80% of the hepatocytes showed cytoplasm vacuolation. The necrosis and vacuolation in hepatocytes were significantly reduced by Silymarin and extracts treatment. Histopathological data of the ethanol intoxicated animals showed liver toxicity including severe centrilobular necrosis, fatty infiltration and lymphocytes infiltration.

The toxic manifestations were significantly decreased in the extracts and Silymarin treated groups.

Figure.7.1. Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the levels of Liver marker enzymes SGOT, SGPT and ALP in rats exposed to CCl_4.

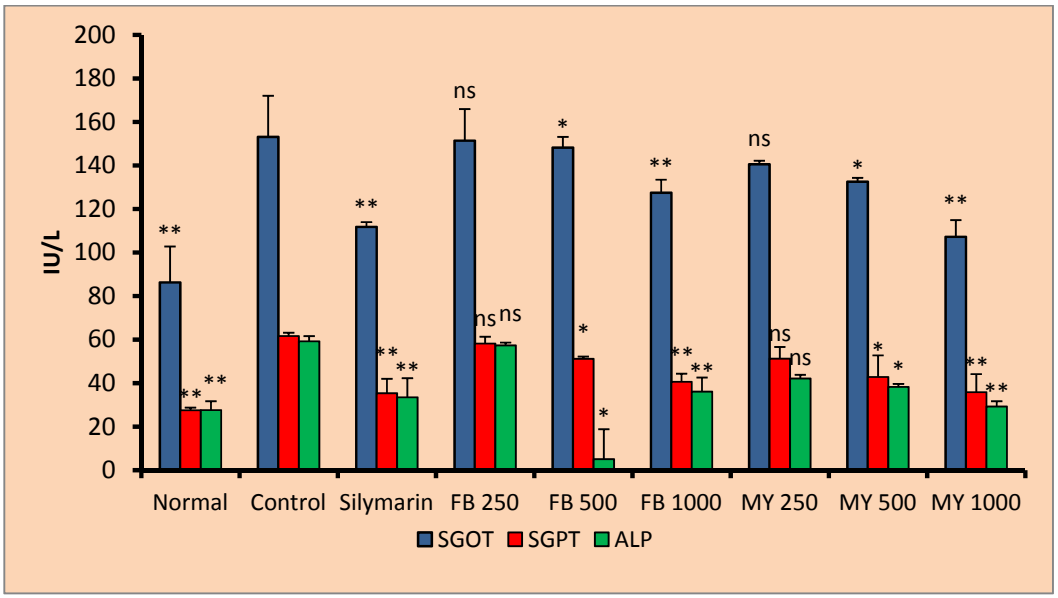


Figure.7.2. Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the levels of Liver marker enzymes SGOT, SGPT and ALP in rats exposed to ethanol.

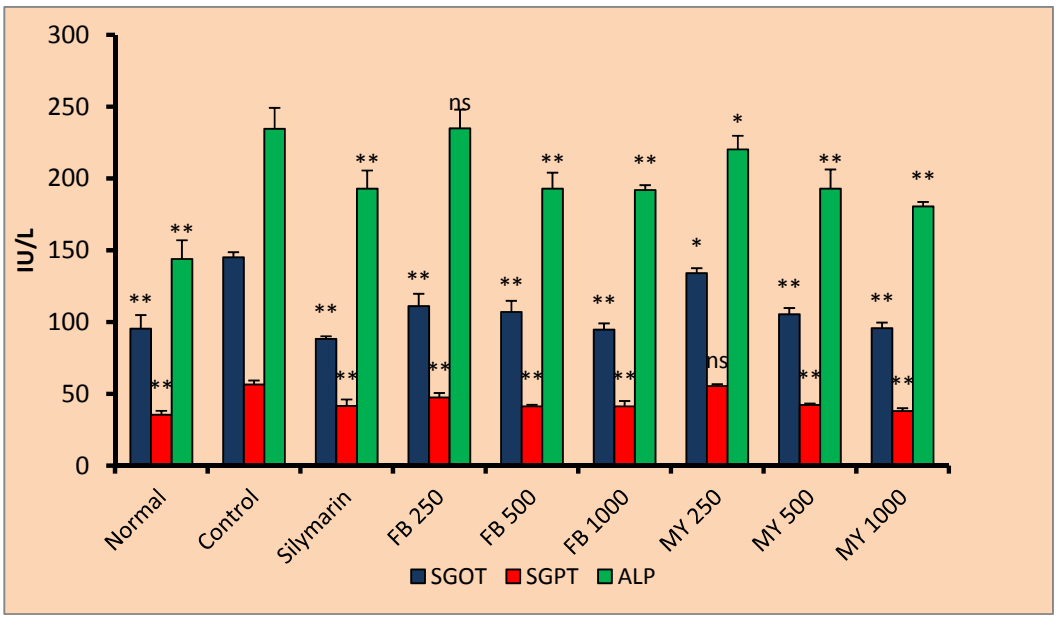


Table .7.1.Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the level of various antioxidant enzymes in rat liver exposed to CCl_4

Groups	CATALASE (U/mg Protein)	SOD(U/mg Protein)	GSH(nmol DTNB/min/mg protein)	GST (n mol CDNB Conjugate formed/min/mg protein)
Normal	110.025 ±9.797**	14.09 ± 0.752**	11.248 ± 0.693**	1334.564 ± 189.46**
Control (CCl_4)	38.252 ± 2.966	3.081 ± 1.107	3.746 ± 0.3103	493.23 ± 13.174
Silymarin (100mg/Kg)	96.443 ± 2.040**	13.0833± 2.295**	9.73 ± 0.647**	1259.64 ± 102.56**
Fruiting body extract(1000mg/Kg)	94.058 ± 7.41**	11.277± 0.4112**	8.45 ± 4.0337**	1213.53 ± 7.274**
Fruiting body extract(500mg/Kg)	89.098 ± 8.390**	10.1866± 0.313**	6.883 ± 0.6940**	912.10 ± 7.311**
Fruiting body extract(250mg/Kg)	84.335 ± 3.647**	7.302 ± 0.609 ns	3.616 ± 0.475 ns	548.191 ± 4.384 ns
Mycelia extract (1000mg/Kg)	93.650 ±17.619**	11.123 ± 0.761**	8.49 ± 0.334**	1231.8 ± 213.89**
Mycelia extract (500mg/Kg)	77.60 ± 9.48**	8.23 ± 1.022 **	6.05 ± 0.327*	822.55 ± 143.04**
Mycelia extract (250mg/Kg)	56.513 ± 3**	3.066 ± 0.913 ns	3.576 ± 0.681 ns	645.70 ± 123.30 ns

All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Table.7.2. Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the level of antioxidant enzymes in rat liver exposed to ethanol

Group	CATALASE(U/mg Protein)	GST(nmol CDNBConjugate formed/min/ mgprotein)	SOD(U/mg Protein)	GSH(nmol DTNB/min/mg protein)
Normal	69.23 ± 4.032**	918.89 ± 36.95**	14.996± 1.01**	8.56 ± 0.408**
Control (Ethanol)	40.63 ± 3.25	468.24± 26.66	3.79 ± 0.347	2.085 ± 0.371
Silymarin (100 mg/Kg)+Ethanol	65.285 ± 3.110**	700.30± 6.66**	12.26± 0.496**	6.61 ± 0.416**
FB(1000mg/Kg)+ Ethanol	64.54 ± 4.10**	837.87 ± 32.63**	11.27± 0.411**	6.36 ± 0.300**
FB(500mg/Kg)+ Ethanol	58.76 ± 1.99**	560.97 ± 18.02 **	10.18± 0.313**	4.788 ± 0.134**
FB(250mg/Kg)+ Ethanol	46.14 ± 2.25 ns	442.30 ± 30.02 ns	7.302 ± 0.66**	2.16 ± 0.106 ns
MY(1000mg/Kg)+ Ethanol	66.09 ± 7.46**	802.84 ± 20.63**	10.77 ± 0.499**	7.3 ± 0.142**
MY(500mg/Kg)+ Ethanol	56.925 ±2.15**	771.92 ± 3.79 **	7.9 ± 0.33**	4.71 ± 0.074**
MY (250mg/Kg)+ Ethanol	49.06 ± 3.22 ns	454.43 ± 8.39 ns	6.7 ± 0.347**	2.3 ± 0.114

All values are expressed as Mean ± SD, n=6, **$p<0.01$ as compared to control considered as significant and ns $p > 0.05$ (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.7.3. Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the level of lipid peroxidation in rats exposed to CCl_4.

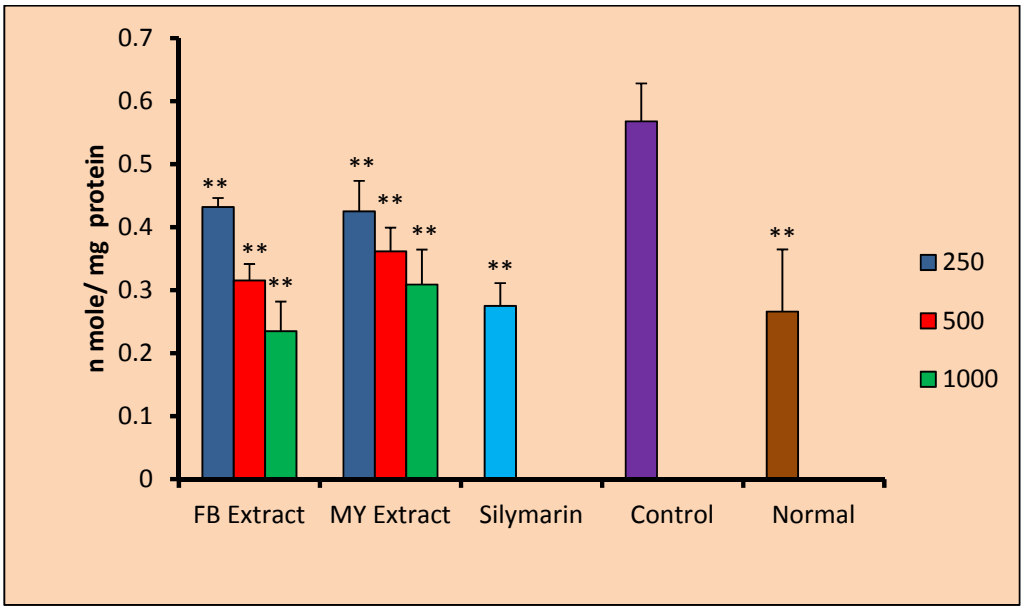
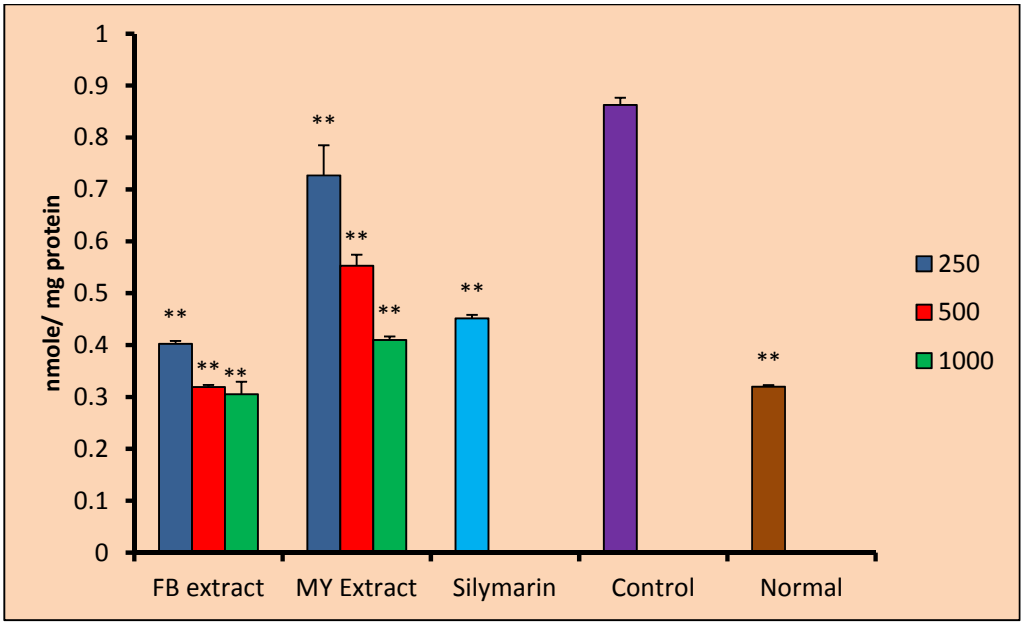


Figure.7.4. Effect of aqueous ethanolic extracts of fruiting bodies (FB) and mycelia (MY) of *H.ulmarius* on the level of lipid peroxidation in rats exposed to ethanol.



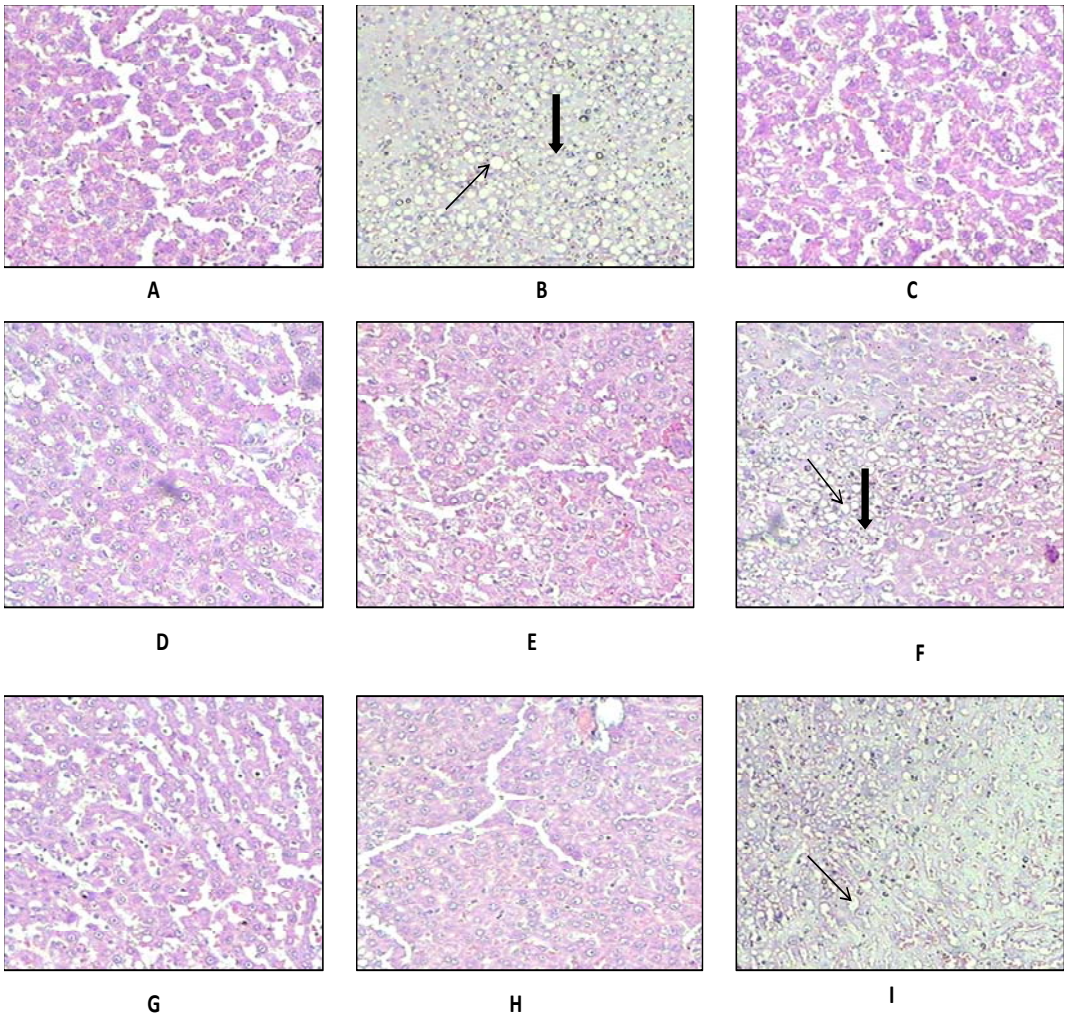


Figure.7.5. Histopathology of liver sections (Staining Hematoxylin- Eosin, 10×).

Black thin arrows: cell necrosis and vacuolation, Black thick arrows : Infiltration of lymphocytes.(A) Normal; (B) Control (CCl_4); (C) Standard (Silymarin,100 mg/kg); (D) Fruiting body extract (1000 mg/kg + CCl_4); (E) 500mg/kg+CCl_4; (F) 250mg/Kg + CCl_4 ; (G) Mycelia extract (1000mg/Kg + CCl_4); (H) 500 mg/kg + CCl_4; (I) 250 mg/kg + CCl_4.

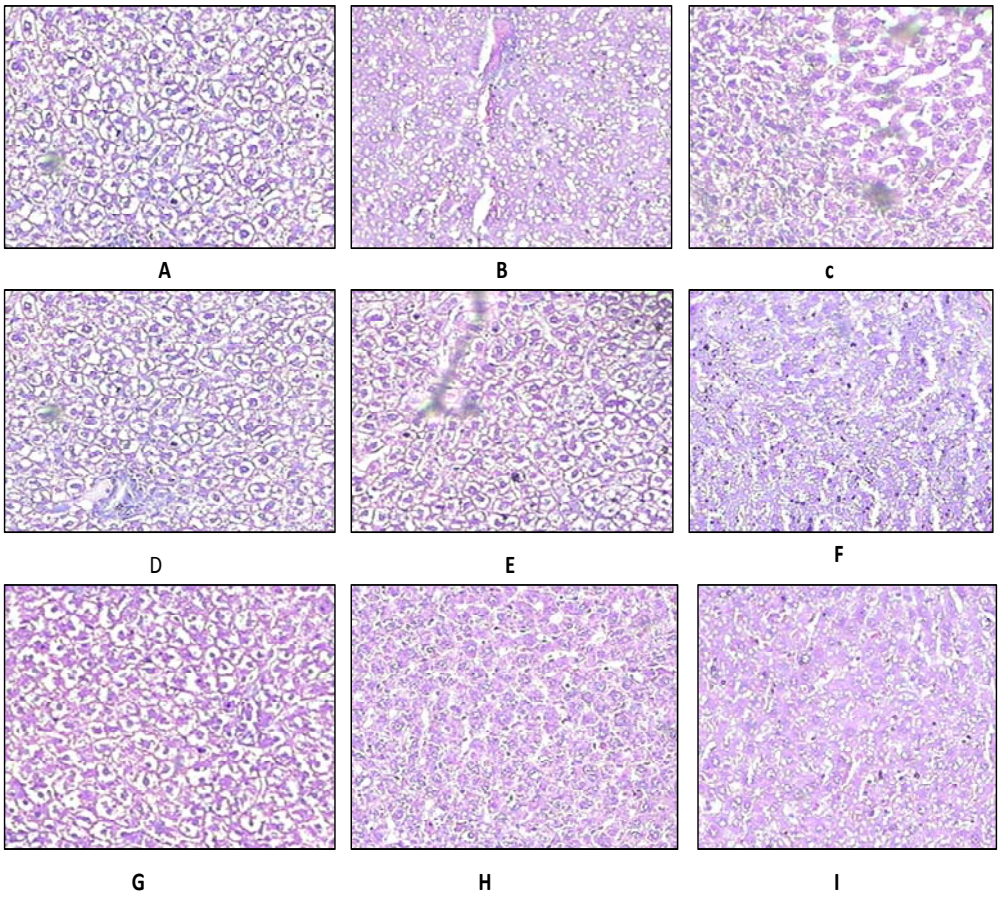


Figure.7.6. Histopathology of liver sections (Staining Hematoxylin- Eosin, 10×).

(A)Normal; (B) Control (Ethanol); (C) Standard (Silymarin,100 mg/kg); (D) Fruiting body extract (1000 mg/kg + Ethanol); (E) 500mg/kg + Ethanol; (F) 250mg/Kg + Ethanol ; (G) Mycelium extract (1000mg/Kg + Ethanol); (H) 500 mg/kg + Ethanol; (I) 250 mg/kg + Ethanol.

7.4. DISCUSSION

The current experimental studies reveal that alcoholic extracts of fruiting bodies and mycelia of *H.ulmarius* have significant hepatoprotective activity against CCl4 and ethanol intoxication. This is evident from evaluation of liver function enzymes and the antioxidant status levels consequent to CCl_4 and ethanol induced hepatotoxicity.

Chemical toxins such as carbon tetrachloride induce acute hepatic injury and a single exposure can rapidly lead to severe centrilobular necrosis, and steatosis. In the initial phase, CCl_4 is metabolically activated by cytochrome P450-dependent oxidases in the endoplasmic reticulum and mitochondria to form highly reactive trichloromethyl and trichloromethyl peroxy free radicals which covalently bind to cellular macromolecules, to induce lipid peroxidation, resulting in the loss of membrane integrity and leakage of microsomal enzymes. Reactive aldehydes such as MDA are released as byproducts of lipid peroxidation that can form protein and DNA adducts leading to hepatotoxicity (Manibusan *et al*.,2007).The second phase involves the activation of Kupffer cells, which is accompanied by the production of pro-inflammatory mediators. Recent reports have demonstrated that induced nitric oxide overproduction occurs in the liver of rats by CCl_4-induced acute liver injury. This suggested that iNOS might act as a mediator in the pathogenesis of hepatotoxicity in rats which up regulates the inflammatory response through specific signalling mechanisms (Bhuyan *et al*., 2018)**.**

Liver diseases are one of the major causes of death in recent years. This was mainly due to the impact of life style changes. Ethanol induced hepatotoxicity is often found to be associated with liver injury. Alcohol dehydrogenase and acetaldehyde dehydrogenase increase the production of acetaldehyde from alcohol which is more toxic than alcohol as it reduces NAD to NADH. The change in NAD/NADH ratio inhibits gluconeogenesis and fatty oxidation, causing liver fatty degeneration. Similarly, acetaldehyde when combined with cellular proteins becomes antigenic enters the cycles of inflammation leading to liver fibrosis and cirrhosis (Wheeler *et al*., 2001; Lu and Cederbaum, 2008). Acetaldehyde is further oxidized to acetate by Xanthine oxidase in liver leading to the production of reactive oxygen species (ROS) through CYP2E1 pathways which result in hepatocyte necrosis, apoptosis and

inflammation (Zhou *et al*., 2003). Findings of histopathological examination of liver samples of ethanol and drug treated animals in the current studies are in agreement with these observations.

Our findings demonstrate that; long term administration of ethanol (36% v/v) in animals causes severe hepatic damage as evident from the significant elevation of aminotransferase enzyme activities which are sensitive markers in the diagnosis of liver diseases. Ethanol administration for 30 days significantly increased all serum marker enzymes, whereas treatments with the aqueous ethanolic extracts of *H.ulmarius* significantly reduced SGOT, SGPT and ALP indicating its hepatoprotective effect (Kew, 2000). Earlier studies suggest that protective effect of the extracts could be the result of stabilization of plasma membrane, preventing the structural integrity of liver cells as well as the repair of hepatic tissue damage caused by ethanol and thus preventing enzymes leakage in to blood circulation.

Glutathione is a major, non-protein thiol in living organisms which performs a key role in co-ordinating innate antioxidant defence mechanisms. It is involved in the maintenance of normal structure and function of cells, probably by its redox and detoxification reactions (Gueeri, 1995). Reduced glutathione (GSH) plays a key role in the detoxification of the reactive toxic metabolites of CCl_4. Liver necrosis is initiated when reserves of GSH are markedly depleted (Ganie et al., 2013; Al Asmari et al., 2015). Thus, the reduced level of GSH was observed in the present investigation in CCl_4 and ethanol alone treated group is consistent with the results of earlier studies. Interestingly, in the present study, group of animals administered with fruiting bodies and mycelia extracts at concentrations 1000, 500 and 250 mg/kg showed significant elevation in GSH level. Earlier studies have reported that when the lipid peroxidation rate is very high, GSH gets depleted because of high rate of scavenging (Nicotera and Orrenius, 1986). Hence the observed low levels of GSH in the CCl_4 and ethanol alone treated group indicates high rate of lipid peroxidation.

Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane polyunsaturated fatty acid (PUFA) (Vaca *et al*., 1988). MDA, a secondary product of lipid peroxidation, is used as an indicator of tissue damage involving a series of chain reactions (Ohkawa *et al*., 1979). It reacts with

thiobarbituric acid, producing red-coloured products. Lipid peroxidation has been implicated in the pathogenesis of increased membrane rigidity, osmotic fragility, reduced erythrocyte survival and perturbations in lipid fluidity. It has been hypothesized that one of the principal causes of CCl4 and ethanol induced hepatotoxicity is lipid peroxidation of hepatocytic membranes (Recknagel *et al*., 1989). The observation of elevated levels of hepatic MDA in Group II rats (administered with CCl4 and ethanol alone) in the present study is consistent with this hypothesis. Thus, the maintenance of near normal levels of hepatic MDA in rats administered with fruiting bodies and mycelia extracts of *Hypsizygus ulmarius* in a dose dependent manner is of great interest since it provides additional evidence to support the hepatoprotective role of fruiting bodies and mycelia extracts of *H.ulmarius.*

Living tissues are endowed with innate antioxidant defence mechanisms, such as the presence of the enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). A reduction in the activities of these enzymes is associated with the accumulation of highly reactive free radicals, leading to deleterious effects such as loss of integrity and function of cell membranes (Reedy and Lokesh, 1992; Krishnakantha and Lokesh, 1993; Sheela and Angusti, 1995). Administration of CCl4 and ethanol leads to generation of peroxy radical, $O2^{-}$ which is associated with inactivation of CAT and SOD enzymes. This explains the significant reduction in the activities of CAT, SOD and GPx observed in rats administered with CCl_4 and ethanol (control). In rats administered with the extracts of *H. ulmarius* the activities of CAT, SOD and GPx enzymes were significantly higher as compared to control. This suggests the hepatoprotective effect of the *H.ulmarius*.

Histopathological studies provide sufficient evidence for the hepatoprotective effect of the extracts of *H. ulmarius* against hepatotoxicity caused by CCl4, and ethanol. In the liver tissue of rats administered with CCl4 and ethanol (Control groups), there was marked disruption in the structure of hepatocytes was noted. There were areas of necrosis, necrotic areas showing plenty of inflammatory cells. Decreases in the disruption of hepatocytes were noted in liver tissues of extracts and Silymarin treated groups. The hepatocytes appeared normal. The areas of necrosis were replaced by inflammatory cells. This minimal disruption of the hepatocyte structure

complemented the levels of the liver function enzymes (SGOT, SGPT and ALP) and the enhanced activities of antioxidants (CAT, SOD, GPx and GSH). The experimental findings indicate that the fruiting bodies and cultured mycelia of *H.ulmarius* possess significant hepatoprotective activity.

Chapter 8:

Hypolipidemic activity of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of Hypsizygus ulmarius

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8.3.3. Effect of *H.ulmarius* on lipid peroxidation in liver and heart

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8.3.6. Status of SGOT and SGPT activity.

8.3.7. Isolation of Lovastatin.

8.3.8. Histopathological observations.

8.4. DISCUSSION

8.1. INTRODUCTION

Hyperlipidemia or hypercholesterolemia is a major risk factor in life style related diseases such as atherosclerosis and related cardiovascular complications including cerebral paralysis and myocardial infarction (Marie and Vladimír, 2001). The modern unhealthy lifestyle, with a high cholesterol diet, lack of physical activities, smoking, diabetes and alcohol abuse significantly contribute to abnormal cholesterol levels (Freedman, 2003). Other illnesses that may elevate cholesterol levels include polycystic ovary syndrome and kidney disease. Hormones like estrogen, drugs like diuretics, beta-blockers and medicines used to treat depression have been found to increase cholesterol levels. Cholesterol levels rise as the person gets older (Lipman *et al*., 2000). Prevention or treatment of cardiovascular disorders can be achieved by targeting the causative hypercholesterolemia through diet and drug administration. (Grundy *et al*, 2004; Larosa *et al*, 1990). Research on natural medicines and their demand in the treatment of various disorders is increasing worldwide. Investigations on natural products might lead to the development of alternative drugs and strategies. Such alternative strategies are required for the effective control of hyperlipidemic disorders, as cost and poor availability of modern therapies make the rural populations particularly in developing countries vulnerable to such ailments.

The consumption of dietary antioxidants might be important in the prevention of cardiovascular diseases as the oxidative modification of LDL (lipids or protein components) play a crucial role in atherogenesis. Increase in lipid synthesis and decrease in its clearance are two reasons behind hypercholesterolemia. In general the reduction in circulating cholesterol is an important factor for preventing the risk of atherosclerosis. Importance of natural products and significance of hypocholesterolemic activity has recently been recognised (Shepherd *et al*, 1995)

Mushrooms are excellent antioxidants and several compounds having antioxidant activity have been isolated from mushrooms. These include polysaccharides, ergosterol, nicotinic acid, triterpenes and phenolic compounds. Oyster mushrooms are ideal dietary supplements for the prevention and treatment of hypercholesterolemia due to high content of dietary fiber, sterol, proteins, and microelements (Hossain *et al*, 2003). Lovastatin and its analogs are inhibitors of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the enzyme that catalyzes the rate-limiting

step of cholesterol biosynthesis leading to decline in biosynthesis of cholesterol. These compounds are early members of statin family, which are secondary metabolites produced by some edible mushrooms like oyster mushrooms including *Pleurotus* species. Meviolin, an inhibitor of HMG CoA reductase have been isolated from *Pleurotus* sp (Gunde - Cimerman, 1999). Statins are the most effective lipid-lowering agents in the market, being commonly used to prevent coronary heart disease. The fact that lovastatin is present in high proportions in oyster mushroom has significant importance for a food supplement for patients suffering from hypercholesterolemia. Lovastatin was found in fruiting bodies and mycelia of edible mushrooms, especially in *Pleurotus* spp. (Alarcon *et al*, 2003).

Edible mushrooms such as *Lentinus edodes, Flammulina velutipes,* and *Pleurotus ostreatus* have been reported to possess hypocholesterolemic property (Kaneda and Tokuda, 1966) and their intake reduces risk of cardiovascular diseases. Studies on the effect of oyster mushroom and its extracts on the lowering of cholesterol levels on laboratory animals have been extensive. The present study is to evaluate the effects of *Hypsizygus ulmarius*, on hypercholesterolemia.

8.2. MATERIALS AND METHODS

8.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

8.2.2. Animals

Male Wistar rats weighing (180 ± 20) were used for the experiments.

8.2.3. Determination of hypolipidemic activity

8.2.3.1. Determination of hypolipidemic activity against Triton WR-1339 induced hyperlipidemia.

Wistar rats were divided in to nine groups of six animals each group

Group I	Distilled water
Group II	Triton WR 1339 (200mg/ Kg. i.p in saline)
Group III	Atorvastatin (2.5 mg/kg)
Group IV	Fruiting bodies extract (250 mg/ kg)
Group V	Fruiting bodies extract (500 mg/ kg)
Group VI	Fruiting bodies extract (1000 mg/ kg)
Group VII	Mycelia extract (250 mg/ kg)
Group VIII	Mycelia extract (500 mg/ kg)
Group IX	Mycelia extract (1000 mg/ kg)

Group I was treated with vehicle (distilled water) served as normal, in all other groups hyperlipidemia was induced by a single intraperitoneal (ip) injection of Triton WR 1339 (Tylaxopol-Sigma Aldrich) (200mg/kg body weight) dissolved in normal saline (pH 7.4). (Okazaki et al., 1990) Group II was kept control, without any treatment. Group III was treated with standard drug atorvastatin (2.5mg/kg body wt.). Group IV, V and VI were treated with fruiting bodies extract and group VII, VIII and IX with mycelia extract of *H. ulmarius* at different concentrations (250mg, 500mg and 1000 mg/kg respectively). The animals were fasted for 12 hours. The extracts and atorvastatin were administered by oral gavage 1 hour before the Triton administration and after 24 hours of treatment, the animals were sacrificed. Blood was collected from the heart and serum was separated and used for the estimation of serum lipid profile. Liver was excised from each animal, washed properly using cold saline till the blood was removed completely and stored at -20^0C until analysis could be completed. The liver samples were collected in order to check the activity of HMG CoA reductase enzyme.

8.2.3.2. Determination of hypolipidemic activity against high cholesterol diet induced hyperlipidemia.

Wistar rats were divided in to nine groups of six animals each group.

Group I	Distilled water
Group II	High Cholesterol Diet (1% cholesterol)
Group III	Atorvastatin (2.5 mg/kg)
Group IV	Fruiting bodies extract (250 mg/ kg)
Group V	Fruiting bodies extract (500 mg/ kg)
Group VI	Fruiting bodies extract (1000 mg/ kg)
Group VII	Mycelia extract (250 mg/ kg)
Group VIII	Mycelia extract (500 mg/ kg)
Group IX	Mycelia extract (1000 mg/ kg)

Group I fed with normal diet and all other groups fed with 1 % cholesterol enriched diet in combination with 0.5 % cholic acid. Group II was given with high cholesterol diet only and kept as control. Group III was treated with atorvastatin (2.5 mg/ Kg). IV, V and VI were treated with fruiting bodies extract of *H.ulmarius* at 250, 500 and 1000 mg/Kg respectively and VII, VIII and IX were treated with mycelia extract at same concentrations. Extracts and atorvastatin were administered once daily for 60 consecutive days. 24 hours after drugs administration animals were sacrificed, serum was separated; liver and heart were excised immediately and stored at -20º C.

8.2.3.3. Estimation of serum lipid profile

The serum samples were used for lipid analysis. Total Cholesterol (Section 3.2.13.), Triglycerides (Section 3.2.14.), and High Density Lipoprotein (Section 3.2.15.) using Agape Diagnostic Kits Pvt., Ltd., India. Very Low Density Lipoprotein was calculated as TG/5 and Low Density Lipoprotein (LDL) Cholesterol was calculated as TC- (HDL+ VLDL). The Atherogenic Index was calculated as follows:

AI = (TC-HDL) / HDL

8.2.3.4. Evaluation of hepatic and cardiac lipid peroxidation

10% homogenate of liver and heart were prepared as described in the section (3.2.3.) and used for the determination of lipid peroxidation (Section 3.2.9.)

8.2.3.5. Determination of antioxidant status in liver and heart

Antioxidants such as Catalase (section 3.2.5), SOD (section 3.2.6), Reduced glutathione (GSH) (section 3.2.7), Glutathione –S- Transferase (GST) (section 3.2.8) were determined.

8.2.3.6. Determination of Hepatic HMG CoA reductase activity.

Hepatic HMG CoA reducatase activity was found out from the HMG CoA/ Mevalonate ratio according to the method of (Rao and Ramakrishnan, 1975)

Principle:

The ratio (absorbance of HMG-CoA/absorbance of mevalonate) is taken as an index of the activity of HMG-CoA reductase which converts HMG CoA to mevalonate in the presence of NADPH. If cholesterol biosynthesis is decreased in a clinical condition, the ratio will increase, and vice versa.

HMG-CoA was determined by reaction with hydroxylamine at pH 5.5 and subsequent colorimetric measurement of the resulting hydroxamic acid by formation of complexes with ferric salts, Because mevalonate interferes in this estimation at acid or neutral pH, alkaline hydroxylamine was used to estimate specifically HMG-CoA only. Possible interference by coenzyme A is also minimal when readings are taken at 540 nm (Durr, and Rudney, 1960). Mevalonate was estimated by reaction with the same reagent, at pH 2.1. At this pH, the lactone form of mevalonate readily reacts with hydroxylamine to form the hydroxamate.

Procedure:

Equal volumes of fresh 10% tissue homogenate and diluted perchloric acid were mixed, kept for 5 min and centrifuged at 2000 rpm for 10 minutes. To 1 ml of supernatant, 0.5 ml of freshly prepared hydroxylamine reagent, pH 2.1 for mevalonate (alkaline hydroxylamine reagent in the case of HMG CoA pH 5.5) was added and mixed. After 5 min, 1.5 ml of ferric chloride was added and shaken well. Readings were taken after 10 min at 540 nm using spectrophotometer against a similarly- treated distilled water blank. The ratio of

HMG Co A to Mevalonate was calculated. Lower ratio indicates higher enzyme activity and vice-versa.

$$\text{HMG CoA reductase activity} = \frac{\text{HMG CoA}}{\text{Mevalonate}}$$

8.2.3.7. Estimation of SGOT and SGPT

Serum Glutamate Oxaloacetate Transaminase (Section 3.2.10) and Serum Glutamate Pyruvate Transaminase (Section 3.2.11) were determined using Agappae Diagnostic Kits, India

8.2.3.8. Histopathological examination of liver.

Portions of the liver tissue were fixed in 10% formalin and used for histological studies (section 3.2.18).

8.2.3.9. Isolation of lovastatin

25g of powdered fruiting bodies and cultured mycelia were weighed and ethyl acetate was added and was kept in cold for 24 hours. The mixture was then stirred vigorously using a magnetic stirrer for 2 hours. The solution was centrifuged at 5000 rpm for 10 minutes. The organic layer was collected and concentrated using a rotary vacuum evaporator. The concentrated extracts were then analyzed by thin layer chromatography. Ethyl acetate extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were used for the detection of lovastatin.

TLC analysis was performed using 20x20 cm Merck silica gel $60F_{254}$ TLC plates. Dichloromethane: Ethyl acetate (70:30) was used as the mobile phase. Lovastatin (sigma Aldrich) was used as standard. Extracts and standard were dissolved in 1ml solvent separately. They were spotted on the TLC plate. The TLC plate was developed in a chamber saturated with the solvent. After the solvent run the plate was removed from the chamber, air dried and placed in a jar containing iodine vapour.

8.3. RESULTS

8.3.1 Effect of *H.ulmarius* on serum lipid level of Triton WR- 1339 induced hyperlipidemic rats.

Serum total cholesterol, triglycerides and LDL cholesterol level in normal group was 80.285±16.4, 95.25±27.09 and 15.1±8.6 respectively. The increase in values of control group with respect to normal were 343.041 ± 11.083, 834.402 ± 226.33 and 147.58 ± 7.89 mg/dL for total cholesterol, triglycerides and LDL cholesterol respectively. The rises in values were accompanied by decline of serum HDL level of 19.84±3.56 mg/dL. The atherogenic index was significantly increased in hypercholestremic control rats (10.82 ± 1.2) as compared to normal group.

The serum total cholesterol had remarkable decline in each groups as compared to control. The decrease in the level of total cholesterol when treated with aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* at doses of 250, 500 and 1000 mg/kg were 239.029± 29.8, 192.47 ± 51.26 and 158.51 ± 42.93 mg/dL and 254.41 ± 21.16, 227.36 ± 26.2 and 185.11 ± 9.83mg/dL respectively. The decrease in the level of triglycerides in the case of fruiting bodies and mycelia extracts at 250, 500 and 1000 mg/kg body wt. were 740.33 ± 127.92, 679.96 ± 177.22 and 420.49 ± 123.46 mg/dL and 832.87 ± 58.17, 595.89 ± 116.17 and 467.96 ± 205.28 mg/dL respectively. The decrease in the level of LDL cholesterol in fruiting bodies and mycelia extracts at 250, 500 and 1000mg/kg body wt. were 33.12 ± 2.3, 25.30 ± 1.1and 23.9 ± 0.12 mg/dL and 77.04 ± 2.3, 59.03 ± 1.23 and 46.1 ± 1.03 mg/dL respectively. The standard reference drug atorvastatin (2.5 mg/kg body wt.) showed 145.44 ± 6.39, 365.37 ± 8.3 and 28.17 ± 0.3 mg/dL decrease in total cholesterol, triglycerides and LDL Cholesterol respectively. (Table 7.1)

The HDL cholesterol level was improved, there was approximately 30.33 ± 8.5, 32.68 ± 12.44 and 41.39 ± 3.59 mg/dl and 28.88 ± 6.7, 31.2 ± 1.4 and 37. 27 ± 5.26 mg/dl increase for 250, 500 and 1000 mg/kg respectively for fruiting bodies and mycelia extracts compared to the control group. The Atherogenic Index was declined after treating with *H.ulmarius* extracts, there were 6.68 ± 0.7, 6.02 ± 0.9 and 3.7 ± 1.1 and 7.6 ± 1.3, 6.56 ± 0.6 and 3.57 ± 0.13 decrease for 250, 500 and 1000 mg/kg groups respectively for fruiting bodies and mycelia extracts compared to control group (Fig .7.1). The Atherogenic Index was declined to 2.34 ± 0.03 in the case of atorvastatin.

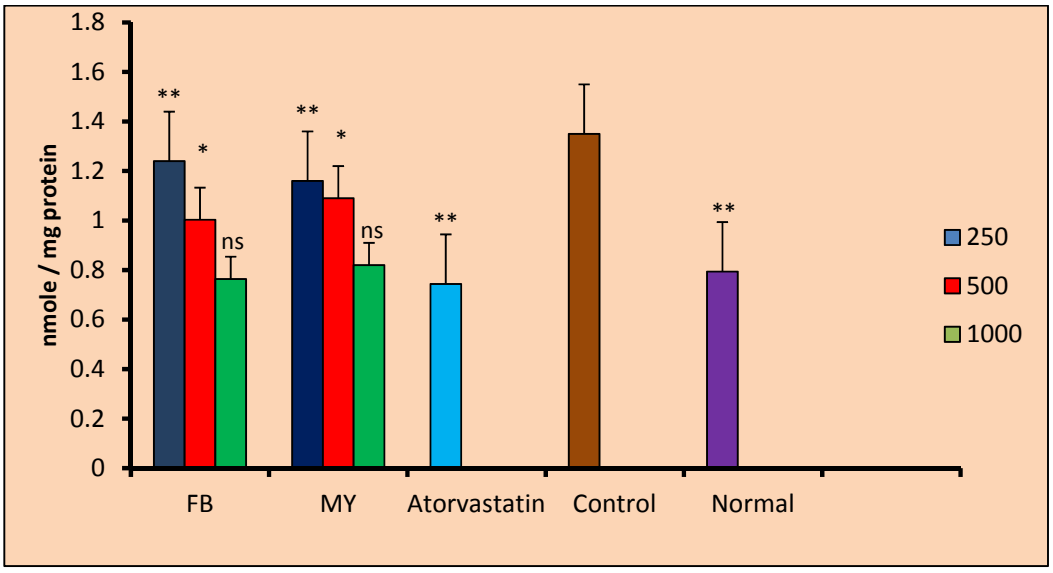
All these data revealed that *H. ulmarius* possessed significant hypocholestremic activity.

Table.8.1. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia extracts (MY) on serum lipid profile of Triton WR 1339 induced hyperlipidemic rats

Groups	Dosage (mg/Kg)	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
Normal	Vehicle	80.285±16.4**	95.25±27.09**	46.87±6.29**	15.1±8.6**
Triton (Control)	200	334.76±11.08	834.40±226.33	19.84±3.58	147.56±3.2
Triton + Atorvastatin	2.5	143.36±6.39**	365.3±8.36**	44.21±2.9**	28.17±11.23**
Triton + FB	1000	158.510±42.9**	420.46±123.46**	41.94±3.59**	23.99±16.3**
Triton + FB	500	192.47±51.26**	679.96±177.22ns	32.23±12.4**	25.306±4.3**
Triton + FB	250	239.02±29.8**	740.33±127.9ns	30.33±8.57**	33.12±.0369**
Triton + MY	1000	183.15±9.83**	467.255±205.2**	37.27±5.26**	46.1±8.36**
Triton + MY	500	227.59±26.2	595.89±116.17*	31.2±1.40*	59.03±3.69**
Triton + MY	250	254.41±21.16**	832.68±58.1ns	28.88±6.74ns	77.82±3.33*

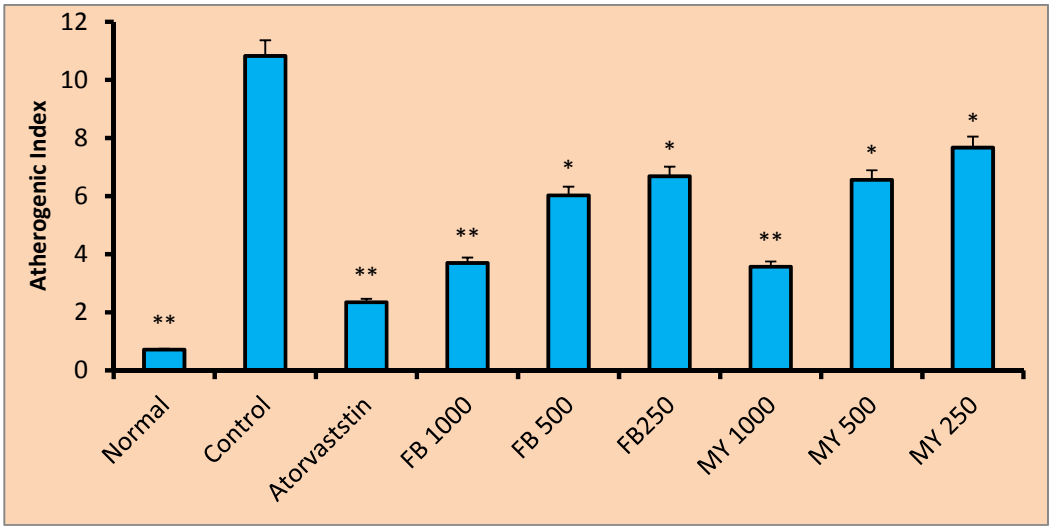
All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.1. Effect of fruiting body (FB) and mycelia (MY) extracts on lipid peroxidation in liver.



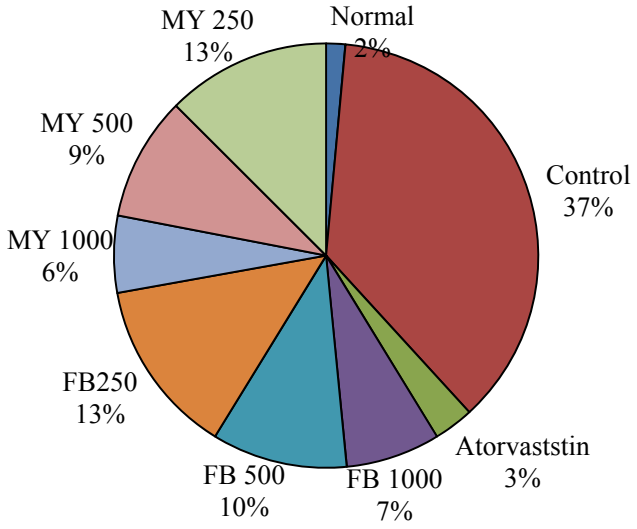
All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.2. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on atherogenic index (AI) in triton induced hyperlipidemic rats



All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.3. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on LDL/HDL ratio in Triton induced hyperlipidemic rats

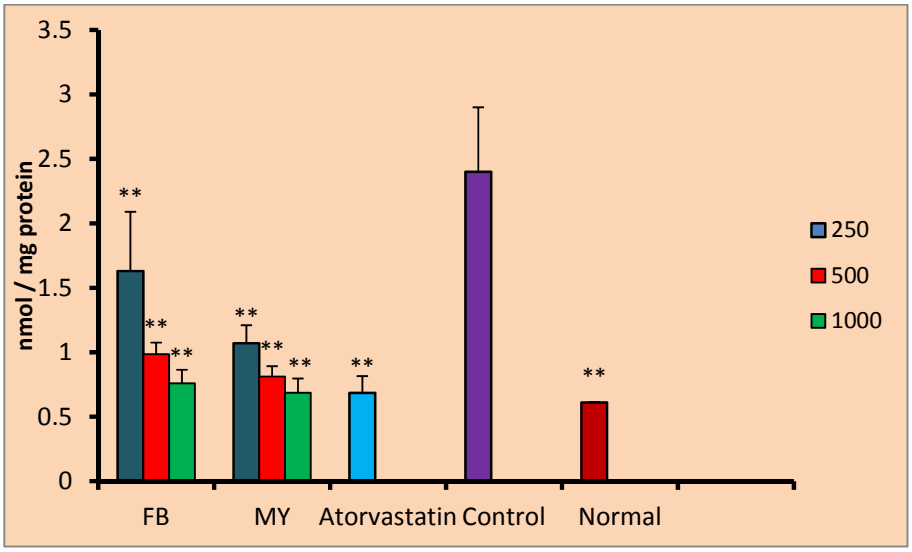


8.3.2. Effect of *H.ulmarius* fruiting bodies and mycelia extracts on serum lipid level of high fat diet induced hyperlipidemic rats.

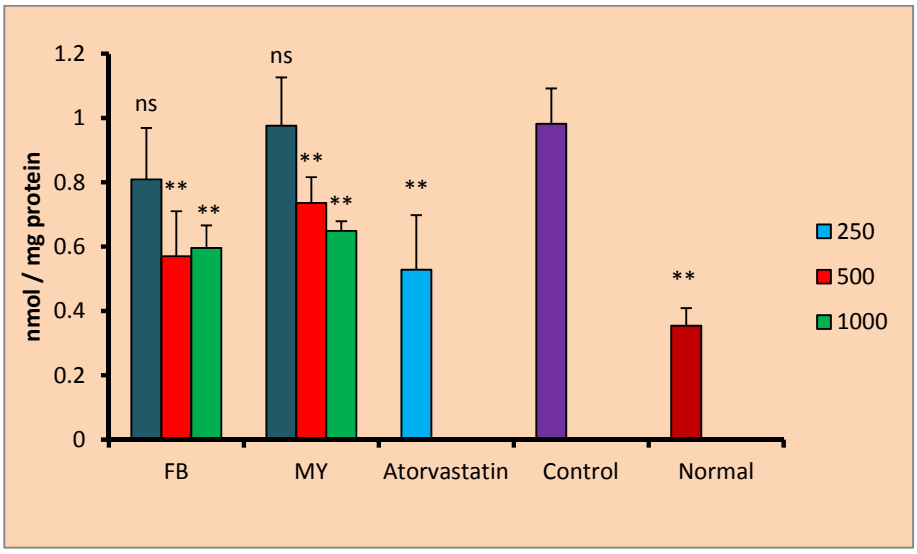
High cholesterol diet administration elevated the level of serum lipid such as total cholesterol, triglycerides and LDL to 130.33 ± 29.7, 163.12 ± 21.52 and 80 ± 0.96 respectively. Treatment of high cholesterol rats with fruiting bodies and mycelia extracts significantly decreased lipid profile. Treatment with aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* at doses of 250, 500 and 1000 mg/kg exhibited a significant reduction of total cholesterol level to 98.1 ± 3.14, 98.23 ± 14.9 and 96.84 ± 7.5 mg/dL and 108.10 ± 20.01, 92.24 ± 15.26 and 89.19 ± 17.63 mg/dL respectively. There was a significant reduction in trigycerides level of fruiting bodies and mycelia treated groups at 250, 500 and 1000 mg/Kg to 117.29 ± 19.25, 95.23 ± 18.97 and 89.6 ± 12.4mg/dL and 103.40 ± 6.6, 103.40 ± 6.3 and 91.42 ± 16.31mg/dL respectively. Fruiting bodies and mycelia treated groups at 250, 500 and 1000 mg/Kg, a marked reduction in LDL to 29 ± 8.3, 26 ± 2.6 and 23.2 ± 1.83 mg/ dL and 34 ± 1.38, 19 ± 3.2 and 15 ± 0.9 mg/dL respectively. Administration of atorvastatin also brought down the elevated level of TC, TG and LDL to 105.79 ± 12.29, 93.66 ± 20.68, and 22.45 ± 1.32 mg/dL respectively.

The High Density Lipoprotein(HDL) level was significantly elevated and there was 49.83 ± 4.6, 50.09 ± 4.08 and 56.94 ± 14.77and 54.703 ± 14.38, 63.35 ± 8.3 and 67.59 ± 17.08 increase in fruiting bodies and mycelia extracts treatment at 250, 500 and 1000 mg/Kg respectively as compared to control (38.62 ± 4.7). Administration of atorvastatin also elevated the HDL level to 65.29 ± 15.93.

Figure.8.4. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on lipid peroxidation in liver and heart



(a) Liver



(b) Heart

All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

8.3.4. Effect of antioxidant status in liver and heart

Table.8.2. Effect of aqueous ethanolic extracts of fruiting body and mycelia of *H.ulmarius* on the level of antioxidant enzymes.

	LIVER			HEART		
	SOD(U/mg Protein)	GST(nmol CDNB Conjugate formed/min/mg protein)	CATALASE(U/mg Protein)	SOD(U/mg Protein)	GST(nmol CDNB Conjugate formed/min/mg protein)	CATALASE(U/mg Protein)
Normal	12.6 ±2.15**	773.80±92.83**	69.22±2.2**	18.88±2.3**	68.05±5.5**	37.2±8.8**
Control (HFD)	2.06±0 .866	374.4±88.7	18.8±4.4	3.6±1.8	25.9±5.9	6.634±6.3
Atorvastatin (2.5mg/Kg)+HFD	9.18±0.829**	574.8±51.30*	51.20±5.4**	11.9±1.7**	66.17±9.11**	25.33±8.2**
FB(1000mg/Kg)+HFD	9.59 ±3.06**	636.2±156.16**	46.8±20.51**	13.65±3.4**	58.9±39.9*	21.2±1.3**
FB(500mg/Kg)+HFD	6.65± 0.93**	417.8±67.376ns	35.57±7.41*	9.15±2.9**	33.5±43.42ns	18.43±2.2*
FB(250mg/Kg)+HFD	6.55 ±1.5**	400.4±86.04ns	25.7±7.96ns	4.1±1.6ns	33.2±3.84ns	8.08±1.7ns
MY(1000mg/Kg)+HFD	11.08±2.02**	739.93±155.5**	46.1±16.09**	13.14±2.2**	54.03±36.17*	25.1±6.3**
MY(500mg/Kg)+HFD	7.8± 2.04**	624.7±133.4**	41.03±12.2**	11.0±4.1**	46.35±11.6ns	21.1±3.7**
MY (250mg/Kg)+HFD	5.4± 8.6**	428.88±29.2ns	30.2±2.1ns	5.68±1.03ns	40.16±4.5ns	14.3±1.2*

All values are Mean ± SD (n=6), **p < 0.01, * p < 0.05 and ns p > 0.05 with respect to control. (One Way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely Significant.

Lipid peroxidation was calculated by determining the amount of malonaldehyde in nanomoles per mg protein. In this study there was an elevation in hepatic and cardiac lipid peroxidation in control group and a decline in the amount of MDA in each treated groups. A significant increase in the level of MDA was found in HFD control group (2.4 ± 0.6 nmol/mg) as compared to normal group which was found to be 0.61 ± 0.04 nmol/mg. Administration of atorvastatin reduced the MDA level to 0.685 ± 0.13 nmol/mg. Treatment with fruiting bodies extract at different concentrations 250, 500 and 1000 mg/kg reduced MDA level to 1.63 ± 0.47, 0.985 ± 0.096 and 0.760 ± 0.104 nmol/mg. Whereas mycelia extract at same concentrations decreased MDA level to 1.07 ± 0.14, 0.812 ± 0.081 and 0.686 ± 0.119 nmol/mg (Fig-7.4)

8.3.5. Effect of *H.ulmarius* on hepatic HMG CoA reducatase activity.

HMG CoA/ Mevalonate ratio is an index of the enzyme, which catalyses the conversion of 3- hydroxyl-3 methyl glutaryl- CoA to mevalonate. There was an increase in HMG CoA to mevalonate ratio in each group as compared to control (1.26 ± 0.31).The ratio was significantly raised in atorvastatin (2.38 ± 0.61). The increase in values for fruiting bodies and mycelia extracts of *H. ulmarius* at 250, 500 and 1000 mg/Kg were1.4 ± 0.27, 2.11 ± 0.39 and 2.21 ± 0.64 and 0.47 ± 0.4, 2.1 ± 0.33 and 2.17 ± 0.59 respectively.(Fig-7.7)

8.3.6 Status of SGOT and SGPT activity.

Excess cholesterol was restored in liver cells and thus caused increase in liver marker enzymes such as SGOT and SGPT in rats. Since liver is the primary organ usually affected by hyperlipidemia there was an elevation in SGOT and SGPT in HFD control group. Whereas the extracts and standard drug atorvastatin treatment attenuated these elevated level of enzymes near normal. (Fig.7.9)

8.3.7. Isolation of Lovastatin

The chromatogram showing the presence of lovastatin in the extracts is shown in Fig 7.10. Lovastatin, a drug used for the treatment of hypercholestremia has been produced by a number of *Pleurotus* species. Fruiting bodies and mycelia of *H.ulmarius* for the production of lovastatin was examined. Lovastatin was detected only in the mycelia. Rf value of the extract of cultured mycelia of *H.ulmarius* is 0.67 which was same as that of lovastatin (Sigma) standard.

8.3.8. Histopathological observations.

Histopathological observation of the liver tissue of triton and high cholesterol challenged group showed necrosis and plenty of inflammatory cells. About 80% of the hepatocytes showed vacuolar degeneration and most of them are fatty vacuolation (Black arrows). The necrosis and fatty vacuolation in hepatocytes were significantly reduced by atorvastatin and extracts treatment. (Fig11 and12)

Figure.8.5. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on LDL/HDL ratio in HCD induced hyperlipidemic rats

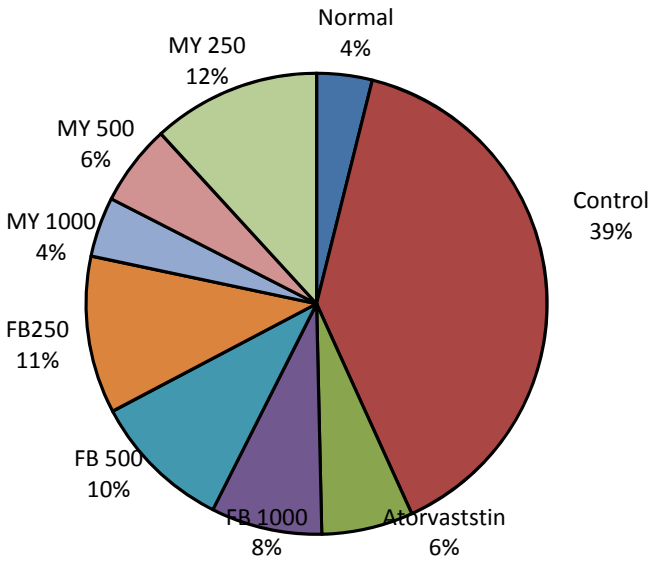
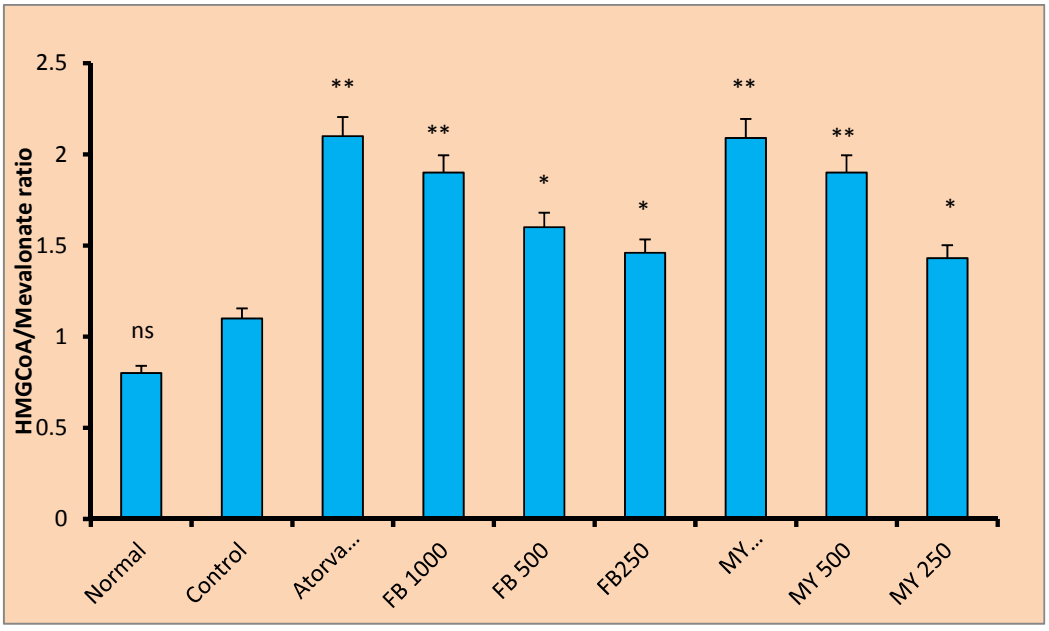
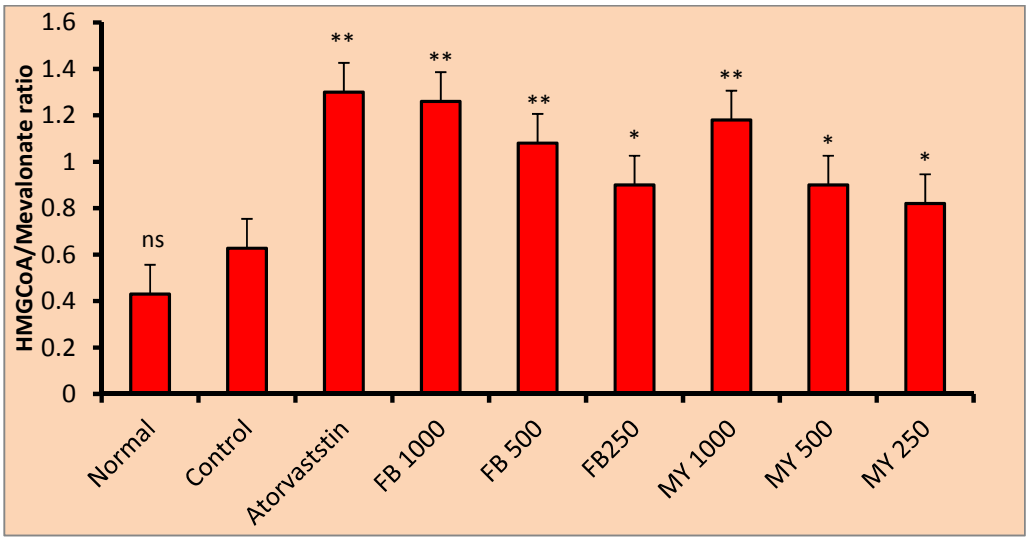


Figure.8.6. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on HMG-CoA/Mevalonate ratio in triton induced hyperlipidemic rats



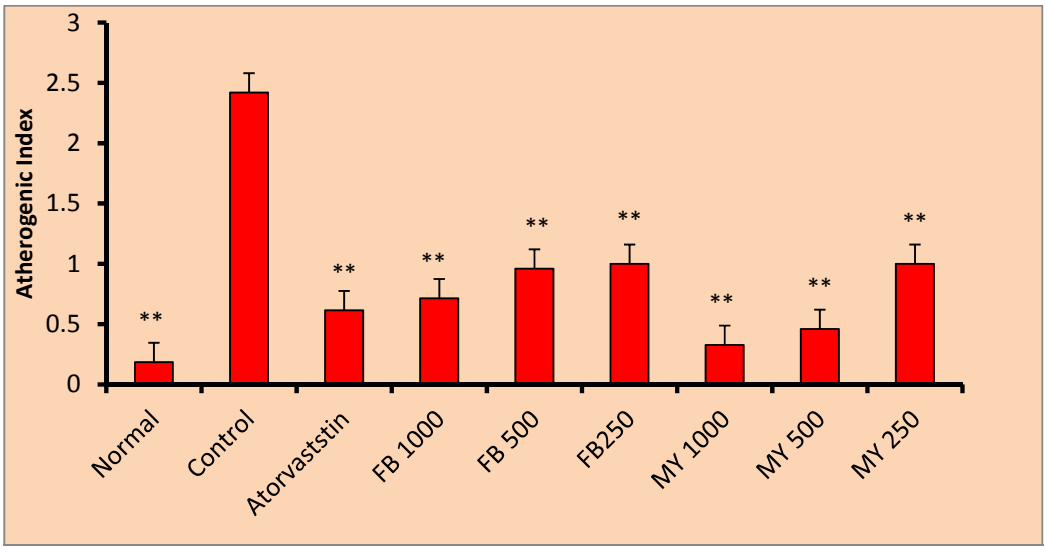
All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.7.Effect of *H.ulmarius* fruiting body (FB) and mycelia (MY) extracts on HMG CoA/Mevalonate ratio in HCD induced hyperlipidemic rats



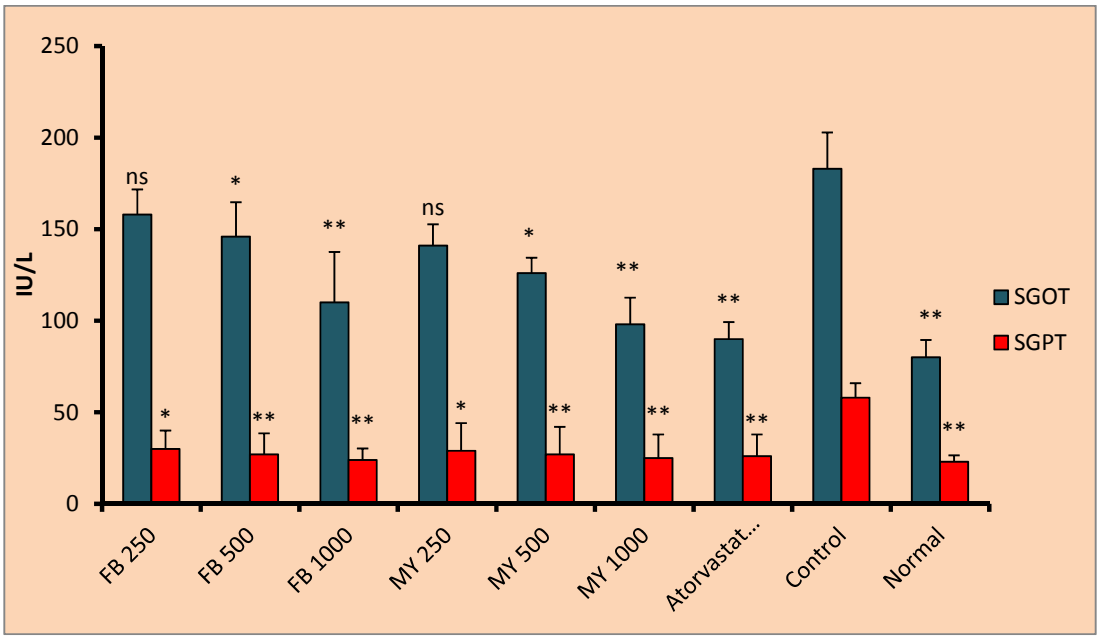
All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.8. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on atherogenic index (AI) in HCD induced hyperlipidemic rats



All values are expressed as Mean ± SD, n=6, **p<0.01as compared to control considered as significant. (One way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.9.Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on SGOT and SGPT in HCD induced hyperlipidemic rats



All values are expressed as Mean ± SD, n=6, **p<0.01;*p<0.05 as compared to control considered as significant and ns p > 0.05 (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

Figure.8.10. Detection of Lovastatin in fruiting bodies (FB) and cultured mycelia (MY) of *H. ulmarius* using thin layer chromatography

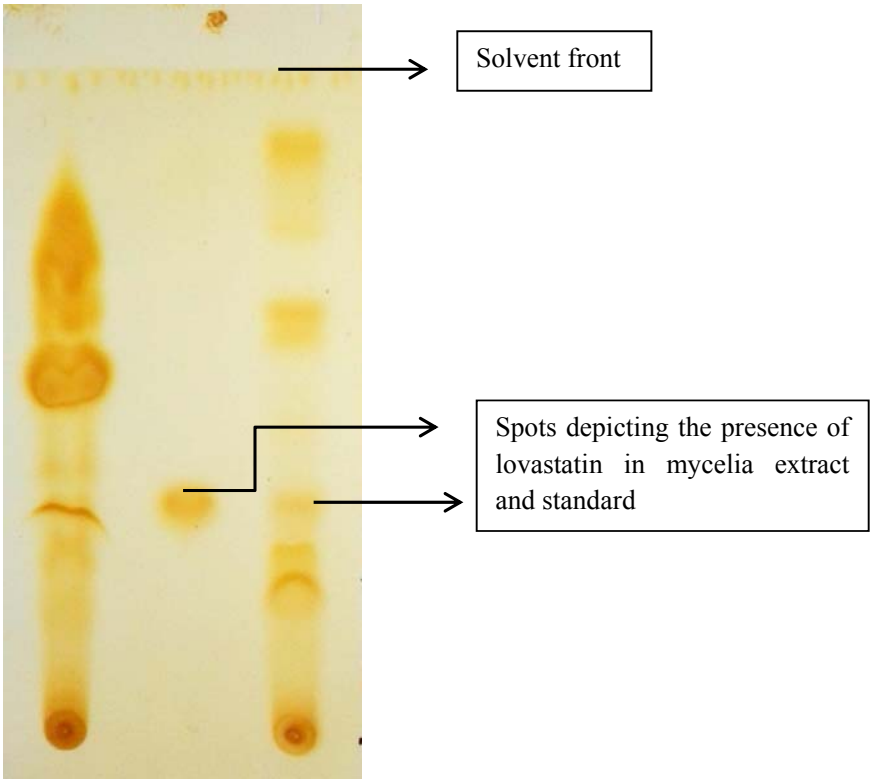
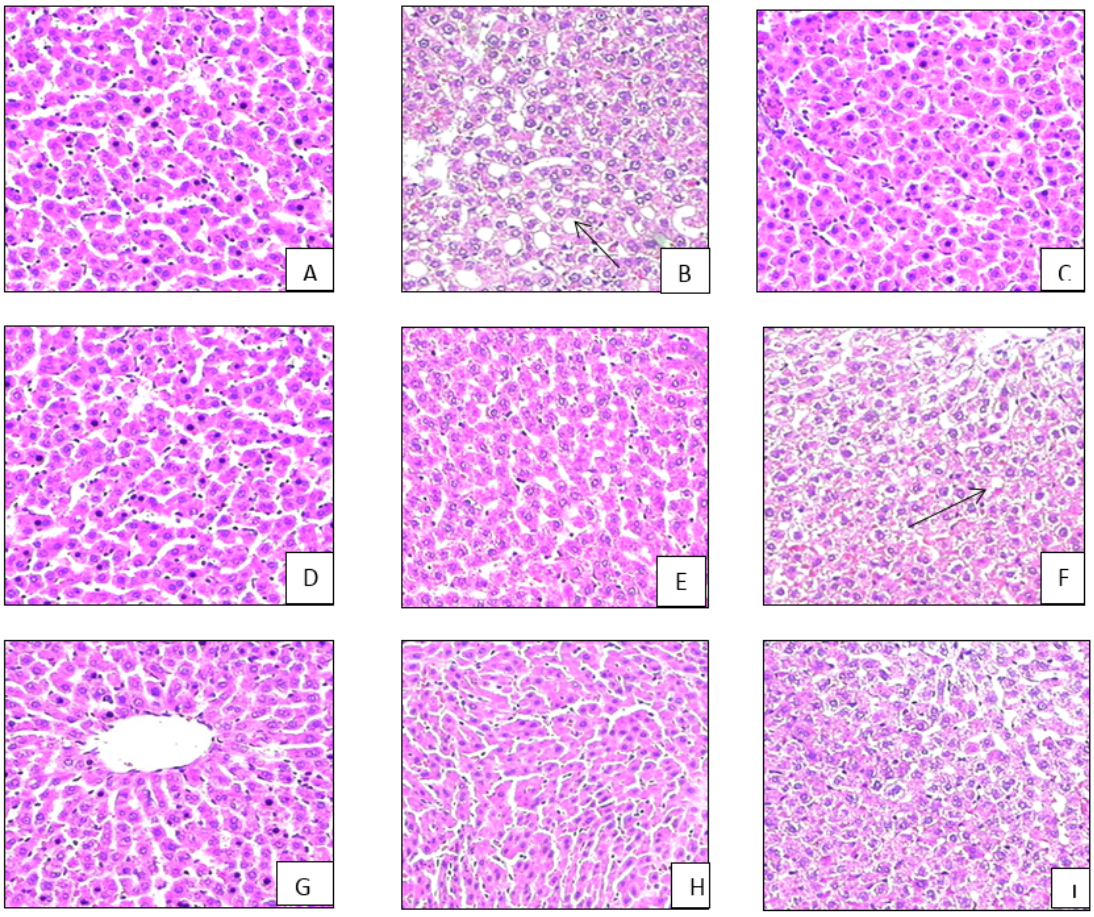


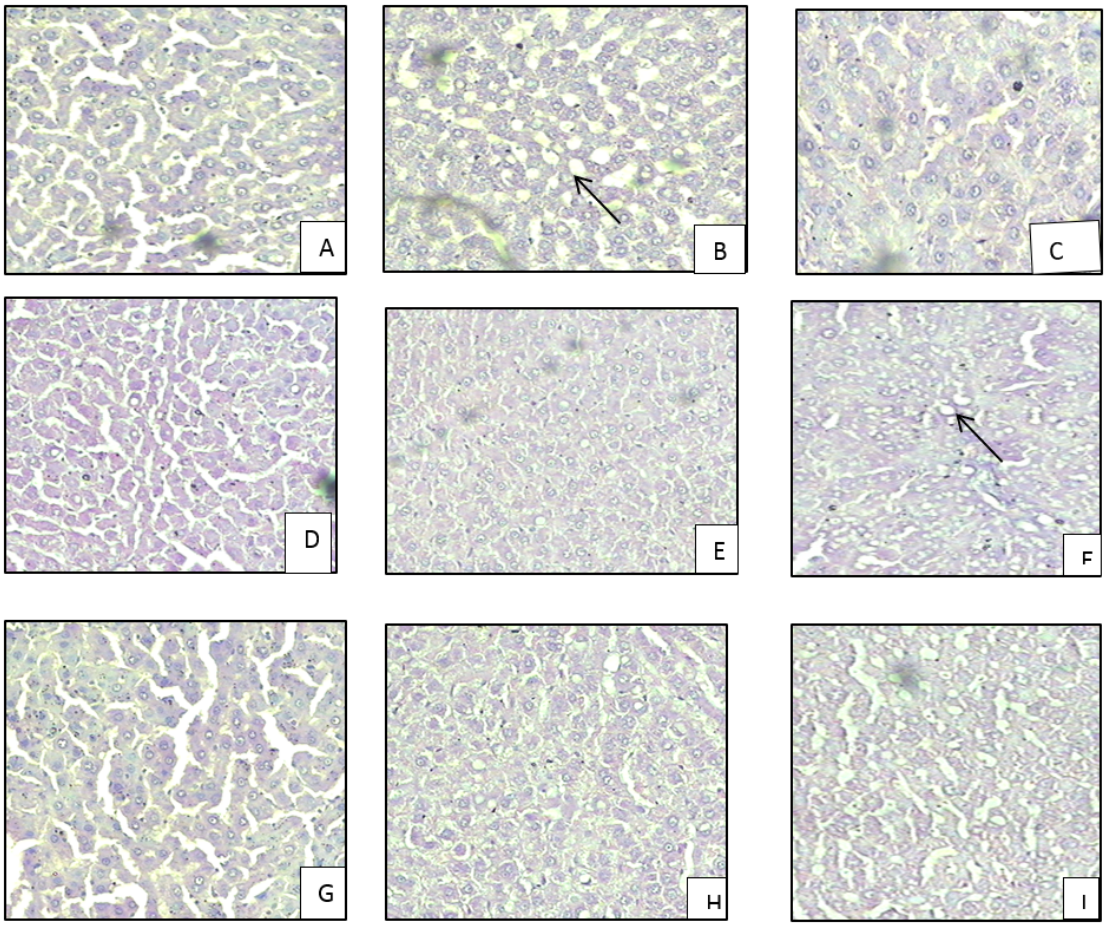
Figure.8.11. Histopathology of liver in triton induced hyperlipidemic rats



Histopathology of liver sections (Staining Hematoxylin- Eosin, 10×).

(A)Normal; (B) Control (Triton); (C) Standard (Atorvastatin 2.5mg/kg + Triton); (D) Fruiting body extract (1000 mg/kg + Triton); (E) FB (500mg/kg + Triton); (F) FB (250mg/Kg + Triton); (G) Mycelium extract (1000mg/Kg + Triton); (H) Mycelium extract (500 mg/kg + Triton); (I)
Mycelium extract (250 mg/kg + Triton).

Figure.8.12.Histopathology of liver in HCD induced hyperlipidemic rat



Histopathology of liver sections (Staining Hematoxylin- Eosin, 10×).

(A)Normal; (B) Control (HCD); (C) Standard (Atorvastatin 2.5mg/kg + HCD); (D) Fruiting body extract (1000 mg/kg + HCD); (E) FB (500mg/kg + HCD); (F) FB (250mg/Kg + HCD); (G) Mycelium extract (1000mg/Kg + HCD); (H) Mycelium extract (500 mg/kg + HCD); (I) Mycelium extract (250 mg/kg + HCD).

7.4. DISCUSSION

Hypercholesterolemia is one of the major risk factors contributing to prevalence and severity of cardiovascular diseases. Ever since health complications caused by obesity were demonstrated, many studies have been conducted in order to identify the main factors that contribute to its development. It is also reported that more than 12 million people die of cardiovascular diseases each year all over the world. Therefore, it is very important to pay attention to early stage prevention and control of hypercholesterolemia in a comprehensive way.

Triton WR-1339, a non-ionic detergent (oxyethylated tertiary octylphenol formaldehyde polymer), has been widely used to produce acute hyperlipidemia in rats which blocks the clearance of triglyceride-rich lipoproteins (Schurr *et al*, 1972). This model is widely used for a number of purposes particularly it has been used for screening natural or chemical hypolipidemic drugs (Harbowy and Balentine, 1997). Triton WR 1339 induced hyper-cholesterolemia has been related to its ability to alter the physico-chemical properties of lipoproteins and thereby prevent their uptake by liver (Byers *et al*, 1963). In the present study, extracts of *H.ulmarius* are effective in minimizing triton induced decrease in HDL as well as increase in LDL and triglycerides. The hypolipidemic efficacy of the extracts also substantiated by the atherogenic index and being the crude extracts, it seems to be quite competitive to the hypolipidemic drug atorvastatin.

In HCD cholesterol rich diet was used to produce hypercholesterolemia in rats. The diet was supplemented with 1% cholesterol, 0.5% cholic acid, gingely oil cake, black gram, hydrogenated ground nut oil, egg yolk, dalda, mineral mix and soyabean meal. Cholic acid facilitates the absorption of cholesterol from intestine. After 60 days, in HCD treated group serum TC, TG and LDL were significantly elevated. But the extracts of *H.ulmarius* and atorvastatin treatment significantly attenuated elevated levels of these lipid parameters.

In the present study, the result shows that the *H.ulmarius* extracts exerts significant antihyperlipidemic effect marked by significant lower serum cholesterol and triglyceride levels in extracts treated groups of animals compared to control group. The extracts seem to have potent antitriglyceridemic effect as it could protect from triton induced hypertriglyceride. It is found that triton elevates serum triglyceride

level essentially by preventing its uptake and clearance by inhibiting catabolising enzymes like lipoprotein lipase (LPL) and lecithin cholesterol acetyl transferase (LCAT) (Otway and Robinson, 1967; Fidge and Poulis; 1975). Apparently the mushroom extracts are able to reduce the inhibition on LPL and LCAT activity making triglycerides available for uptake and metabolism by tissues. The antilipidemic drug atorvastatin seems more potent in preventing the elevation of triglyceride levels.

The exact mechanism by which fruiting bodies and mycelia extracts reduced the serum cholesterol is not clear. But the increased HDL-C levels could be one of the possible mechanisms for decrease in serum lipid levels by extracts. The increased HDL-C facilitates the transport of triglycerides or cholesterol by a pathway termed 'reverse cholesterol transport' from serum to liver where it is catabolised and excreted out of the body. Increase of HDL-C is attributed to the mobilization of cholesterol from peripheral cells to the liver by the action of LCAT (Puri, 2011). Also, high HDL-C levels may compete with LDL receptor sites on arterial smooth muscle cells and thus inhibit the uptake of LDL. In addition, the increase in HDL-C concentration could protect the LDL against oxidation *in vivo* because lipids in HDL are preferentially oxidized before those in LDL (Young *et al*., 2004). LDL/HDL ratio had direct correlation with cardiovascular diseases. If the ratio increases there is high risk for CVD. In current study the HCD fed group showed increased atherogenic index (AI), LDL/HDL ratio as compared to normal diet fed group. Administration with fruiting bodies and cultured mycelia extracts treated group markedly decreased AI index, LDL/HDL ratio due to its action on total cholesterol.

In the current study, hypocholesterolemic activity of the extracts could be attributed to its inhibition of hepatic HMG-CoA reductase activity. HMG-CoA reductase is a key rate limiting enzyme involved in the cholesterol biosynthetic pathway in such manner the extract may exert its hypocholesterolemic effect through the mechanism of atorvastatin (standard drug used in this study). In both HFD and triton-treated group, cholesterol synthesis was increased and the HMG-CoA/ mevalonate ratio was decreased. On the other hand in extracts treated groups, cholesterol biosynthesis in liver was decreased while the HMG-CoA/mevalonate ratio was increased as compared to the hyperlipidemic control group. The results are statistically significant in all conditions as compared to the hyperlipidemic controls. Thus the HMG-

CoA/mevalonate ratio agrees with the results that would be anticipated on actual assay of the enzyme activity.

Level of malondialdehyde was significantly elevated in liver tissue of rats in which hyperlipidemia was induced using triton. Lipid peroxides produced as the end products of lipid peroxidation from membrane damage which are elevated in rats with induced hyperlipidemia. In the present study, rats fed with a high-cholesterol diet showed a significant ($p < 0.05$) increase in the liver TBARS level. Elevated cardiac and hepatic lipid peroxidation status in untreated hypercholesterolemic rats indicate increased oxidative injury and is consistent with reports from similar studies that showed that hypercholesterolemia leads to increased lipid peroxidation (Dutta *et al*., 2009; Ma *et al*., 2011) However, both fruiting bodies and mycelia extracts caused significant ($p < 0.05$) reduction in the TBARS levels when compared with the control (Fig.7.1 and 7.4). This clearly indicates a marked improvement in the *in vivo* antioxidant status following supplementation with the fruiting bodies and mycelia extracts of *H.ulmarius* that are rich in antioxidant phytochemicals such as phenolic compounds.

Hypercholesterolemia has been reported to be related to enhanced oxidative stress and increased lipid peroxidation, and an increase in oxidation of LDL was identified as a major contributor to the vascular damage caused by high cholesterol levels. Therefore, inhibiting oxidative stress in the hypercholesterolemic state is considered an important therapeutic approach. A major type of defense system in living tissues against oxidative damage is the use of antioxidant enzymes to convert ROS into non-toxic compounds and the tissue activities of those enzymes have been reported to be changed in response to the oxidative stress (Wispe *et al*., 1992; Jaruga *et al*., 1994). In this study, the diminished antioxidant defense system in HFD and triton rats leads to lipid peroxidation and oxidative damage as characterized by significant increase in TBARS level and significant decrease in tissue enzymes SOD, catalase and GST. ROSs attacks on membrane lipoproteins and polyunsaturated fatty acids results in formation of numerous oxygenated compounds, particularly malondialdehyde, MDA (Esterbauer *et al*., 1991)**.** High cholesterol diet also causes oxidative stress (enzymatic and non-enzymatic) in rats, thus, increases oxidation of low density lipoprotein (LDL) which plays key role in genesis of atherosclerosis. Antioxidants are known to effectively prevent this kind of damage (Vijayakumar *et al*., 2004). The presence of

strong antioxidant activities of *H.ulmarius* may offer additional benefit in combating the oxidative stress caused by high cholesterol.

Liver is the main site for cholesterol homeostasis and a high fat diet increases the lipid content of the liver. Previous studies have demonstrated that a cholesterol-rich diet can induce steatohepatitis in animal models (Kucera *et al*., 2011)). Hepatic damage is related to deterioration of the hepatocyte membrane, which generates a reduction of its antioxidant and detoxification capacity (Maki *et al*., 2013). For a good lipid lowering therapy, a drug should be able to significantly lower LDL and increase HDL cholesterol concentration and this appreciably decreases the fatty vacoulated cells in liver parenchyma and prevents hepatic necrosis and this correlates with the present study (Steinberg *et al*., 1999). Results obtained from this study show that rats fed on hypercholesterolemic diet had increased liver enzymes AST and ALT meanwhile rats fed on that hypercholesterolemic diet but supplemented with extracts of *H.ulmarius* and atorvastatin recorded improvement in liver function enzymes. The increased levels of serum AST and ALT indicated the increased permeability and necrosis of hepatocytes since hypercholesterolemia was reported to cause production of free radicals which in turn led to lipid peroxidation of membrane lipids (Goldberg and Watts, 1965).

Phytochemical analysis of fruiting bodies and mycelia extracts of *Hypsizygus ulmarius* revealed the presence of tannins, alkaloids, phenolics, and steroids (Greeshma *et al*., 2016). Alkaloids have been identified as a potent hypolipidemic bioactive compound in experimental studies. It promotes an increase in faecal sterols, which in turn leads to a decreased absorption of dietary cholesterol. Alkaloids and phenols may also contribute to the hypolipidemic activity by increasing the cholesterol metabolism and by modulating the enzymes involved in cholesterol metabolism, such as HMG-CoA reductase, lecithin cholesterolacyl transferase and acyl- CoA: cholesterol acyltransferase (Kumar *et al*., 2010). Decreased LDL-C and increased HDL-C levels, removes cholesterol from peripheral tissue to liver for excretion (Weggmans and Trautwein, 2003). The hypocholestrolemic effect is attributed to the HMG-CoA reductase inhibiting effect of extracts.

Pleurotus is a nutritional food with valuable therapeutic use. The best known therapeutic agent of potential use for lowering hypercholesterolemia is lovastatin and

its analogues. *Pleurotus* species are reported to be a good source of this drug (Endo and Klin, 1998). Lovastatin was detected in cultured mycelia of *H.ulmarius*. TLC analysis of the extract supports this conclusion. Thus it was found that the mycelia of *H. ulmarius* had sufficient amount of lovastatin which could inhibit HMG-CoA reductase enzyme and thus decrease the cholesterol biosynthesis.

Fruiting bodies and mycelia extracts of *H.ulmarius* administration may be an effective treatment option against hypercholesterolemia which is a great risk factor for cardiovascular diseases. The extracts are also able to ameliorate reactive oxygen species, serum biochemical parameters and lipid profile. The current experimental findings reveal that, administration of diet rich in the natural antioxidant specially mushrooms is highly beneficial for protection against oxidative stress, and cardiac vascular diseases resulting from hyper-lipidemia, hypercholesterolemia and arteriosclerosis.

Chapter 9:

Toxicity studies of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *Hypsizygus ulmarius*

5.1. INTRODUCTION

5.2. MATERIALS AND METHODS

5.2.1. Preparation of the extracts

5.2.2. Animals

5.2.3. Acute toxicity study

5.2.4. Subacute toxicity study

5.3. RESULTS

5.4. DISCUSSION

5.1. INTRODUCTION

Toxicity studies are carried out for the identification of the hazard of drugs and limitation of doses, so that precautions can be taken to protect humans from the adverse effects of products used in therapy.

Edible mushrooms are healthy food and nutrient sources and some of them are reported to have remarkable bioactivities. (Fan *et al*., 2006). Many mushrooms have been used widely throughout Asia for medicinal purposes. Experimental investigations to evaluate the toxicity of mushrooms are rare. However *H.ulmarius* is a high yielding excellently edible mushroom and its cultivation is gaining popularity throughout the worldwide. Hence the toxicity studies have significant importance.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

5.2.2. Animals

Male Swiss Albino mice weighing (20 ± 2) were used for the experiments.

5.2.3. Acute toxicity study

Swiss albino mice were divided into seven groups of six animals each and experiment was performed as follows

Group I	Normal (Distilled Water)
Group II	Fruiting bodies extract (500 mg/ kg) of *H.ulmarius*
Group III	Fruiting bodies extract (2500 mg/ kg) of *H.ulmarius*
Group IV	Fruiting bodies extract (5000 mg/ kg) of *H.ulmarius*
Group V	Mycelia extract (500 mg/ kg) of *H.ulmarius*
Group VI	Mycelia extract (2500 mg/ kg) of *H.ulmarius*
Group VII	Mycelia extract (5000 mg/ kg) of *H.ulmarius*

Group I was treated with distilled water and kept as normal, single dose of extracts were administered orally and group II, III, IV were treated with 500, 2500 and

5000mg/Kg concentrations of fruiting bodies extracts respectively whereas group V, VI and VII with same concentrations of mycelia extracts. Animals were observed for toxic symptoms and mortality for 72 hours (Walum, 1998).

5.2.4. Subacute toxicity study

Swiss albino mice were divided into five groups of six animals each and extracts were administered orally once daily for 30 days (Parchment, 1998).

Group I	Normal (Distilled Water)
Group II	Fruiting bodies extract (250mg/ kg) of *H.ulmarius*
Group III	Fruiting bodies extract (500mg/ kg) of *H.ulmarius*
Group IV	Mycelia extract (250mg/kg) of *H.ulmarius*
Group V	Mycelia extract (500mg/kg) of *H.ulmarius*

Body weight of the animals were recorded weekly and observed for toxic symptoms and mortality. After the last dose of extracts administration animals were sacrificed. Blood was collected and used for the analysis of hematological parameters, total leucocyte count (3.2.17), Hb (3.2.16) and Differential count (6.4.12). Serum was separated for the analysis of liver function enzymes such as SGOT (3.2.10) and SGPT (3.2.11).

5.3. RESULTS

5.3.1. Acute toxicity of extracts

Animals treated with aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* did not show any symptoms of toxicity and mortality up to a dose of 5000 mg/Kg.

5.3.2. Subacute toxicity of extracts.

Treatment with different concentrations (500 and 250mg/Kg) of fruiting bodies and mycelia extracts for 30 days did not produce any toxic symptoms. Mortality rate was negligible and there was no significant difference in the hematological (RBC, WBC, Hb) (Table 9.2) and biochemical parameters (SGOT and SGPT) as compared to

normal group (Fig 9.1). Body weight of the animals also remained more or less same in the studies (Table 9.1)

The administration of extracts did not produce any significant changes in the liver function enzymes. The SGOT and SGPT activities in the normal animals were found to be 98.15 ± 11.23 and 20.06 ± 1.25 IU/L. Treatment with aqueous ethanolic extracts of fruiting bodies and mycelia of *H. ulmarius* at doses of 250 and 500 mg/kg exhibited a significant reduction of SGOT, SGPT, levels to 108.18 ± 13.5, 34.24 ± 1.9 IU/L and 101.335 ± 2.36, 28.285 ± 0.9 IU/L and 110.06 ± 5.3, 30.25 ± 2.33 IU/L and 100.335 ± 1.26, 22.45 ± 1.1 IU/L respectively.Body weight is a factor that determines the health of the animal. Low bodyweight indicates the side effects. In this study there was no much difference in body weight of treated groups after 30 days as compared to initial body weight.

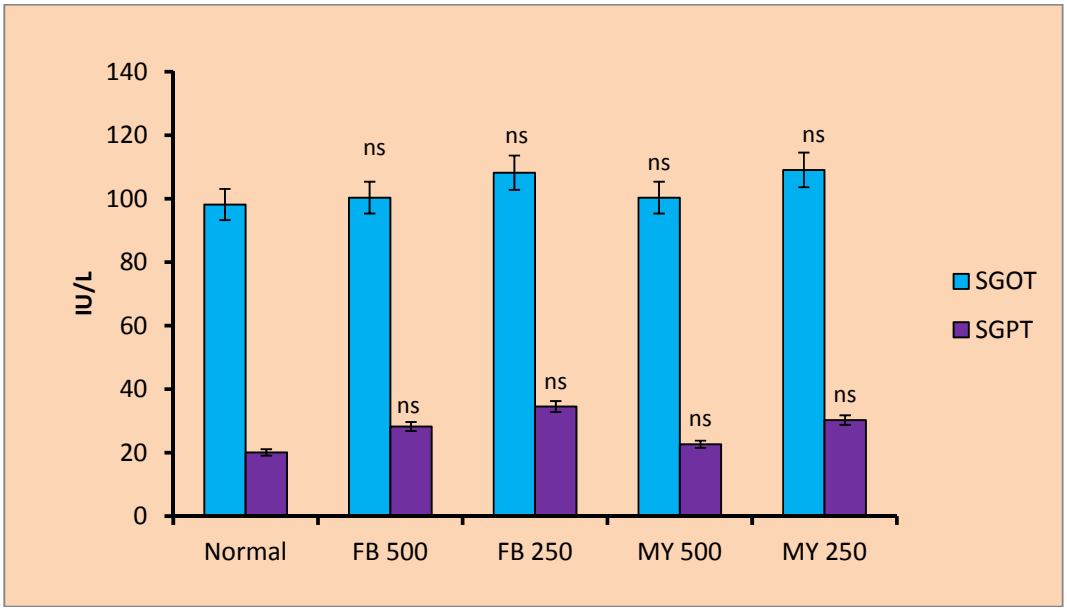
Table.9.1. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on the body weight of animals before and after treatments

Groups	Initial body weight	Final body weight	Mortality
Normal	**26.59 ± 2.3**	**27.56± 4.5**	**0**
FB(500mg/Kg)	**27.48 ± 2.66**	**27.72 ± 1.68**	**0**
FB(2500mg/Kg)	**31.6 ± 3.38**	**32.14 ± 2.4**	**1**
FB(5000mg/Kg)	**28.6 ± 4.8**	**28.44 ± 3.25**	**0**
MY(500mg/Kg)	**28.4 ± 4.7**	**30.32 ± 2.8**	**1**
MY(2500mg/Kg)	**27.6 ± 3.3**	**28.22 ± 2.206**	**0**
MY (5000mg/Kg)	**29 ± 2.0**	**30.12 ± 1.6**	**0**

Table.9.2. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on the hematological parameters of treated animals

Groups	Hb (g/dl)	WBC (Cells/mm^3)	RBC (Cells/ mm^3)	Neutrophils(%)	Lymphocytes (%)	Eosinophils (%)
Normal	9 ± 2.1	5300± 513.6	3.4± 0.32	42± 4.65	54± 2.3	4± 0.01
FB(250mg/Kg)	7.8 ±1.69	10900 ± 119.23	2.9± 0.91	38± 1.36	53± 2.2	4± 0.02
FB(500mg/Kg)	10.8 ± 0.36	13100± 226.29	3.7± 0.001	43± 3.3	68± 5.4	4± 0.14
MY(250mg/Kg)	11.6± 1.10	5100± 311.35	4± 0.11	39± 4.02	52± 2.87	6± 0.06
MY(500mg/Kg)	12.3 ± 0.62	6500± 656.12	4.2± 0.26	42± 3.66	56± 1.9	5± 0.32

Figure.9.1. Effect of *H.ulmarius* fruiting bodies (FB) and mycelia (MY) extracts on the activity of liver function enzymes



All values are expressed as Mean ± SD, n=6, ns when p > 0.05 as compared to control considered as non significant (one way ANOVA followed by Dunnett's test). The p value < 0.001 is considered extremely significant.

5.4. DISCUSSION

The purpose of toxicity testing is to provide information on the bio toxicity of substances, so that precautions can be taken to protect humans, animals and the environment from the adverse effects of products used in medicine, industry, agriculture, and the household (Weil and Scala, 1971). In toxicity studies, animals were administered with extracts at various doses for a period of one month. Animals were observed for toxic symptoms and mortality. Some of the symptoms observed in toxicity testing include changes in skin, fur, eyes, mucous membranes, loss of appetite, dehydration, nasal discharge, and diarrhoea. But there were no observed symptoms in animals. Also, biochemical analysis was performed to investigate major toxic effects in tissues, specifically, effects on kidney and liver. The results of toxicity tests can be used to predict the risk of adverse effects resulting from the exposure of humans and to decide an effective measure to protect human health.

Current investigation reveals that the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* do not show any toxicity up to a dose of 5000 mg/Kg body weight. There was no significant difference in hematological profile such as total leucocyte count, differential count, RBC and Hb as compared to normal group. After 30 days, a slight increase in serum SGPT and SGOT in the extracts treated groups were observed as compared to normal value. But these changes were statistically non-significant. This finding reveals the therapeutic significance of *Hypsizygus ulmarius* as a potent nontoxic pharmaceutical.

Chapter 10:

Phytochemical analysis of aqueous ethanolic extracts of fruiting bodies and cultured mycelia of Hypsizygus ulmarius

10.1. INTRODUCTION

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RESULTS

DISCUSSION

10.1. INTRODUCTION

Mushrooms are functional food and are a source of biologically and therapeutically valuable components that offer great potential for the prevention and control of several diseases such as cardiovascular diseases, cancer, diabetes etc. Mushroom components are gaining increasing recognization in their application as potent therapeutic agents. A large number of mushroom-derived bioactive compounds, both cellular components such as glycoproteins, polysaccharides and secondary metabolites like phenolic compounds, terpenoides, alkaloids, sterols and polyphenols have been isolated from fruiting bodies, mycelia and culture broth. Most research studies conducted on the pharmacological potential of mushrooms were assayed using crude mushroom extracts or mixture of mushroom metabolites. These studies will require the isolation and identification of the bioactive compounds in order to determine the bioactive effect of each compound.

Higher basidiomycetes especially mushroom such as *Pleurotus* species possess unlimited and useful therapeutic bioactiveses. Theses bioactive constituents include polysaccharides, lipopolysaccharides, proteins, peptides, phenols, glycoproteins, carbohydrates, alkaloids, nucleosides, triterpenoids, lectins, lipids and their derivatives (Yashvant *et al*., 2012). The presence of bioactive compounds makes these mushrooms popular to consume as food sources and also valuable candidates for drug development. Due to the presence of biologically active compounds of medicinal value medicinal mushrooms are used as anticancer, antiviral, hepatoprotective, immunopotentiating and hypocholesterolemic agents. Investigations carried out in our laboratory showed that aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* possessed significant antioxidant, anti-inflammatory, antitumor activities (Greeshma *et al* ,2016), hepatoprotective activity and lipid lowering activity. These biological activities are correlated to metabolites present in the crude mushroom extracts. Hence investigations were carried out to analyse the bioactive principles present in the fruiting bodies and mycelia extracts of *H.ulmarius*.

10.2. MATERIALS AND METHODS

10.2.1. Preparation of the extracts

Aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* were prepared as described in section 3.2.2.

10.2.2. Phytochemical analysis of the extracts

Phytochemical screening of fruiting bodies and cultured mycelia extracts of *H. ulmarius* were carried out by standard methods. (Harborne, 1973)

10.2.2.1 Quantitative estimation of total carbohydrate.

Quantitative estimation of carbohydrate was carried out by Anthrone method (Yemm and Wills, 1954). Anthrone reagent was prepared by dissolving 200 mg anthrone in 100ml ice cold 95% sulphuric acid. To a small amount of extract dissolved in 1 ml of distilled water, 4 ml of anthrone reagent was added. The reaction mixture in a test tube was heated on a boiling water bath for 10 minutes. The tube was cooled rapidly and the absorbance of green coloured solution was measured at 630 nm.

10.2.2.2. Quantitative estimation of phenolic compounds

The total phenolic content was determined by Folin-Ciocalteu method (Ainsworth and Gillespie, 2007), with slight modifications. Briefly, 0.5 mL of the fruiting bodies and mycelia extracts were mixed with 2 mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm. The amount of total phenolic compounds was calculated as milligram of gallic acid equivalents (GAE) from the calibration curve of gallic acid standard solution (concentration range 10-80 μg/mL) and expressed as mg gallic acid/g dry weight of the mushroom material.

10.2.2.3. Test for proteins

The presence of protein and its concentration was assayed using Bradford's method (Section 3.2.4)

10.2.2.4. Test for alkaloids

10.2.2.4. a. Wagner's test

The extracts were treated with Wagner's reagent containing 1.27g of iodine and 2 g of potassium iodide are dissolved in 5 ml of distilled water and made up to 100ml with distilled water. Formation of brown flocculent precipitate indicates the presence of alkaloids.

10.2.2.4. b. Dragendorff's Test

The dragendroff's reagent was prepared by mixing solution A (containing 0.6g of bismuth subnitrate in 2ml of Con.HCl and 10ml of distilled water) solution B (containing 6g of potassium iodide in 10ml of water) together with 7 ml of Con. HCl and 15ml distilled water and the whole solution was diluted to 100ml with distilled water to form Dragendroff's reagent. The extracts were treated with Dragendorff's reagent. Formation of orange red precipitate indicates the presence of alkaloids.

10.2.2.4. c. Mayer's Test

The extracts were treated with Mayer's reagent. Reagent was prepared by mixing solution A containing 1.36g of Mercuric chloride dissolved in 60 ml of distilled water and solution B containing 5g of potassium iodide in 10 ml distilled water and it was then diluted to 100ml with distilled water. Formation of a white precipitate indicates the presence of alkaloids.

10.2.2.5. Test for steroids/ Terpenoides

Liebermann-Burchard test

A few milligrams of extracts were dissolved in chloroform and an ice cold mixture of acetic anhydride and sulphuric acid (4:1) was added through the sides of the test tubes. A green colour formation indicates the presence of steroids and pink colour indicates the presence of terpenoides.

10.2.2.6. Test for tannins

A little of the extracts were dissolved in 2ml distilled water and a few drops of lead acetate solution was added. Formation of white precipitate indicates the presence of tannins.

10.2.2.7. Test for cumarins

The extracts were dissolved in methanol, on adding alcoholic KOH or NaOH gives yellow colour which disappears on adding Con. HCl.

10.2.2.8. Test for saponins

A little of the extracts were shaken well with water and the formation of froth in the test tube, which persist for a few minutes, showed the presence of saponins.

10.2.2.9. Test for carbohydrates

A little of the extracts were dissolved in distilled water and few drops of Molish's reagent was added. To this mixture Conc. H_2SO_4 was added slowly through the sides of the test tube so that it forms a ring at the middle of the two solutions. The formation of violet ring indicates the presence of carbohydrate.

10.2.2.10. Test for phenolic compounds

The extracts were dissolved in methanol and 1% methanolic ferric chloride was added. A dark brown or green colour formation indicates the presence of phenols.

10.2.2.11. Test for anthraquinones

A little of extracts dissolved in chloroform and added magnesium acetate solution.
The formation of a pink colour indicates the presence of anthraquinons.

10.2.2.12. Test for flavanoides (Shinodas test)

Extracts were dissolved in methanol and magnesium turnings were added followed by Con. HCl. Pink colour indicates the presence of flavonoids

10.2.3. HPTLC analysis

HPTLC analysis of aqueous ethanolic extracts of fruiting bodies and cultured mycelia extracts of *Hypsizygus ulmarius* were carried out. Samples were dissolved in

methanol (10 mg/ml) and was applied to a Silica gel 60 F_{254} TLC plate (E. Merck, Germany) (7 cm x 10 cm) using Linomat V sample applicator. Plate was then developed up to 80 mm in a twin trough glass chamber using the mobile phase Chloroform-Methanol-Water (30:4:1) and then derivatised using anisaldehyde sulphuric acid reagent or vanillin sulphuric acid reagent heated at 110˚C for 10 min. Plate was then scanned densitometrically at 580 nm using TLC Scanner 3 equipped with Win Cats software.

10.2.3.1. Spray Reagents

10.2.3.1. a. Anisaldehyde- sulphuric acid reagent.

0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid. The reagent has less stability and no longer usable as its colour changes to red violet. The plate was sprayed with 10 ml reagent and then heated at 110˚C for 10 minutes, then observed under UV light and in visible light.

10.2.3.1. b. Vanillin- sulphuric acid reagent

The plate was sprayed vigorously with 10 ml of 1% ethanolic vanillin reagent and followed by 5-10 ml of 10% ethanolic sulphuric acid. After heating at 100˚C for 5-10 minutes, the plates were observed under UV light and visible light.

RESULTS

Preliminary phytochemical assay.

Preliminary phytochemical analysis showed the presence of polysaccharides, steroids/terpenoids, phenolics, tannins and alkaloids in both the extracts (Table 1).

Total carbohydrate, phenolic and protein content in the aqueous- ethnolic extracts of *H. ulmarius*

Total polysaccharide and protein present in the fruiting body extract of *H. ulmarius* was 20% and 28.5% respectively whereas that in the mycelia extract the content was 18.9% and 25.6% respectively. The phenolic content in the extract of fruiting bodies

was found to be 4.47% gallic acid equivalent and that in the mycelial extract was 5.49% gallic acid equivalent.

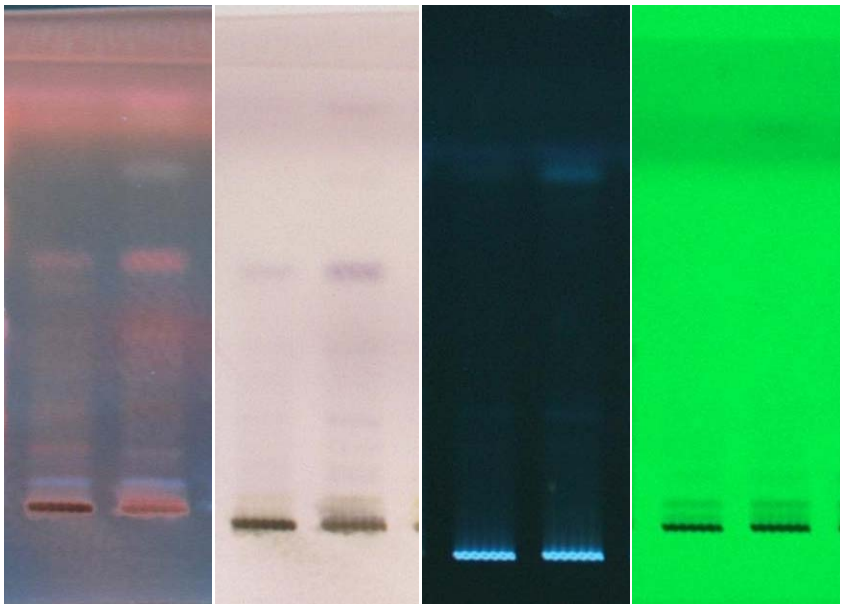
HPTLC analysis

The HPTLC analysis revealed that the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *Hypsizygus ulmarius* contain a large number of major and minor compounds. Nine major compounds were obtained in both the extracts but with more concentration in mycelia extract as compared to fruiting bodies extract. Under UV illumination blue coloured bands were obtained. Presence of different compounds with varying concentrations was detected in HPTLC analysis (Fig.10.1). Fig. 10.2 and Fig.10.3 represents HPTLC profile of the extracts with solvent system Chloroform-Methanol-Water (30:4:1)

Table.10.1.Phytochemical screening of aqueous- ethanolic extracts of *H.ulmarius*

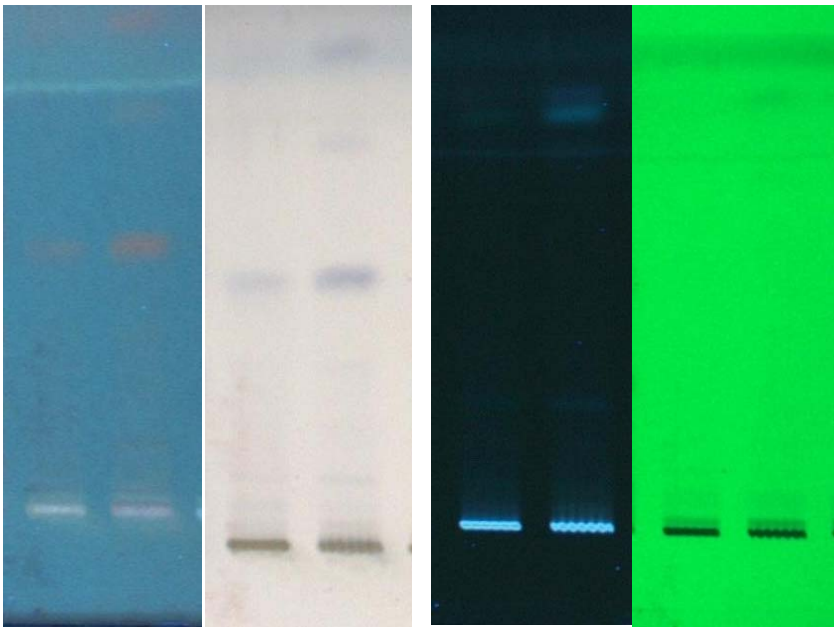
Compound	Fruiting body extract	Mycelia extract
Phenolics	+ +	+ + +
Alkaloids	+ + +	+ + +
Saponins	-	-
Flavanoids	-	-
Coumarins	-	-
Tannins	+ +	+ +
Quinone	-	-
Steroids/terpenoids	+ +	+ +
Polysaccharides	+ + +	+ + +
Proteins	+ + +	+ + +

Figure .10.1. HPTLC plates of aqeous ethanolic extracts of fruiting bodies and cultured mycelia of *Hypsizygus ulmarius*. (A)Using Anisaldehyde- sulphuric acid (B) Vanillin- sulpuric acid spray reagent.



(A)

1 2 3 4

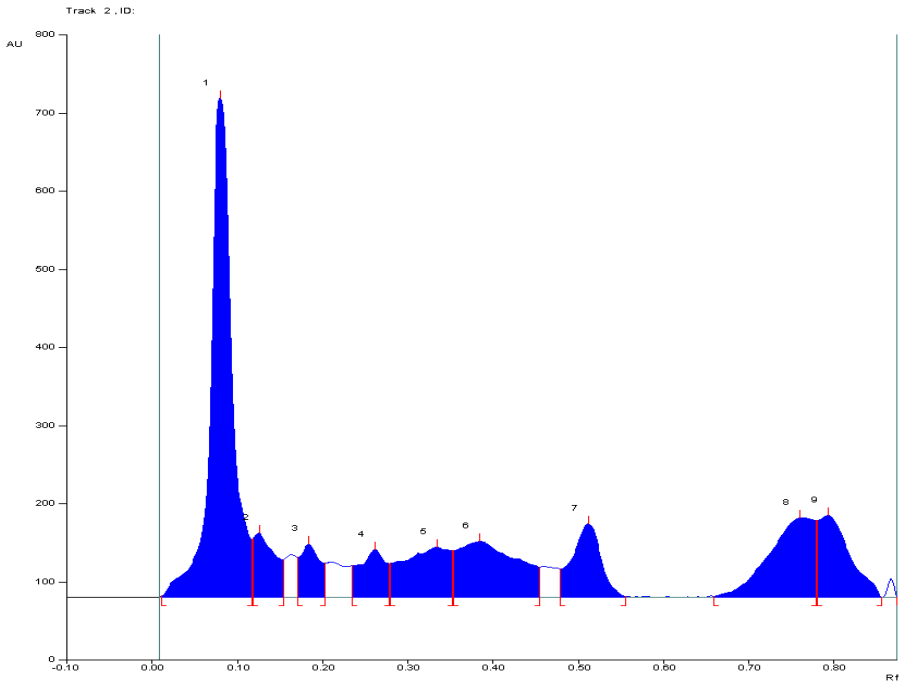


(B)

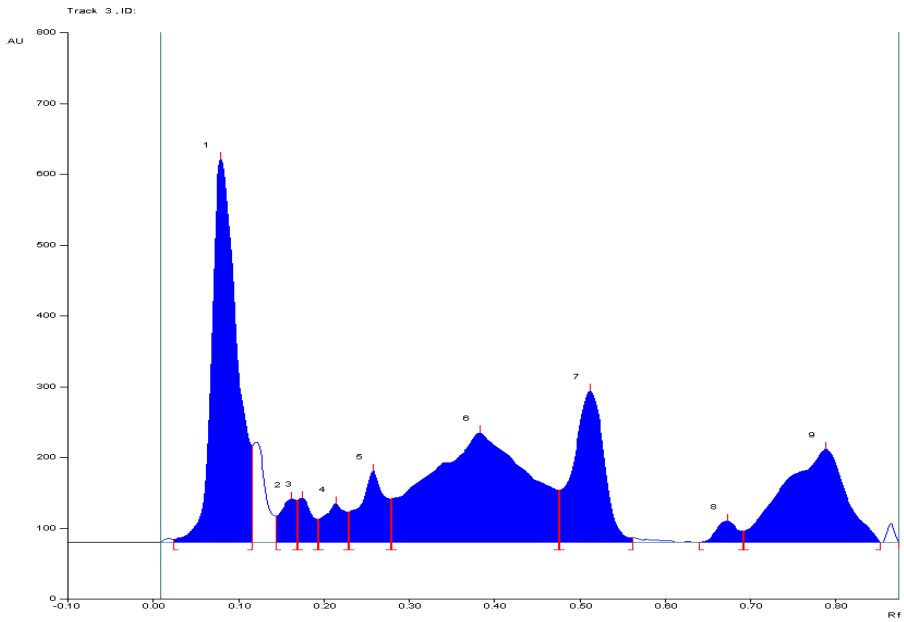
1 2 3 4

1. Derivatised plate at 366 nm
2. Derivatised plate white light
3. Developed plate at 366 nm
4. Developeded plate at 254 nm

Figure.10.2. Graphical representation of the HPTLC pattern of aqueous ethanolic extracts of *Hypsizygus ulmarius*. Derivatised plate at 580 nm scan.bmp using anisaldehyde-sulphuric acid spray reagent. (A) FB extract (B) MY extract

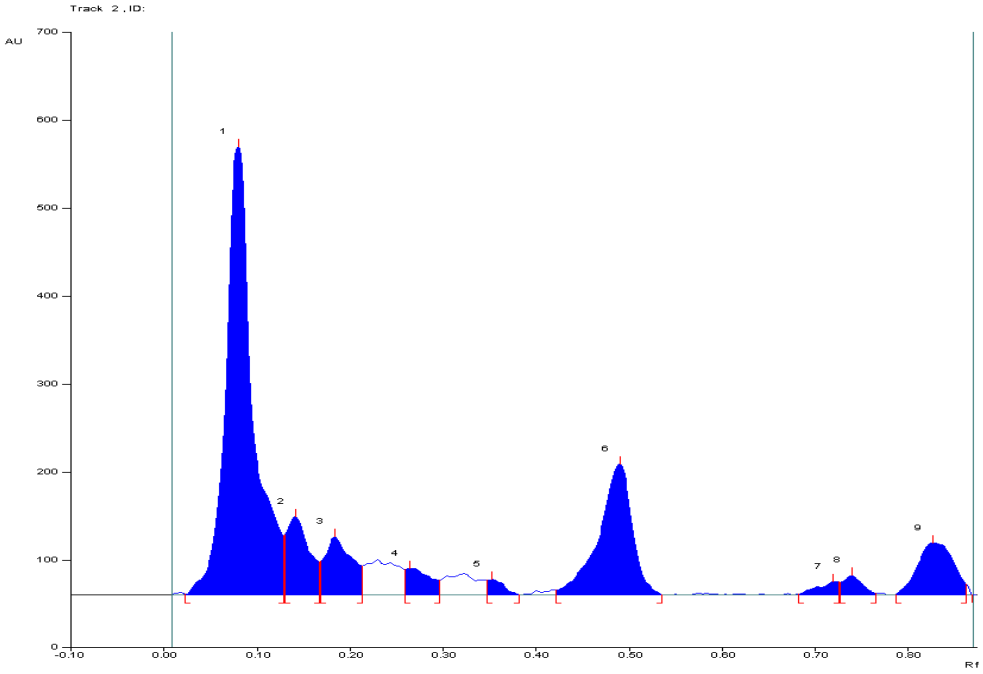


(A)

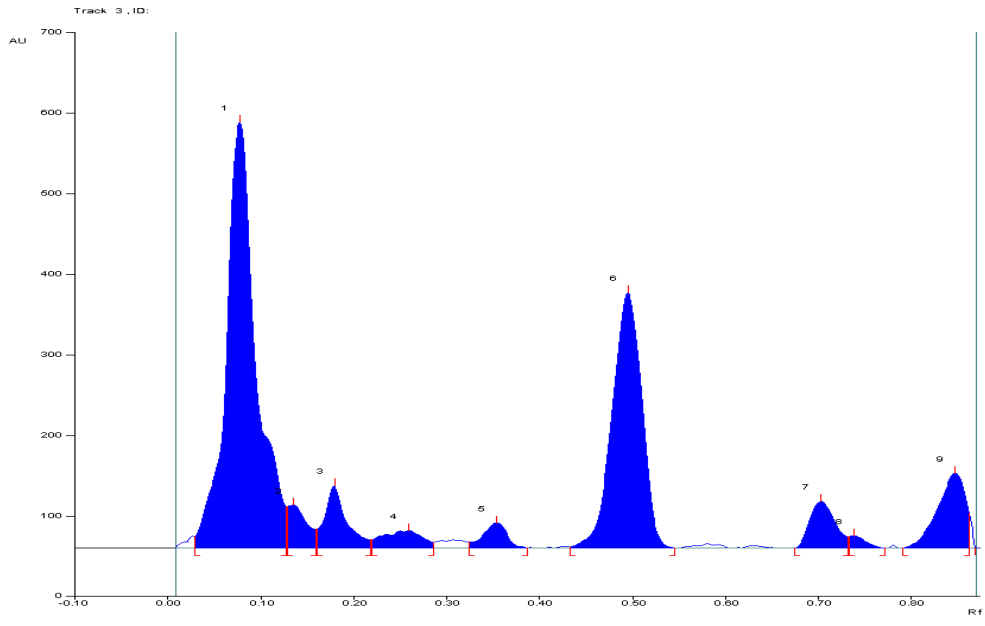


(B)

Figure.10.3. Graphical representation of the HPTLC pattern of aqueous ethanolic extracts of *Hypsizygus ulmarius*. Derivatised plate at 580 nm scan.bmp using vanillin- sulphuric acid spray. (A) FB extract (B) MY extract



(A)



(B)

DISCUSSION

Preliminary phytochemical analysis confirmed the presence of several bioactive compounds such as polysaccharides, phenolic compounds, proteins, steroids, alkaloids and tannins.

Many previous studies have reported antitumor activity of medicinal mushrooms was due to the presence of polysaccharides, alkaloids, polysaccharide-protein complexes, dietary fiber, certain types of proteins, terpenoids, steroids, phenols, etc (Ivanova *et al.*, 2014). Among these Polysaccharides are the best known and most potent bioactive compound with antitumor and immunomodulating properties in mushrooms (Wasser, 2002)**.**

Phenolic compounds are powerful chain breaking antioxidants; their radical scavenging ability is due to hydroxyl groups and exhibits a wide range of spectrum of medicinal properties such as anti-cancer, anti-inflammatory and diabetic effects (Hamzah *et al.,* 2013). The phenolic compounds present in mushrooms have the ability to scavenge radicals such as hydroxyl, superoxide etc. Alkaloids have been reported in the treatment of many human diseases like AIDS, cancer, and lung diseases (Manadal *et al*., 2008). Alkaloids are also known to have inhibitory effects on angiogenesis and therefore are useful in inhibiting the growth of cancerous cells (Aniszewski, 2015).

Bioactive compounds including polysaccharides and terpenoids were isolated from mushroom and evidence from *in vitro* and clinical studies has indicated that mushrooms exhibit cancer-preventive and anticancer activity, which might be due to its antioxidative and radical-scavenging effects, inhibition of metabolic activation and enhancement of detoxification of carcinogens, direct cytotoxicity, antiproliferation, and modulation of signaling transduction molecules, induction of cell-cycle arrest and apoptosis, and enhancement of host immune function.

Among the several bioactive compounds isolated from mushrooms, polysaccharides, terpenes and phenolic derivatives are the most implicated and known to show anti-inflammatory bioactivity. Hence, mushrooms have valuable therapeutic compounds, thus mechanism of action need to be fully elucidated. Experimental and preclinical

studies are needed in order to confirm the effectiveness of some of these mushroom-based compounds for the development of mushroom-based nutraceuticals or drugs.

Value of medicinal mushroom intake has become a matter of great significance, particularly in preventing or treating serious chronic disease conditions such as cancer and cardiovascular disease. The previous studies reveal that aqueous ethanolic extracts of *H.ulmarius* possessed significant antioxidant, anti-inflammatory, antitumor, hepatoprotective and hypocholesterolemic activities. Presence of bioactive phytochemicals detected in the extracts might be responsible for these biological activities of *Hypsizygus ulmarius.*

Summary and conclusion

Mushrooms are increasingly being used as sources of antioxidants because of their bioavailability and lesser side effects. *In vitro* antioxidant activity of the aqueous ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* was evaluated using various assays such as DPPH radical, $ABTS^{+}$ radical, hydroxyl radical, FRAP assay, nitric oxide radical scavenging assays. The results of the present investigation reveal that the ethanolic extracts of *H. ulmarius* possessed significant capacity to inhibit free radical formation and scavenging activity. Both the extracts showed high free radical scavenging activity in a dose dependent manner. This shows the ability of the extracts to scavenge stable free radicals.

Inflammation is a natural response of the body to foreign agents characterised with edema formation. The anti-inflammatory effect of the fruiting bodies and mycelia extracts of *H.ulmarius* showed a dose dependent activity. Carrageenan induced acute inflammation in animals is used to screen anti-inflammatory agents and is one of the most suitable procedure. Formalin induced paw edema model is suitable to screen chronic anti-inflammatory agents as it closely resembled human arthritis (Greenwald, 1991). The present investigations reveal that aqueous ethanolic extracts of *H.ulmarius* possessed profound anti-inflammatory activities in the anti-inflammatory response induced by carrageenan and formalin in mice**.** *In vitro* analysis of COX activity indicated that both fruiting bodies and mycelia extracts of *H.ulmarius* significantly inhibited the cyclooxygenase enzyme activity which reflected the anti-inflammatory potential of this mushroom. Inflammatory response induced by inflammatory mediators generated through up-regulation of inducible pro-inflammatory genes COX-2 and iNOS. (Vane *et al.,* 1994; Jong *et al.,* 2006). Since *H. ulmarius* is an excellent edible mushroom, the medicinal properties of this mushroom have great important practical application. Because, consumption of this mushroom might be useful to prevent several diseases which are mediated through free radicals as evident from its significant antioxidant properties. Anti-inflammatory activity of this mushroom also will be advantageous, as long term consumption of this mushroom will be able to provide relief to patients suffering from inflammation and arthritis.

The current anti-cancer drugs available in market are not target specific and have been demonstrated to possess several side-effects and complications as compared with natural anticancer materials, which highlight the urgent need for novel effective and less-toxic agents from natural products (Fu *et al*., 2013). Mushrooms are known to

complement chemotherapy and radiation therapy by lowering the side-effects of cancer. As such, medicinal mushrooms and their synthetic derivatives are expected to play an important role in developing innovative agents for prevention of human cancer. Considering the high prevalence and incidence of cancers in the country, natural antioxidant products prepared from mushrooms may find a place in the future. The scientific community is consistantly working on natural additives for the treatment of cancer. Only a small percentage of bioactive compounds from mushrooms have been discovered and used as anticancer agents. Extracts of *H.ulmarius* possessed significant antitumor activity. Although the extracts was unable to prevent the tumour development completely, but it was able to reduce the average tumour size significantly in a dose dependent manner. Water soluble polysaccharides have been isolated from the fruiting bodies and mycelia of *Hypsizygus ulmarius* and it significantly reduced the tumour volume induced by DLA cells in mice. Cytotoxicity was evaluated on cancer cell lines which serve as *in vitro* model in cancer research. Ethanolic extracts of fruiting bodies and cultured mycelia exhibited significant cytotoxicity towards Dlaton's lymphoma ascites cancer cells and non-cytotoxic to normal cells. Hence this mushroom may represent a practical and promising approach in anti-cancer therapy based on current experimental data from *in vitro* and *in vivo* studies. The results of the current studies thus suggest the potential therapeutic use of *H. ulmarius* as an adjuvant in the treatment of diseases such as cancer**.** Preliminary chemical analysis of the mushroom extracts showed the presence of phenols, alkaloids, steroids, polysaccharides, tannins and proteins. Many of such compounds are reported to have antitumor activity (Carlo *et al*., 1999). There is not a clear explanation available on the mechanism of tumour inhibiting activity of these compounds. Hence identifying such compounds and their mechanism underlying the cytotoxic and antitumor potential will provide novel drug candidates for treatment of cancer.

Liver is the largest internal organ which plays an essential role in the metabolism of toxic compounds in the body. Over the past several years, the rate of incidence of hepatic diseases has increased manifold. Results of experimental studies showed that aqueous ethanolic extracts of *H.ulmarius* effectively protected the liver from hepatotoxicity induced by CCl_4 and ethanol in rats. Extracts efficiently decreased the elevated serum GOT, GPT and ALP activities in a dose dependent manner. The

treatment with the extracts also restored the depleted levels of antioxidants in liver. The histological observations of liver tissues also supported the protective effect. The results indicated that aqueous-ethanolic extracts of fruiting bodies and cultured mycelia of *H.ulmarius* possessed significant hepatoprotective activity. Being an excellently edible mushroom, the findings suggest potential therapeutic use of elm oyster mushroom for preventing liver disorders. Hence significant use in the development of dietary supplements or nutraceuticals, for therapeutic use. The hepatoprotective activity elicited by the extracts might be due to its free radical scavenging activity and ability to activate antioxidant enzymes.

Fruiting bodies and mycelia extracts of *H.ulmarius* administration may be an effective treatment option against hypercholesterolemia which is a great risk factor for cardiovascular diseases. The extracts are also able to ameliorate reactive oxygen species, serum biochemical parameters and lipid profile. The current experimental findings reveal that, administration of diet rich in the natural antioxidant specially mushrooms is highly beneficial for protection against oxidative stress, and cardiac vascular diseases resulting from hyper-lipidemia, hypercholesterolemia and arteriosclerosis. This could be helpful to find out the significance of adding mushrooms to our diet both as a preventive or curative measure of treating hyperlipidemia and cardiovascular diseases. This would be a more healthy and reliable solution to get rid of harmful side effects created by synthetic drugs. Thus, there is a scope for expanding the present study with the aim of deciphering the antihypercholestremic and antioxidant properties of other edible mushrooms.

Bioactive compounds of natural origin have been the most consistent and successful source for new drugs. Exploration of natural sources for novel bioactive compounds for medicine has been an emerging area of research over the past decades. Among natural sources, microbes especially fungi enjoy a unique position as an important source for the production of many bioactive compounds. In this mushrooms have continued to generate a lot of interest particularly in its consumption as food, in nutrition and cure of diseases and in bioremediation. They are important products of commerce all over the world due to their nutritional, antioxidant and therapeutic values. A large number of mushroom-derived bioactive compounds, both cellular components such as glycoproteins, polysaccharides and secondary metabolites like phenolic compounds, terpenoides, alkaloids, sterols, polyphenols etc. have been

isolated from fruiting bodies, mycelia and culture broth. Their presence makes the mushrooms popular to consume as good food sources and valuable drug. Due to the presence of biologically active compounds of medicinal value, they are used as anticancer, antiviral, hepatoprotective, immunopotentiating and hypocholesterolemic agents.

In conclusion, the present study reveals that the aqueous ethanolic fruiting bodies and mycelial extracts of *Hypsizygus ulmarius* possessed profound therapeutic activities. These activities elicited by the extracts might be due to its free radical scavenging activity and ability to activate antioxidant enzymes. The finding thus suggests the potential therapeutic use of *Hypsizygus ulmarius.* But further studies are required to isolate and characterise the active principle responsible for these activities and their mechanism of bioactivities.

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List of publications

- Greeshma PV, Ravikumar KS, Neethu MN, Meera, Zuhara KF, Janardhanan KK. Antioxidant, Anti-Inflammatory, and Antitumor Activities of Cultured Mycelia and Fruiting Bodies of the Elm Oyster Mushroom, *Hypsizygus ulmarius* (Agaricomycetes). *International Journal of Medicinal Mushrooms*. 2016; 18(3): 235–244.

- PV Greeshma, J Feba, KF Zuhara, KK Janardhanan. *Hypsizygus ulmarius* (Bull.:Fr.), elm Oyster mushroom attenuates carbon tetrachloride-induced hepatic injury in Wistar rats. *Ind J Exp Bio*. (Accepted)

- PV Greeshma, KK Janardhanan. *In vivo* hepatoprotective activity of aqueous ethanolic extracts of elm oyster mushroom *Hypsizygus ulmarius* (Bull.:Fr.) against ethanol induced hepatotoxicity. (Communicated).