1-10-2024

Medicinal Mushrooms: The Extraction and Analysis of Bioactive Compounds from Hericium erinaceus and Ganoderma lucidum Mushrooms.

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By

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A Thesis

Submitted to the Faculty of Graduate Studies

through the Department of Chemistry and Biochemistry

in Partial Fulfillment of the Requirements for

the Degree of Master of Science

at the University of Windsor

Windsor, Ontario, Canada

2023

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ABSTRACT

Medicinal mushrooms, such as *Hericium erinaceus* (lion's mane) and *Ganoderma lucidum* (reishi), have a rich history of traditional use in natural remedies, garnering attention for their bioactive compounds and nutritional benefits. Renowned for being protein-rich and low in fat, these fungi exhibit antimicrobial, anti-tumor, antioxidant, anti-diabetic, and anti-hypercholesterolemic properties attributed to their diverse bioactive components, including polysaccharides, proteins, terpenes, sterols, vitamins, polyphenols, and fatty acids.

This study aimed to optimize extraction techniques, comparing maceration, ultrasound-assisted maceration, and Soxhlet extraction on fresh and dried lion's mane and reishi mushrooms. Varying solvent polarities and extraction times revealed that 8-hour extractions with dried samples, particularly using methanol for Soxhlet extraction, yielded the highest mass recoveries. Maceration with water extraction for 72 hours produced optimal results with dried samples.

Proximate analysis showcased lion's mane with 54% moisture, 7.4% ash, 11.02% fiber, 1.04% fat, 23.94% protein, 57.45% carbohydrate, and 312.72 kcal/100g total energy. Reishi exhibited 7.28% moisture, 0.76% ash, 4.38% fiber, 3.87% fat, 18.03% protein, 65.67% carbohydrate, and 355.44 kcal/100g total energy.

Assays for protein concentration, β-glucan content, and antioxidant activity demonstrated higher soluble protein and β-glucan in lion's mane extracts, particularly with methanol and dried samples. Fresh samples, however, exhibited superior antioxidant abilities.

Quantification via HPLC-DAD revealed the degradation of ergosterol to Vitamin D2 over time, with higher content in dried samples. Ganoderic acid A was absent in lion's mane but efficiently extracted with ethyl acetate in reishi. Polyphenol composition will be further investigated for accurate results. Fatty acid profiling using GC-FID, with oleic acid as a standard and olive oil as a positive control, provided additional insights.

In conclusion, these analyses and extraction protocols offer valuable insights into the medicinal potential of lion's mane and reishi mushrooms. This knowledge paves the way for developing promising remedies for various ailments and diseases.

DEDICATION

To my cherished family and those whose unwavering support has been my anchor.

ACKNOWLEDGEMENTS

I extend my heartfelt gratitude to my family for their unwavering support, patience, and understanding throughout the entirety of this academic endeavor. Their unwavering encouragement has been the driving force behind my pursuit of knowledge and dedication to this research.

A special acknowledgment is reserved for Dr. Mary Egbuta, whose exceptional leadership and guidance have played a pivotal role in shaping my academic and research trajectory. As a postdoctoral mentor, Dr. Egbuta's generosity in sharing her wealth of knowledge and expertise in medicinal mushrooms has significantly enriched my learning experience. Her mentorship has not only refined my research skills but has also inspired a deeper appreciation for the intricate world of Mushrooms and their medicinal potential.

I express my deepest gratitude to my supervisor, Dr. John Trant, for his continuous support, scholarly insights, and unwavering encouragement. Dr. Trant's mentorship has been instrumental in navigating the challenges of research and academia. His commitment to excellence and passion for advancing scientific knowledge have been a guiding light throughout my thesis journey.

In addition to my family and mentors, I would like to acknowledge my research group for their invaluable contributions to my research. Their support, whether through insightful discussions, technical assistance, or emotional encouragement, has significantly enriched the quality and depth of this thesis.

This thesis stands as a testament not only to my personal dedication but also to the collective efforts of those who believed in me and contributed to my growth as a researcher. To everyone who has been a part of this journey, I offer my sincere appreciation. Thank you for being an integral and inspiring part of my academic adventure.

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LIST OF ABBREVIATIONS

AA: Arachidonic acid

ABTS: 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

AD: Alzheimer's Disease

CDS: Chromatography data system

COX: Cyclooxygenase

CUPRAC: Cupric reducing antioxidant capacity

CVD: Cardiovascular diseases

DAD: Diode array detectors

DB: Degree of branching

DCM: Dichloromethane

DHA: Docosahexaenoic acid

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

ER: Endoplasmic Reticulum

FAMES: Fatty Acid Methyl Ester

FID: Flame ionization detection

FIPs: Fungal immunomodulatory proteins

FLAP: Five-lipoxygenase activating protein

FRAP: Ferric reducing antioxidant potential

GAA- Ganoderic Acid-A

GC: Gas chromatography

GLC: Gas-liquid chromatography

GLT: *Ganoderma lucidum* triterpene

GSC: Gas-solid chromatography

HAT: Hydrogen atom transfer

HDL: High-density lipoprotein

HIV: Human immunodeficiency virus

HPLC: High-Performance Liquid Chromatography

IGFR: Insulin-like growth factor receptor

IR: Insulin receptors

LDL: Low-density lipoprotein

LSD: Lysergic acid diethylamide

MIC: Minimum inhibitory concentration

MM: Medicinal mushrooms

MRSA: Methicillin-resistant *Staphylococcus aureus*

MS: Mass Spectroscopy

MW: Molecular weight

NGF: Nerve growth factor

NK: Natural killer

ORAC: Oxygen radical absorbance capacity

OS: Oxidative stress

PDA: Photodiode array detectors

PUFAs: Polyunsaturated fatty acids

RIPs: Ribosome Inactivating Proteins

Rt: Retention time

SET: Single electron transfer

TCDs: Thermal conductivity detectors

TFA: Trifluoracetic Acid

UV: Ultraviolet

VEFGR: Vascular endothelial growth factor receptors

VEGF: Vascular endothelial growth facto

CHAPTER 1: Introduction

1.1 Mushrooms: Scientific Origin, Geographical History, Uses in Traditional Medicine

1.1.1 Scientific/ Medicinal Significance of Mushrooms

Mushrooms are macro-fungi, commonly identified by their significant differences in shapes, sizes, spore colour and chemical compositions. They are described "as a macrofungus with a distinctive fruiting body, which can be epigeous or hypogeous and large enough to be seen with the naked eye and picked by hand"[1]. In reality, what we call a mushroom is only the fruiting body of the fungus. They can either be exotic (rare or not indigenous to the area they are observed) or non-exotic. Exotic mushrooms, such as edible and medicinal varieties, belong to the group of eukaryotic, heterotrophic organisms known as fungi. While fungi were initially categorized within the Plant Kingdom, they are now acknowledged as a distinct group called the Kingdom Mycetea[1].

Despite mushrooms being cherished as a valuable ingredient in gourmet cuisine worldwide, only approximately 25 out of the 2000 species found in nature are recognized as food sources [2]. The number of them that are being cultivated for commercial purposes is even less. Mushrooms are celebrated for their culinary excellence, rich nutritional content, and functional benefits[2]. They are not only considered a delicacy but are also acknowledged as nutraceutical foods. Their appeal lies in their sensory attributes, medicinal potential, and economic importance[2]. However, its important to note that it isn't necessarily easy distinguishing between edible and medical mushrooms because many mushrooms commonly consumed as food also offer therapeutic properties, and several used in traditional medicine are also edible[2].

The most commonly grown edible mushroom globally is the *Agaricus bisporus*, with *Lentinus edodes*, *Pleurotus* spp,, and *Flammulina velutipes*, following closely[3]. Mushroom cultivation has seen a consistent rise, and China stands out as the leading producer worldwide. Nonetheless, wild mushrooms are gaining increasing significance due to their nutritional value, sensory appeal, and notably, their pharmacological properties[2].

***Agaricus bisporus* (Button mushroom)**

***Lentinus edodes* (Shiitake mushroom)**

***Flammulina velutipes* (enoki mushroom)**

***Pleurotus species* (oyster mushroom)**

Figure 1: The most common edible mushroom species[2].

The utilization of medicinal mushrooms has a long and varied history that cuts across multiple cultures and civilizations. These fungi have been harnessed for their perceived health advantages for countless centuries, forming an integral part of traditional healing traditions. This historical application is firmly grounded in the practices of traditional medicine, reflecting the enduring recognition of mushrooms for their potential therapeutic properties.

Mushrooms have been utilized as traditional ancient therapies since the Neolithic age and are apart of many cultures[4]. In Europe, the amadou mushroom (*Fomes fomentarius*) was classified by Hippocrates, the Greek physician, circa 450 BC, as a powerful anti-inflammatory as well as for

cauterizing wounds. Similarly, the world's oldest wet mummy, Ötzi, the Ice Man, lived nearly 5300 years ago[5]. It was said that he carried around amadou mushroom and a birch polypore tethered in a pouch to help him survive in the Alps of northern Italy. In Asia, a well-known alchemist from the 5th century, Tao Hongjing, described the usage of many medicinal mushrooms, some of which were even used by Shennong, who is an known farmer in ancient Chinese culture[5]. These include ling zhi (*Ganoderma lucidum*) and zhu ling (*Dendropolyporus umbellatus*) [5]. Also, the First peoples of North America used puffball mushrooms (*Calvatia genus*) as wound healers[5].

(*Fomes fomentarius*) Amadaou

ling zhi (*Ganoderma lucidum*)

zhu ling (*Dendro Polyporus umbellatus*)

Giant Puffball (*Calvatia gigantea*)

Figure 2: Some common mushrooms used in tradition medicine[6-9]

There is also a long history of the usage of the genus *Psilocybe* as curatives in Mesoamerica[4]. These mushrooms are a source of naturally occurring psychedelics and were used for religious and

spiritual ceremonies. Also referred to as “magic mushrooms”, they are classified as hallucinogens that produce mind-altering and reality-distorting effects, known as hallucinations, once ingested[10]. Today, they are being formulated into drugs and used as a therapeutic option for neuropsychiatric conditions[10]. Typical psychedelic (serotonergic) drugs, like LSD and psilocybin, interact with serotonin receptors (5-HT receptors) and their various subtypes found in the brain[10]. These receptors are densely distributed in the brain and have a role in regulating emotions (including anxiety and aggression), cognitive processes, sexual behavior, learning and memory, appetite, and various other physiological, neurological, and neuropsychiatric functions[10]. Furthermore, these serotonin receptors are not limited to the brain but can also be found in the central and peripheral nervous systems. As a result, they serve as targets for a variety of recreational and pharmaceutical drugs, including hallucinogens, empathogens, antipsychotics, antidepressants, drugs for preventing nausea (antiemetics), medications for managing migraines (antimigraine agents), and appetite suppressants (anorectics)[10].

Much of the knowledge gained from medicinal mushrooms (MM) which has been passed down since the stone age has been validated and documented using modern day research. Under the banner of Natural Product chemistry, an interdisciplinary field of science that focuses on MMs has been developed that showcases many of the unique natural products that are extracted from a variety of mushroom species over the last 30 years. Many Asian countries such as Japan, China, Korea and Russia heavily utilize mushroom derived compounds in their modern clinical practices[4.]

The escalation in interest of mushrooms is not only related to its rich cultural history of use, but it is also fueled by modern advancements in mycelium tissue culture techniques and innovative methods for assessing the effects of individual components and their interactions[5]. For instance, *Ganoderma lucidum*, also known as reishi, is known to possess over 16,000 genes, responsible for the synthesis of more than 200,000 compounds[5]. Among these, approximately 400 are identified as "active constituents"[5]. To date, over 150 novel enzymes have been identified from mushroom species so far, demonstrating that there are many natural products made by mushrooms that merit medical study[5].

Mushrooms have the potential to serve as an alternative reservoir of novel antimicrobial substances, particularly secondary metabolites like terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolones[2]. They can also yield some primary metabolites such as oxalic

acid, peptides, and proteins with antimicrobial properties[2]. Among mushroom species, *Lentinus edodes* has garnered the most research attention and appears to exhibit antimicrobial effects against a broad spectrum of bacteria, including both gram-positive and gram-negative strains[2].

Apart from microbial properties, mushroom have excellent nutritional value attributing to their high protein content including essential amino acids and dietary fiber. They have low fat content but contain essential fatty acids in substantial amounts. Additionally, edible mushrooms are rich in various vitamins, including B1, B2, B12, C, D, and E. As a result, they have the potential to serve a an excellent resource for a variety of nutraceuticals and incorporating them in everyday diet would promote health due to the combined effects of the numerous bioactive compounds they contain[2].

A wide array of mushrooms has been historically employed in various cultures to support overall health and to prevent and treat diseases, due to their immune-regulating and anti-cancer properties. Thereby, it is being said that mushrooms are like "miniature pharmaceutical factories", generating compounds with remarkable biological attributes[2]. Additionally, since we have gained new knowledge in understanding the underlying processes that govern the development and spread of tumours, mushrooms provide the opportunity to explore and discover drugs that may counteract the abnormal molecular and biochemical signals that contribute to cancer[2].

Mushrooms and fungi offer over 100 medicinal functions, with their primary uses encompassing antioxidative, anticancer, antidiabetic, antiallergic, immunomodulatory, cardiovascular protection, cholesterol reduction, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective effects, as well as safeguarding against tumor growth and inflammatory processes[2]. In addition to the valuable compounds mentioned previously in mushrooms, a plethora of bioactive molecules are found in their fruiting bodies, cultured mycelium, and culture broths[2]. These include polysaccharides, proteins, fats, minerals, glycosides, alkaloids, volatile oils, terpenoids, tocopherols, phenolics, flavonoids, carotenoids, folates, lectins, enzymes, ascorbic acid, and organic acids, among others. Among these, polysaccharides, with β-glucan being the pinnacle as it is most significant in modern medicine due to its biological versatility[2].

Modern science is now recognizing the role of mushrooms in naturopathic medicine and utilizing them to reinforce conventional medical practices. Using solvents other than water for extraction has opened up a whole new range of compounds that were not available to our ancestors[5]. Scientists are now finally fully involved in swiftly evolving scientific transformation in the realm of medicinal mushrooms.

There is a range of exotic mushrooms, encompassing those that are edible and those with medicinal properties. Among the edible varieties are Grey Oyster mushrooms (*P. ostreatus*), Shiitake mushrooms (*Lentinula edodes*), Lion's Mane mushrooms (*Hericium erinaceus*), and Maitake mushrooms (*Grifola frondose*); all of which are known for their therapeutic qualities[11]. In addition, two species, *G. lucidum* (reishi) and *Trametes versicolor* (Turkey Tail), are exclusively considered medicinal mushrooms and are not intended for consumption[11]. However, this study will primarily concentrate on *G. lucidum* and *H. erinaceus* to delve into their advantages and applications taking into consideration their chemical composition

1.1.2 Species of Mushrooms: Hericium erinaceus (Lions Mane) and Ganoderma lucidum (Reishi).

Hericium erinaceus (Lions Mane)

H. erinaceus also known as Yamabushitake (Japanese), Houtou/猴头菇 (Chinese) or Lion's Mane, is a type of edible fungus that holds considerable importance in the field of medicine[12]. The most well-known name, "Lion's Mane" is due to its distinctive appearance, which resembles a cascading mane adorned with white, typically long, dangling spines. When mature, they are white at first, then becoming yellowish, later brownish with age. It is a basidiomycete belonging to the family Hericiaceae, order Russulales and class Agaricomycetes, formerly recognized as *Hydnum erinaceum*. This mushroom stands out as one of the few that imparts a taste resembling that of lobster or shrimp when prepared as a dish[12]. Classified as a saprotroph or weak parasite, lions mane mushroom typically grows on decaying wood, but occasionally, its fruiting bodies can emerge from knotholes or crevices in living hardwood trees[12]. This behavior could suggest that it may also have an endophytic way of life, residing inside the living tree. While it's not commonly found in

Europe and rarely found in tropical or polar regions, this species thrives in various regions across Asia and North America[12]. In Poland, all the species belonging to the *Hericiaceae* family are legally safeguarded because of their scarcity[12]

Hericium erinaceus **(Lions Mane)[13]**

In terms of growth and cultivation, monokaryotic mycelia of Lion's Mane mushroom tend to grow more slowly compared to dikaryotic cultures[14]. Optimal conditions for sporulation include increased temperature and reduced relative humidity under natural conditions[14]. However, in a laboratory setting, sporulation is favored at 85-95% relative humidity and temperatures of 24-27°C, ceasing at higher temperatures[14]. Growth regulators such as 2,4-D and gibberellin and low-intensity light stimulate spore germination and mycelium growth[14]. Additionally, the use of argon and helium lasers accelerates fructification and increases fruitbody weight and yield[14]. For optimal growth, temperatures of 21-24°C and substrate moisture content of 50-70% are favoured[14]. For the fruiting period, a constant temperature of 23°C is recommended, with pH ranging from 5.8 to 6.2[14].

Cultivating lions mane mushroom can either be extensive or intensive[14]. Applied on a wide scale in China, extensive cultivation is simple and involves spawning wood logs or stumps with mycelium overgrown wood fragments[14]. Fruiting occurs under natural, uncontrolled conditions, which can lead to long periods before harvest. Conversely, intensive cultivation methods are used to achieve higher yields and better quality[14]. These methods typically involve the use of bottles or bags with sterilized substrates and controlled conditions[14]. Bottles are made from heat-resistant materials like polypropylene and equipped with filters for gas exchange, while cultivation in polypropylene bags is simpler and cheaper but may yield smaller fruitbodies compared to bottles[14]. Lion's Mane mushroom can be cultivated on various substrates, including wood from coniferous trees like Pinus species. Certain fungal species are used to prepare the wood for Lion's Mane

cultivation. Substrate compositions may vary, with examples like sawdust supplemented with cereal bran, cotton chaff, wheat bran, corn meal, and gypsum[14]. Various combinations have been successful for promoting mycelium growth and fruitbody yields. Rice straw with high nutrient content can be added to enrich the substrate and accelerate primordium formation[14]. Other additives, such as vegetable oils, fatty acids, sunflower husks, Mn (manganese), and NH_4 (ammonium), have been used to enhance mycelium growth and fruiting body yields[14].

Lion's Mane mushroom offers a nutritional profile rich in various macronutrients and amino acids. Additionally, it contains a diverse array of aroma compounds, with 1-octen-3-ol being a prominent component of its aroma profile (56–60% of the total aroma substances)[14]. Both the fruitbodies and mycelium of lion's mane mushroom contain health-promoting compounds. Cultivation methods have been optimized to obtain selected substances from the fruiting bodies. The most important groups of compounds include polysaccharides, hericenones, and erinacines[14].

Bioactive polysaccharides from mushrooms, including lion's mane, have been extensively studied for potential pharmaceutical and functional food applications. Polysaccharide content in the fruiting bodies of lions mane mushroom are considerably higher in the fruiting bodies (26.63%) than in the mycelium (18.71%)[14]. There have been 12 polysaccharides that have been successfully isolated from *H. erinaceum* extract, with enzymatic extraction resulting in a significant 67.6% increase in number of polysaccharides compared to hot water extraction. Studies conducted on the qualitative and quantitative composition of polysaccharides in the fruitbodies show that arabinose is the most abundant, followed by glucose and rhamnose[14]. Lion's mane mycelia, stimulated with Lipopolysaccharides (LPSs) have also displayed significant antioxidative activities by increasing hepatic glutathione levels in mice, while β-glucans reduce lipid peroxidation levels, indicating their potential in enhancing antioxidant defenses[15, 16]. Interestingly, β-glucans also show anti-skin aging properties by inhibiting matrix metalloproteinase (MMP)-1 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 activities in aged rat models[17].

There are also low molecular weight secondary metabolites isolated from the fruiting bodies of lions mane mushroom[12]. They have poor solubility in water and their extraction requires the use of organic solvents such as methanol or ethyl acetate[12]. Often exhibiting non-specific activities in biological systems, these include hericenones and erinacines. Hericenones A, B, C, D, E, F, G, H

(Figure 3) are benzyl alcohol derivatives predominantly found in the fruiting body of mushroom. Studies showed that Hericenones A and B exhibited cytotoxic properties against HeLa cells, inhibiting their growth[12 9]. Additionally, hericenones C, D, and E stimulated the synthesis of nerve growth factor (NGF), with hericenone E being the most potent in stimulating NGF production. Erinacines, including A, B, C, E, F, H are diterpenoid derivatives found in the mycelium of the mushroom[12 9](Figure 4). They are known to demonstrate strong activity in inducing NGF synthesis, both in vitro and in vivo[12, 14]. *Hericium erinaceus* is considered a biotransformation model species, as it is well-known for efficient terpenoid production of the unique cyathane type. This includes erinacine E, which is a unique terpenoid in the cyathane family.

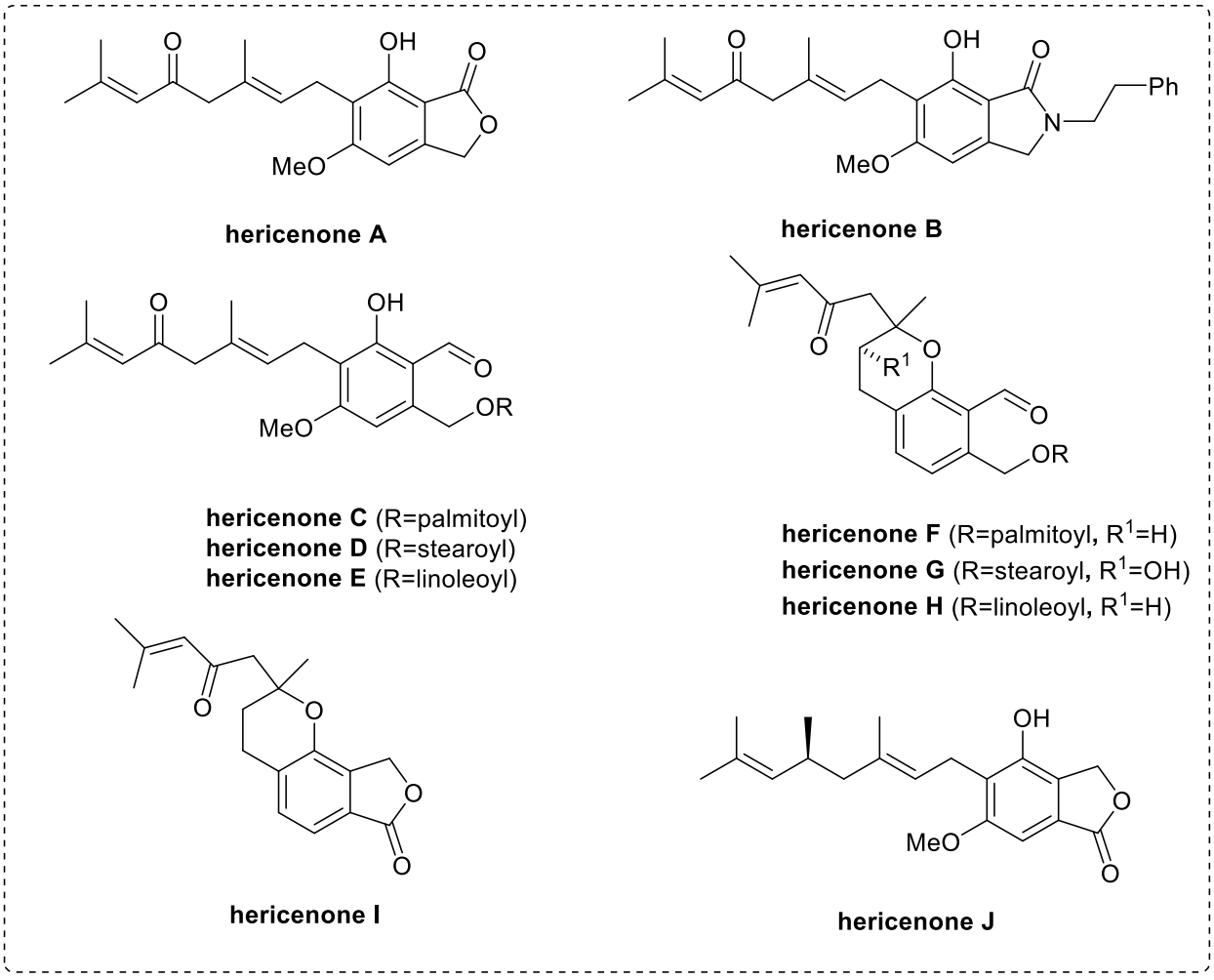

Figure 3: Chemical structures of selected hericenone derivatives

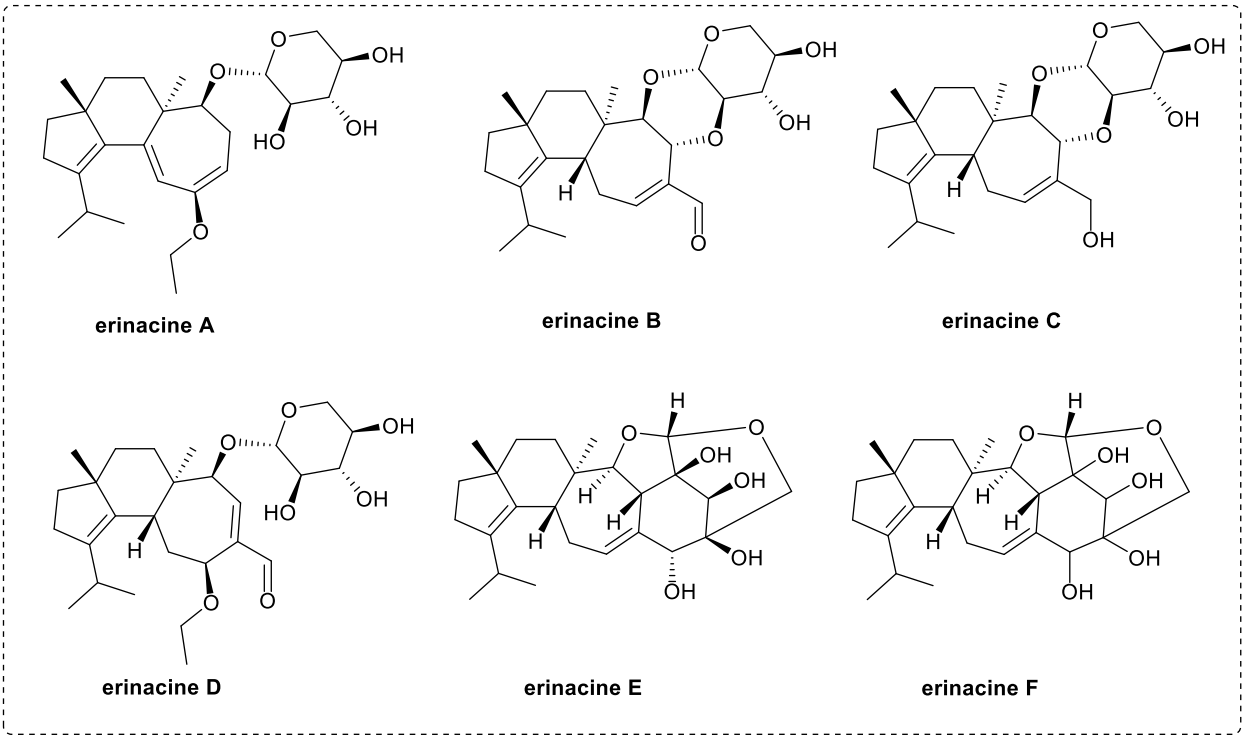

Figure 4: Chemical structures of erinacines and other cyathane diterpenes with neuroprotective effects from H. erinaceus

Lion's mane mushroom has a long history of traditional medicinal use and is now the subject of extensive research to uncover its potential health benefits. Chinese and Japanese healers utilized the fruiting bodies and mycelium in both cuisine and medicine, particularly in the treatment of conditions like gastritis. Moreover, Chinese medical practitioners have recommended the use of *H. erinaceus* as a preventive measure against gastrointestinal cancer[5]. Recently, this species is getting more attention because it has been proposed as a natural alternative to treat symptoms of dementia and cognitive decline in the elderly[18]. Research has been conducted, including experiments with extracted compounds on mice in a controlled environment, with these studies presenting encouraging outcomes[14]. The extracts have demonstrated potential for the treatment of cancer, liver-related conditions, and neurodegenerative diseases like Alzheimer's and Parkinson's by achieving surrogate endpoints in *in vitro* and *in vivo* preclinical studies[19].

Preparations derived from *Hericium erinaceus* may hold promise in the treatment of several types of cancer, including esophageal, intestinal, pancreatic, and stomach cancers[12]. Studies have shown that patients who received *H. erinaceus*-based treatments experienced notably fewer adverse side effects compared to those undergoing radiotherapy and chemotherapy[12]. Another study reported

that extracts consisting of crude water-soluble polysaccharides from the mushroom demonstrated significant anticancer activity. This includes effects against a range of cancer cell lines, including hepatocytes, mammary carcinoma, lymphoma, esophageal cancer, and others[20]. The results indicated that the polysaccharides found in lions mane have an anti-tumor impact by stimulating various immune cells. They do this by increasing the expression of specific cytokines like IL-1ß and TNF-ß and by promoting the production of nitric oxide (NO)[20]. These polysaccharides, more specifically, the β-glucans, also exhibit immunomodulatory effects. Liu et al[21], reported significant effects on artificial pulmonary metastatic tumor in mice[21]. They enhance the immune response by increasing the activity of immune cells, such as natural killer (NK) cells, T cells, and macrophages. This enhanced immune response can play a crucial role in fighting cancer as they are cytotoxic to tumour cells[21].

Lions mane mushroom is also reported to have antihyperglycemic (lowering high blood glucose) and anti-hypercholesterolemic (reducing high cholesterol) activities. This is due to being rich in various bioactive compounds, including D-threitol, D-arabinitol, palmitic acid, and α-D-glucan, that have demonstrated its potential to improve blood glucose control and lipid profiles in animal studies[22, 23]. These findings suggest that *Hericium erinaceus* may offer therapeutic benefits in the context of metabolic health and cardiovascular disease prevention. Additionally, this mushroom's methanolic mycelial extracts have shown protective effects against liver damage induced by substances like CCl4[24].

Hericenones and erinacines in *H. erinaceus* extracts have been reported to demonstrate the ability to stimulate NGF synthesis, potentially aiding in the treatment of Alzheimer's disease (AD). These compounds are low-molecular-weight and can cross the blood-brain barrier, making them effective candidates for neuroprotection and regeneration. Furthermore, studies have shown that these extracts can also reduce endoplasmic reticulum (ER) stress and amyloid-β peptide toxicity, which are factors contributing to neurodegenerative diseases[25]. Additionally, the mushroom's extracts promote myelin genesis, supporting nerve tissue development[26].

Phenol-like and fatty acid-like compounds derived from lions mane exhibit antifungal and antibacterial activity. Some specific compounds, including 4-Chloro-3,5-dimethoxybenzyl alcohol, 4-chloro-3,5-dimethoxylbenzaldehyde, and chlorinated orcinol, have been recognized for their antimicrobial properties[27]. Phenolic compounds from this mushroom also contribute to its

antioxidant ability, with mycelial extracts showing the highest total phenolic content and ferric reducing antioxidant power (FRAP)[28, 29]. Additional research in the 2000s revealed more antimicrobial compounds for the mushroom that showed effectiveness against a broad spectrum of microorganisms, including fungi, protozoa, and various pathogenic gram-positive and gram-negative bacteria, one of which is Methicillin-resistant *Staphylococcus aureus* (MRSA). The mycelium extract exhibited a minimal inhibitory concentration (MIC) with an EC_{50}value of 5.5 µl/ml[30]. Another study reported that metabolites from the fruiting body of *H. erinaceus* exhibited antimicrobial activity by stimulating the immune system to inhibit the growth of *Salmonella*[31]. There was also a direct inhibitory effect of ethanol and ethyl acetate extracts against *Helicobacter pylori*, a bacterium associated with chronic gastritis and gastric ulcers[32].

Despite being one of the well-known medicinal mushrooms, ongoing research continues to unveil new metabolites and compounds in Lion's Mane mushroom, broadening our understanding of its potential applications and benefits.

Ganoderma lucidum (Reishi)

The Latin term "lucidum," meaning shiny or brilliant, is a fitting description of the **reishi** mushroom's fruiting body, which boasts a varnished and artistically sculpted appearance. Recognized by different names, the Chinese and Koreans refer to it as "Ling Zhi," signifying the mushroom of herb and immortality, while the Japanese know it as "Reishi" or "Mannentake," translating to “the 10,000-year mushroom” [33]. Throughout the ages, *Ganoderma lucidum* extracts have been revered and passed down through generations, believed to possess remarkable qualities such as being a potential "cancer cure." It serves as a symbol of positive omens, good fortune, robust health, long life, and even immortality. This mushroom's significance can be traced back to the Yuan Dynasty (1280–1368 A.D.), where it found its way into numerous forms of art, including paintings, carvings, furniture designs, and various artistic expressions, becoming a prevalent motif wherever artistic inspiration could flourish. However, its first historical mention can be traced back to the era of United China's first historical emperor, Shinghuang of the Ch'in Dynasty (221–207 B.C.). Following this, depictions and references to this remarkable fungus became ubiquitous in Chinese literature and art. In North America and Europe, some people recognize it as one of the "artist's conk" fungi, although it's important to note that the true artist conk is a different species, *Ganoderma applanatum*[33].

***Ganoderma lucidum* (Reishi)[34]**

Traditionally, this basidiomycete fungus has been recognised for its medicinal purposes by Chinese medicinal professionals. In China and Japan, it is used to address a variety of health issues, including insomnia, cancer, high cholesterol, and hypertension. This has earned *G. lucidum* a reputation as the mushroom with the most medicinal properties[35]. When used as a treatment for cancer, *G. lucidum* appears to stimulate the production of cytokines and antibodies, leading to the suppression of various tumors[36]. Moreover, it exhibits antimicrobial properties, effectively combating Helicobacter pylori and even HIV[35]. It possesses antioxidant properties, capable of neutralizing harmful free radicals[37]. Due to these beneficial attributes, *G. lucidum* is currently grown on a large scale for commercial purposes and widely used as a dietary supplement[35].

Out of over 2,000 known reishi mushroom species, only six have been extensively examined for their potential health benefits. These six types include red, black, blue, white, yellow, and purple reishi. Among them, red and black reishi have demonstrated the most substantial health-enhancing effects, making them prominent in the global health supplement market. Black reishi, scientifically known as *Ganoderma sinensis*, can grow up to ten inches in diameter, although most mature specimens are around six inches. Many products that claim to contain "wild" reishi utilize or contain black reishi. Even though black reishi still offers some moderate herbal benefits, it is considered inferior to red reishi because of its lower polysaccharide content. Wild purple reishi is exceedingly rare and closely resembles red reishi in appearance, but it has a distinct purple coloration at the center of the mushroom cap. Research on this type of reishi has been limited, primarily due to the scarcity of genuine purple reishi specimens[38]. This study focuses on experiments using Red reishi.

Blue-Reishi

Purple-Reishi

Yellow-Reishi

White-Reishi

Black-Reishi

Red-Reishi

Figure 5: Different types of reishi mushroom[39-44].

Taxonomically, reishi belongs to the Fungi Kingdom, Basidiomycota Division, Agaricomycetes Class, Polyporales Order, *G. lucidum* taceae Family and the *G. lucidum* Genus with a species name of *G. lucidum* [45]. Often considered the most potent medicinal mushroom, this mushroom boasts a rich history of use spanning over two millennia[46]. Despite its scarcity in nature, this wood-decaying fungus with a penchant for causing white rot in various tree species has a global presence in green ecosystems[46]. It thrives in both temperate and subtropical regions due to its ability to withstand hot and humid conditions, but it is more prevalent in tropical areas. It can be found in

India and Asia, where it enjoys a dominant presence. Consequently, it is most widely cultivated in countries such as China, Japan, Malaysia, Korea, Taiwan, and North America[46]. In its natural habitat, Reshi is saprotrophic. It grows on deciduous trees such as oak, maple, elm, willow, sweet gum, magnolia, and locust and is rarely found on coniferous trees. In Asian regions, this mushroom species predominantly thrives on plum trees. You can also discover it growing on tree stumps, typically close to the ground's surface, and sometimes even on soil that forms atop buried tree roots[33].

In the early 1970s, successful artificial cultivation of *Ganoderma lucidum* was achieved, and since 1980, particularly in China, its production has grown significantly. Today, various methods are now employed for its commercial production, including wood log, short wood segment, tree stump, sawdust bag, and bottle procedures. Log cultivation methods involve natural logs and tree stumps inoculated with spawn under natural conditions. A more advanced technique employs sterilized short logs, which are approximately 12 cm in diameter and 15 cm long, resulting in a shorter growing cycle, higher biological efficiency, and superior fruiting body quality. This method, while beneficial, is more complex and costly compared to natural log and tree stump methods. For short-log cultivation, logs are prepared from broad-leaf trees, preferably oak, during the dormant period, ensuring an optimal log moisture content of about 45–55%. The process involves tree selection, sawing log segments, transferring them to plastic bags, sterilization, inoculation, spawn growth, log burial in soil, tending to fruiting body development, harvesting, drying, and packaging. The logs are typically buried in soil within a greenhouse or plastic shed, with well-balanced soil conditions of drainage, air permeability, and water retention, avoiding excessive humidity. Cultivation substrates using plastic bags or bottles may include combinations of sawdust (most common), wheat bran, gypsum, soybean powder, bagasse, cane sugar, cotton seed hull, corn cob powder, cereal straw ash, and more. After sterilization, these plastic bags can be placed horizontally on beds or the ground to facilitate fruiting[47].

To cultivate *Ganoderma lucidum* successfully, specific growth conditions must be maintained. These conditions include temperature, humidity, light and oxygen levels. Spawn production has an ideal temperature between 25-32˚C, with the sawdust substrate requiring an optimal moisture content of 65-70%. For primordial induction, humidity needs to be kept high, ideally between 90-100%, then down to 80-95% for cap formation. In the final stages of fruit body development, lower

the humidity to 30-40%. For primordia formation, temperature ranges from 18-24˚C, with high carbon dioxide (CO2) levels, between 20,000-40,000 ppm and 4-8 hours of light at 200-500 lux. Meanwhile, optimal fruiting body development falls between 21-27˚C, with lower CO_2 levels of < 2000 ppm and 12-hour light cycles at 750-1500 lux. Considering all these parameters, it takes approximately 14-28 days to complete primordial formation, while fruiting body development requires up to 60 days[48].

Even though reishi is considered too tough to be edible, similar to Lions Mane mushroom, it has a high nutritional value, but its medicinal value is largely attributed to its many bioactive compounds[33]. Its major bioactive constituents are polysaccharides, triterpenoids, proteins and amino acids. Research suggests that the potent components in reishi responsible for enhancing the body's immune system are these polysaccharides, particularly those with anti-tumor properties, which have been structurally analyzed[38]. It also contains nucleotides, sterols, steroids, and fatty acids.

The major bioactive *Ganoderma* polysaccharides species are β-1-3 and β-1-6-d glucans. The structure is β-1-3 d-glucopyronan with 1–15 units of β-1-6 monoglucosyl side chains. These polysaccharides are known for their high molecular weights, which increase their water solubility and enhance their effectiveness in anti-tumor activity. Interestingly, some water-insoluble polysaccharides also exhibit anti-tumor properties. The branching of polysaccharides plays a role in determining their activity. Research into the anti-tumor and immune-modulating effects of *G. lucidum* dates back to 1957, and more recently, extensive studies have focused on identifying the key anti-tumor components, particularly polysaccharides and protein/peptide-bound polysaccharides. In the early 1980s, several glucans isolated from water and alkali extracts were discovered to be bioactive[49].

Ganoderma lucidum contains over 140 different triterpenes, with the majority being bitter-tasting compounds, primarily ganoderic acids. These are the main active terpenoids in this mushroom. Recently, a new triterpenoid called ganosporeric acid A was found in the spores of the mushroom[50]. Terpenoids can be classified into four groups based on their properties and chemical structure: (a) volatile mono- and sesquiterpenes (essential oils) consisting of 10 or 15 carbon atoms, (b) less volatile diterpenes with 20 carbon atoms, (c) non-volatile triterpenoids and sterols containing 30 carbon atoms, and (d) carotenoid pigments with 40 carbon atoms.

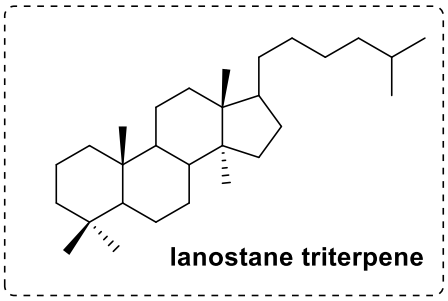

Figure 6: Chemical structure of lanostane triterpene

Triterpenes share a chemical structure based on lanosterol, an essential intermediate compound. The diverse structures of over 130 lanostane-type triterpenoids have been described, starting with the discovery of ganoderic acid A and B[49]. Studies have identified six new lanostane-type triterpenes, particularly in the spores, such as ganoderic acids g, d, e, z, Z, and y.

The spores are notably rich in ganoderic acids compared to other parts of the fungus, and the composition of triterpenes in the fruiting body varies depending on its growth location. Additionally, the spores contain triterpene lactones, and known triterpenoids have been categorized into ten groups based on their structural similarities and recognized medicinal properties[33].

Proteins with bioactivity can be found in reishi mushroom including LZ-8, which has been successfully isolated. When its sequence was analyzed, it was found to share similarities with the variable region of immunoglobulin heavy chains in terms of sequence and predicted secondary structure. It exhibits significant biological activities resembling lectins, such as stimulating the growth of mouse spleen cells and human peripheral lymphocytes and causing agglutination of sheep red blood cells in laboratory tests. Importantly, LZ-8's agglutination reactions were not inhibited by the sugars tested, suggesting that it does not function as a typical lectin. While LZ-8 does not agglutinate human red blood cells, it can act as a potent suppressor of bovine serum albumin-induced anaphylaxis in CFW mice in experimental settings[51]. These findings indicate that LZ-8 is related to an ancestral protein of the immunoglobulin superfamily. Reishi also contains sterols, amino acids, soluble proteins, oleic acid, cyclo-octasulfur[33]

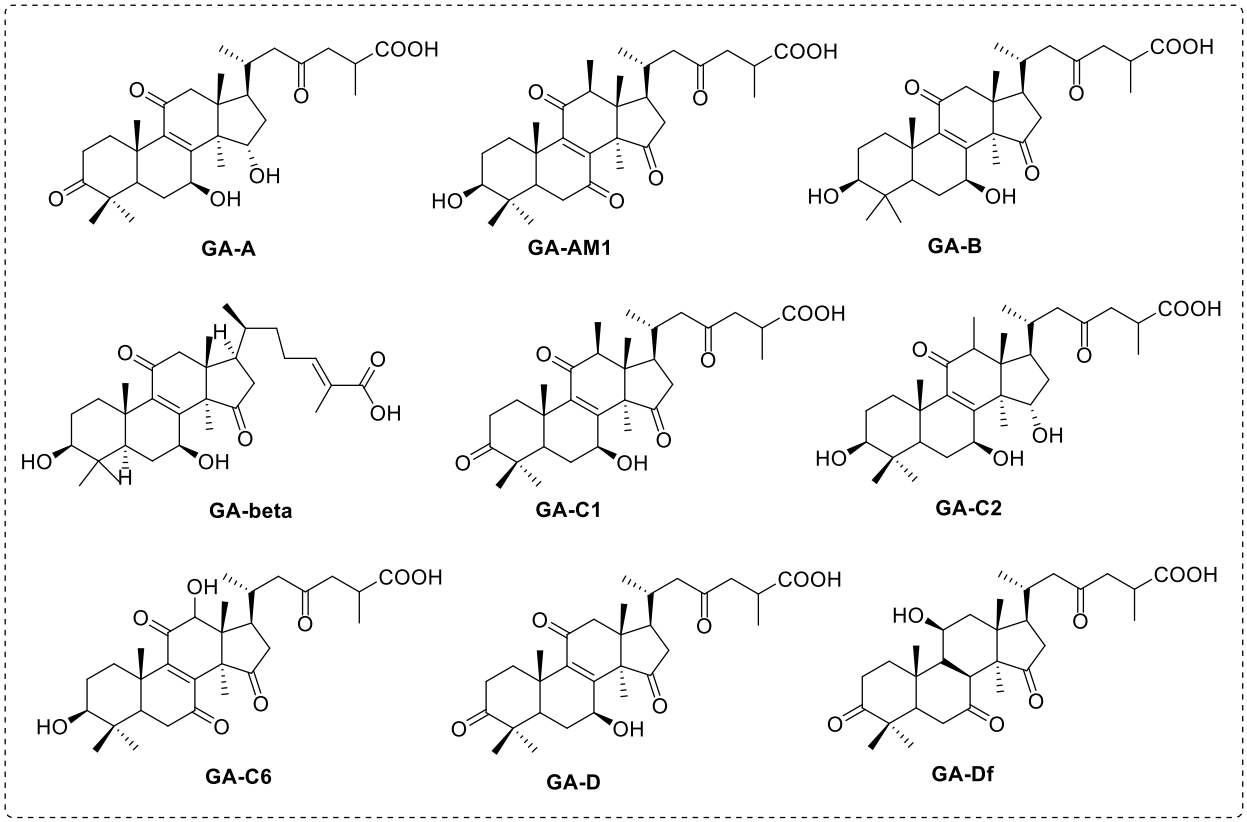

Figure 7: Different derivatives of ganoderic acid.

Ganoderma lucidum also exhibits potent antitumor effects attributed to various active compounds. Polysaccharides, particularly active β-D-glucans, play a key role in enhancing the immune system's function and are responsible for the antitumor activity[33]. They activate immune cells like macrophages, T-helper cells, natural killer cells, and others, leading to increased production of cytokines (such as TNF-α, interleukins, and interferon), nitric oxide, and antibodies. This immune response contributes to tumor regression, as it damages tumor blood flow and causes necrosis through activated T cells and local TNF-α production[52]. In addition to immune potentiation, other mechanisms are involved in the antitumor effects of *G. lucidum* [33]. Research has shown that reishi extracts can inhibit the proliferation and induce apoptosis (programmed cell death) in leukemia, lymphoma, myeloma, and acute myeloblastic leukemia cells[49]. It can induce the differentiation of cancer cells into more mature forms. Compounds from *G. lucidum* inhibit DNA polymerase, modify oncoproteins, and may involve organic germanium. These multifaceted mechanisms collectively contribute to its antitumor activity. While *G. lucidum* products have been used in clinical settings, including in combination with other herbal medicines or chemotherapy,

comprehensive randomized, placebo-controlled, multicancer clinical studies involving *G. lucidum* as a standalone treatment are relatively scarce[33].

G. lucidum extracts, primarily containing polysaccharides and triterpenoids, possess protective effects against liver injuries induced by toxic substances like carbon tetrachloride (CCl_4) and Bacillus Calmette-Guerin (BCG) plus lipopolysaccharide (LPS). A randomized placebo-controlled clinical study demonstrated that treatment with *G. lucidum* polysaccharides for 12 weeks led to a reduction in hepatitis B e antigen (HBeAg) and HBV DNA in a subset of patients with HBV infection[52]. The mechanisms underlying the hepatoprotective effects of *G. lucidum* are not fully elucidated but may involve several potential mechanisms.

Recent research has explored natural products as potential anti-HIV agents, including various structural classes such as coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthraquinones, and polysaccharides[33]. Among these, *G. lucidum* has shown promise in inhibiting HIV. Several triterpenoids from *G. lucidum* have demonstrated strong inhibitory activity against HIV. For instance, lucidenic acid O and lucidenic lactone, found in its fruiting body, not only inhibited calf DNA polymerase-a and rat DNA polymerase-b but also showed inhibitory effects on HIV-1 RT. Ganoderiol F and ganodermanontriol from the fruiting bodies also effectively inhibit HIV-1 growth with an IC_{100} of 7.8 μg/ml. Moreover, other triterpenoids, including ganoderic acid C1, 3β-5α-dihydroxy-6β-methoxyergosta-7,22-diene, ganoderic acid-a, ganoderic acid H, ganoderiol A, and ganoderic acid-b, have shown moderate to significant anti-HIV-1 protease activity. Notably, ganoderic acid A, B, and C1 have minor inhibitory effects against HIV protease[53].

Further preclinical and clinical studies are necessary to fully validate the potential of *G. lucidum* in cancer prevention and therapy, immunomodulatory, hepatoprotective and antioxidant properties, as well as other health applications.

1.1.3 Commercial relevance/ Value of Lions Mane and Reishi Mushroom

Hericium erinaceus, has been traditionally recognized for its therapeutic properties. It is noted for its ability to strengthen the spleen and nourish the stomach, promote a tranquilized mind, and exhibit potential anticancer effects. This makes it a versatile remedy for various conditions, including conditions associated with body deficiency, dyspepsia, insomnia, gastric and duodenal ulcers, chronic gastritis, and digestive tract tumors. The efficacy of Lion's Mane mushroom is

attributed, in part, to its primary bioactive components, polysaccharides. The oral liquid derived from it, particularly its polysaccharide content, is a key element in commercial health products. These products, such as fresh *H. erinaceus* oral liquid, have been utilized for health promotion, including support for learning and memory. Additionally, *H. erinaceus* oral liquid demonstrates positive effects on gastric mucosal injury[54]. Innovative applications involve the development of *H. erinaceus* polysaccharides-encapsulated curcumin nanoparticles. These nanoparticles, prepared through nanoprecipitation, exhibit enhanced solubility in water, increased drug delivery efficiency, and improved in vitro antitumor activity compared to free curcumin[55].

The medicinal benefits of *H. erinaceus* are further harnessed in various listed drugs, where *H. erinaceus* extracts serve as the primary medicinal ingredient. These drugs play a significant role in promoting human health, particularly in nourishing the stomach and intestine and enhancing immune function. Notably, a range of patent health products, including meal replacement powder, chewable tablets, and solid beverages containing *H. erinaceus* polysaccharides, contribute to health improvement without adverse side effects[54].

The *Ganoderma lucidum* mushroom generates a diverse range of products categorized into nutraceuticals, pharmaceuticals, and cosmetics. Various components of *G. lucidum*, such as mycelia, spores, and fruit bodies, are commercially accessible, with products available in different forms like powders, dietary supplements, and herbal teas. A glimpse into the cosmetic industry reveals a variety of *G. lucidum* -based products. The commercial landscape boasts an impressive array of over 100 well-known brands offering Ganoderma-derived products and as a result, remarkably, the global market value of *G. lucidum* and its derivatives has surged to around USD 4 billion[56].

Diverse reishi products, derived from various parts of the mushroom, are now accessible, with manufacturing processes varying. The simplest type involves grinding intact fruiting bodies into powder, which is then processed into capsules or tablets. Other "non-extracted" products originate from dried and powdered mycelia harvested from submerged liquid cultures, combinations of substrate, mycelia, and mushroom primordia, or intact fungal spores. Spore preparations, despite being researched and promoted vigorously, still face controversy regarding any additional medicinal effects attributed to the removal or breakage of spore walls—an extra and often costly step in production. Some products involve extracting materials like polysaccharides and triterpenes

from fruiting bodies or mycelia, typically using hot water or ethanol, followed by evaporation to dryness and encapsulation. The introduction of supercritical fluid CO2 extraction technologies has expanded the range of extracted substances due to the low processing temperatures involved. Additionally, certain products are formulated as mixtures of powdered Ganoderma and other mushrooms, either as binary, ternary, or more complex blends[57].

1.2 Nutritional Benefits

1.2.1 Macronutrients found in Lions Mane and Reishi Mushrooms

Mushrooms are very useful for vegetarian diets because they provide all the essential amino acids for adult requirements; also, mushrooms have higher protein content than most vegetables[2]. Most mushrooms are composed of around 90% water by weight. The remaining 10% consists of approximately 10–40% protein, 2–8% fat, 3–28% carbohydrate, 3–32% fiber, 8–10% ash, and some vitamins and minerals, with potassium, calcium, phosphorus, magnesium, selenium, iron, zinc, and copper accounting for most of the mineral content. It also contains lower amounts of iron, sodium, zinc, copper, manganese, strontium, and trace heavy metals like lead, cadmium, and mercury. [58].

Lion's mane mushroom is characterized by its relatively high nutritional value. In terms of dry matter content, the fruit bodies of this mushroom consist of 57% carbohydrates, 3.52% fats, 7.81% fiber, 22.3% protein, and 9.35% ash. Additionally, soluble sugars such as arabitol, glucose, mannitol, inositol, and trehalose were identified in varying amounts[59]. Detailed analyses of amino acid composition revealed the presence of 14 amino acids in lion's mane mushroom fruit bodies. L-alanine and L-leucine were the most abundant, at 2.43 mg/g and 2.38 mg/g dry matter, respectively, while L-tryptophan and L-phenylalanine had the lowest concentrations at 0.10 mg/g and 0.20 mg/g dry matter, respectively. Notably, L-isoleucine and L-tyrosine were not detected[59]. This mushroom is a source of essential minerals, with notable amounts of potassium (254 mg/100 g dry matter) and phosphorus (109 mg/100 g dry matter)[60]. However, manganese, copper, and zinc are present in trace amounts. It's important to note that the mycelium of lion's mane mushroom tends to have higher levels of heavy metals, including arsenic, lead, copper, and cadmium, compared to the fruit bodies[61]. In terms of aromatic compounds, lion's mane mushroom exhibits a diverse profile. 1-octen-3-ol is the dominant compound, constituting 56–60% of the total aroma substances. Other studies have identified compounds like 2-methyl-3-furanthiol, 2-ethylpyrazine,

and 2,6-diethylpyrazine as dominant contributors to the aroma profile. Overall, 16 aroma substances with various chemical characteristics, including nitrogen or sulfur, aldehydes, ketones, alcohols, and esters, were identified in fruit bodies of lion's mane mushroom[14, 62].

Ganoderma lucidum is composed mainly of protein, fat, carbohydrates, and fiber. When comparing artificially cultivated varieties with their wild counterparts, the nutritional content is quite similar[63]. In a study of the nutraceutical composition of *G. lucidum* , it was found that the mushroom (% of dry weight) contains 2.01% ash, 82.47% carbohydrate, 0.53% crude fat, 3-32% crude fiber, and 15.04 % crude protein[64, 65]. The study also revealed that *Ganoderma lucidum* is a notable provider of dietary fiber, constituting approximately 50.19 ± 3.91% of its composition. Comparative studies have demonstrated substantial variability in the fiber content across various mushroom species, with values ranging from 6.11% - 54.12%. Importantly, *Ganoderma lucidum* stands out with the highest observed fiber content of 54.12% among the mushrooms examined in these studies. Majority of its fiber comes from cellulose and other indigestible cell wall polymers. Fiber is indigestible, however it plays significant nutritional role since, it helps clean and maintains the proper motility of the intestinal tract [66].

Ganoderma lucidum, particularly in log-cultivated fruit bodies, is rich in various minerals, including phosphorus, silica, sulfur, potassium, calcium, and magnesium. Wild Ganoderma spp. collected from the wild have a mineral content of 10.2%, with potassium, calcium, and magnesium as the major components[67]. The germanium content of reishi has garnered attention, being the fifth highest among detected minerals. It is present in many plant-based foods, including ginseng, aloe, and garlic. At low doses it has been credited with immunopotentiating antitumor, antioxidant, and antimutagenic activities[68],[58].

The protein content was found to be around 15.04 %, which is lower than that of many other mushrooms. Bioactive proteins, including LZ-8, a peptide preparation (GLP), and a 15-kDa antifungal protein called ganodermin, contribute to its reported medicinal properties. With a carbohydrate content of 82.47%, reishi contains lectins. These are nonenzymatic proteins or glycoproteins that bind carbohydrates. a novel 114-kDa hexameric lectin has be isolated, which was revealed to be a glycoprotein having 9.3% neutral sugar and showing hemagglutinating activity on pronase-treated human erythrocytes[58].

It's important to note that the chemical composition of both lions mane and reishi mushroom products can vary in terms of both quality and quantity. These variations depend on factors such as the strain, geographical origin, extraction methods, and the conditions under which the mushrooms are cultivated[33].

1.2.2 Proximate Analysis

In the present day, it is a requirement for virtually all food products to feature standardized nutritional labels. This obligation ensures that consumers have clear insights into the nutritional makeup of foods, empowering them to make informed choices about their diet. These labels also foster fair competition among food companies in the market. The standardized nutritional labels are obligated to include information on five essential components—protein, fat, moisture, ash, and carbohydrates. These components, collectively known as "proximates," are determined and presented through the process known as "Proximate Analysis."[69]

Proximate analysis is a methodology used to ascertain the levels of macronutrients in food samples. Typically, these values are disclosed as nutritional facts on the labels of the final food products or are assessed during the production process. The roots of nutritional analysis date back to 1861, and over time, this method has undergone ongoing development, modification, and improvement[69]. It is a technique employed to determine the approximate quantities of protein, lipid, water, ash, and carbohydrate in a given sample. In the context of feed or food, it is referred to as proximate composition, encompassing components like moisture, crude protein, ether extract, crude fiber, crude ash, and nitrogen-free extracts. These components are expressed as percentages in the sample. Notably, protein, lipid, and carbohydrate contribute to the overall energy content of an organism, whereas water and ash solely contribute to mass[70].

This analysis holds significant importance in the commercial realm, particularly for food manufacturing companies. These companies must ensure that their products comply with relevant laws and legal disclosure requirements. Additionally, they need to address safety considerations for end consumers when their products reach the market. To meet industry standards and maintain competitiveness, food manufacturing companies must integrate reliable analytical techniques into their production processes for ongoing monitoring and analysis[69].

Moisture content is determined by a series of drying and weighing the sample to a constant weight. Water and other volatiles are evaporated under high temperature, and the loss is expressed as the percentage moisture content of the sample. Ash content is determined by incinerating the sample at high temperature for a longer duration. This is done to convert sample into ash and it is expressed as a percentage of the dry sample. Crude fat, often referred to as ether extract or free lipid content is determined by a Soxhlet extraction followed by weighing the extracted fat and expressing it as a percentage of the sample. This represents the unrefined mixture of fat-soluble components present in a sample. This traditional measure of fat in food products encompasses various lipid materials, including triglycerides, diglycerides, monoglycerides, phospholipids, steroids, free fatty acids, fat-soluble vitamins, carotene pigments, and chlorophylls. The typical method for determining total crude fat involves leveraging the solubility of lipids in non-polar organic solvents such as hexanes, petroleum ether, or supercritical liquid carbon dioxide, with or without a solvent modifier[70]. Crude protein content is determined by the Kjeldahl method. It was first developed in 1883 by a Danish chemist, Johan Kjeldahl, for determining the amount of protein in samples taken from a wide variety of organisms. This procedure relies on the oxidation of the organic compound facilitated by strong sulfuric acid and a catalyst. As the organic material undergoes oxidation, the carbon within it is transformed into carbon dioxide, while the hydrogen is converted into water. Simultaneously, the nitrogen present in the amine groups of the peptide bonds within the polypeptide chains is converted into ammonium ions (ammonium sulphate). The nitrogen is then distilled from an alkaline medium and absorbed (in the form of ammonia gas) in boric acid[71]. The ammonia is then determined by titration with a standard mineral acid to determine percent nitrogen. This value is then multiplied by a conversion factor to determine percentage crude protein. For mushrooms, this value is 4.38 as mushrooms contain significant amounts of non-protein nitrogen. Fiber content is determined by digesting the sample in acid and base as to get rid of the matrix, followed by incineration. Percentage crude fiber is determined as the difference between the mass loss before and after incineration. Carbohydrate content (%) is then determined by the difference between 100 and the summation of other proximate parameters[72]. Total energy content in kcal/100g is also determined by calculation[73]. Generally, mushrooms are low-calorie foods, but their energy value is not exclusively due to their fat content but also to the other macronutrients[74].

1.3 Medically beneficial/ Bioactive Compounds found in Reishi and Lions Mane Mushrooms.

1.3.1 Proteins

Protein is a crucial nutrient essential for maintaining health, serving not only as an energy source but also playing key roles in various bodily functions such as forming tissues and acting as hormones or enzymes. Plant sources, including mushrooms, offer additional health benefits through dietary fiber, contributing to the prevention of cardiovascular diseases, colon cancer, diabetes, and constipation. Edible mushrooms offer a valuable source of high-quality protein as they typically boast a complete essential amino acid profile. Moreover, their production is often quicker and more cost-effective compared to animal and plant proteins. An additional advantage lies in their eco-friendly cultivation, utilizing various substrates, usually waste from industries like wood, paper, and agriculture. Mushroom protein content varies based on factors such as species, strain, maturation stage, substrate, and environmental conditions. Despite this variability, mushrooms are seen as providing a favorable protein-to-energy ratio compared to other protein sources. Mushrooms exhibit a protein content ranging from 18.87% to 36.96% of their dry weight. This means that 100 grams of mushrooms on a dry basis can fulfill a significant portion of the Recommended Dietary Allowance (RDA), covering approximately 29.41% to 66.00% of the RDA for men and 35.80% to 80.35% for women[75].

While the polysaccharides found in edible mushrooms are often credited for their various biological activities, it's important to note that mushrooms also serve as a reservoir of peptides and proteins with biological activities. These bioactive compounds have the potential to play a significant role in enhancing health and could be explored for their therapeutic and preventive properties against diseases [75]. The primary bioactive proteins found in mushrooms include lectins, fungal immunomodulatory proteins (FIP), ribosome inactivating proteins (RIP), antimicrobial/antifungal proteins, ribonucleases, and laccases[76].

Lectins are described as “nonimmune proteins or glycoproteins that bind specifically to cell surface carbohydrates, with ability in cell agglutination”[76]. Various mushroom lectins have been extensively studied, with a particular focus on those exhibiting antiproliferative, antitumor, and immunomodulatory activities[76]. For instance, a xylose-specific lectin isolated from the wild mushroom *Xylaria hypoxylon* demonstrated potent antiproliferative activity against tumor cell

lines and anti-mitogenic effects on mouse splenocytes[77]. Another lectin, CNL, obtained from the edible mushroom *Clitocybe nebularis*, displayed immunomodulatory properties along with antiproliferative activity against human leukemic T cells[78]. Lectins from mushrooms such as *Hericium erinaceum,* have also exhibited antiproliferative activity against various cancer cells, highlighting their potential as bioactive compounds with therapeutic implications[76].

Ribosome Inactivating Proteins (RIPs) found in mushrooms have gained attention in the past decade. These mushroom-derived RIPs exhibit diverse bioactivities, including inhibitory effects on HIV-1 reverse transcriptase, antifungal properties, and antiproliferative activities against hepatoma Hep G2 and breast cancer MCF-7 cells[76].

Proteins targeting immune cells or FIPs (Fungal immunomodulatory proteins) are a new family of bioactive proteins isolated from mushrooms. More than ten years ago, about six FIPs (LZ-8, gts, jap, fve, vvo and gsi) have been found and identified. More specifically, Kino et al isolated the immunomodulatory protein, ling zhi-8 (LZ-8), from *Ganoderma lucidium* and its biochemical and immunological properties were evaluated. It was found that it enhances T helper type 1 antigen-specific humoral and cellular immune responses [79]. Furthermore, researchers have been also making great efforts to investigate the mechanisms of FIPs in anti-tumor and immunomodulation. Lin et al investigated the immune modulatory effects of rLZ-8 on human monocyte-derived DCs. Their experiments demonstrated that rLZ-8 can enhance the cell-surface expression of CD80, CD83, CD86, and HLA-DR, the production of cytokines IL-12 p40, IL-10, and IL-23, and suppress the capacity for endocytosis in DCs (dendritic cells). Further studies showed that rLZ-8 was able to augment IKK, NF-kappa B activity, and also, I kappa B alpha and MAPK phosphorylation. These results demonstrated that rLZ-8 can effectively enhance the activation and maturation of immature DCs via the NF-kappa B and MAPK pathways[80].

1.3.2 Polysaccharides (β-Glucans)

Mushrooms are acknowledged as functional foods due to their bioactive compounds, with one particularly potent component being β-glucan. Mainly sourced from the fruiting bodies of mushrooms, β-glucans are polysaccharides composed of D-glucose monomers linked by β-glycosidic bonds. This type of dietary fiber is found in cereals, yeasts, mushrooms, certain bacteria, and seaweeds. Mushroom-derived β-glucans consist of β-(1 → 3) and (1 → 6) linkages, forming a backbone of glucose residues linked by β-(1 → 3)-glycosidic bonds with attached β-(1 → 6)

branch points. Unlike β-glucans from oats and barley, mushroom-originated β-glucan exhibits antitumor and immune-stimulating properties, contributing to its effectiveness in anti-tumor defense and immune system enhancement. The biological activities of β-glucan, influenced by factors such as primary structure, solubility, degree of branching (DB), molecular weight (MW), polymer charge, and structure in aqueous media, can vary among different sources[81].

Enzymes known as endoglycosynthases play a crucial role in synthesizing β-glucan molecules. They catalyze reactions that facilitate the self-condensation of sugar donors, leading to the in vitro synthesis of a regular polysaccharide[82]. The extraction of β-glucan is a challenging process that demands special attention to ensure consistent raw material quality. The extraction procedure significantly impacts the molecular weight (MW) of β-glucan, influencing its functional behavior. Common extraction methods rely on the solubility of β-glucan in hot water and alkaline solutions. Techniques involve separating dissolved proteins through isoelectric precipitation and precipitating β-glucan using substances like ammonium sulfate, 2-propanol, or ethanol[81].

β-Glucans, found in the cell walls of fungi, including mushrooms, exhibit structural diversity. Mushroom β-glucans typically consist of linear β-(1→3)-linked backbones with β-(1→6)-linked side chains of varying length and distribution. These β-glucans can form complex tertiary structures stabilized by interchain hydrogen bonds. Examples include schizophyllan from *S. commune* and scleroglucan from *S. glucanicum*, both with a β-(1→3)-linked backbone and an average of one β-(1→6)-glucose substitution every three backbone residues[81]. Dong et al, characterized a novel β-D-glucan (GLSA50-1B) from *G. lucidum* , featuring a 1,6-linked β-D-glucopyranosyl backbone with branches of different lengths[83]. Another study isolated two polysaccharides (AF2S-2, BF2S-2) from the fruiting bodies of *H. erinaceus*. They were composed of a backbone of *β*-(1 → 6)-linked D-glucopyranosyl residues, and had *β*-(1 → 3) and *β*-(1 → 6) glucosidic linkages[84].

The water solubility of β-glucans is intricately linked to their structure and origin. Generally, an increase in temperature enhances the solubility of β-glucans in water. The bioactivities of polysaccharides, including β-glucans, can be influenced by factors such as chain conformation and the introduction of suitable ionic groups with an appropriate degree of substitution.

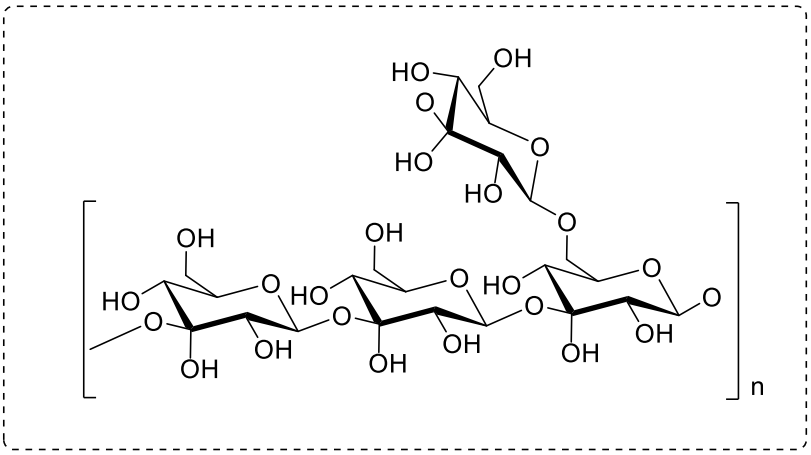

Figure 8: Structure of (1 → 3) β-glucan backbone with (1 → 6) glucosidic linkages.

Water-soluble polysaccharides, in contrast to their insoluble counterparts, exhibit significant bioactivity. Sulfated derivatives of water-insoluble polysaccharides, in particular, demonstrate high antitumor and/or antiviral activities. The introduction of sulfate groups and improved water solubility are key factors contributing to enhanced antitumor activities. One study found that sulfated derivatives exhibited higher in vitro antitumor activity against the human hepatic cancer cell line HepG2 compared to the native water-soluble hyper-branched β-glucan[85]. Soluble β-glucans display stronger immuno-stimulatory effects than their insoluble counterparts, although the exact reasons for this phenomenon remain unclear[81].

Leveraging the capabilities of modern structure elucidation, β-glucans have been early recognized as highly potent components with various biological activities, including biological response modifier and anti-carcinogenic properties in mushrooms. Enhancing the β-glucan content in mushrooms could significantly elevate their nutritional and economic values. However, achieving a reliable and rapid quantitative analysis of β-glucans remains challenging due to the considerable diversity within this group of compounds. As a result, the methods for detecting β-glucan in mushrooms include: (1) enzymic method or McCleary method (Megazyme kit), (2) enzyme-linked immunosorbent assay (ELISA) method to determine high molecular weight β-glucans, using grifolan and lentinan as prototypes, (3) fluorimetric method with aniline blue that provides selectivity among various 1,3-β-glucan species, and (4) colorimetric method with Congo red that measures β-1,3–1,6-glucans [81].

Mushrooms are acknowledged as functional foods primarily due to the presence of β-glucans, which confer significant health benefits. Studies have explored various receptors for β-glucans and elucidated immune responses initiated by them in both vertebrates and invertebrates during fungal infections[86]. Another study highlighted that the mechanisms of action of fungal β-glucans involve binding to cell receptors like dectin-1, CR3, LacCer, and scavenger receptors[87]. Chanput et al investigated immunological aspects of β-glucans from different sources, concluding that their immuno-modulating properties vary based on structural and compositional characteristics[88]. The use of mushroom β-glucans in colon cancer is also investigated. Emphasis was placed on their potential to decrease tumor size via immune system stimulation and direct cytotoxicity[89]. β-Glucans have been associated with various health-promoting effects, including immuno-modulation, antitumor, antiviral, cardiovascular, liver protection, anti-inflammatory, radioprotection, antidiabetic, antioxidant, antibacterial, and antiobesity activities. Overall, β-glucans from mushrooms contribute significantly to promoting health and preventing various diseases[81].

1.3.3 Ergosterol

Mushrooms are known for many bioactive compounds. Ergosterol, a prominent sterol integral to the cell membrane in fungi, possesses the capability to trigger the expression of various defense genes, enhancing plant resistance against fungal pathogens. It is predominantly found in fungi in both free and esterified forms, with the ratio between free and esterified ergosterol varying across different species, with its content reduced with aging of the mushrooms. Research indicates that ergosterol and its peroxidation products offer potential health benefits and play essential physiological roles. These roles include reducing inflammation associated with pain, lowering the risk of cardiovascular disease, inhibiting cyclooxygenase (COX) enzymes, and exhibiting antimicrobial, anticomplementary, and antitumor activities[90].

Ergosterol, the most abundant sterol in fungal cell membranes, including those of edible mushrooms, is crucial for maintaining membrane integrity, akin to cholesterol in animal cells. This compound possesses three double bonds and β-hydroxy groups at positions 5, 7, and 22, forming a 1,2-cyclopentanoperhydrophenanthrene ring nucleus, conferring amphipathic lipid characteristics. Present in both free and esterified forms, ergosterol undergoes photolysis when

exposed to ultraviolet light (280–320 nm). This process transforms it into pre-vitamin D2 (pre-ergocalciferol), subsequently undergoing thermal conversion into vitamin D2 (ergocalciferol)[91].

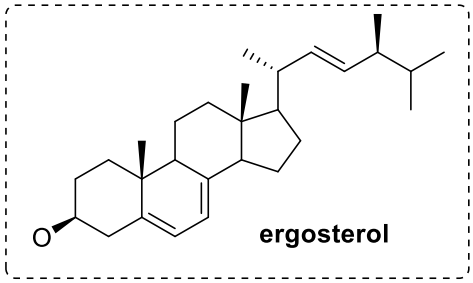

Figure *9: Structure of ergosterol*

The most commonly used analytical method for total ergosterol analysis in mushrooms is the AOAC method 2002.05, which involves alkaline saponification in ethanol solution, followed by extraction with hexane and C18 reversed phase HPLC separation with UV detection at 282 nm. Modifications include combining methanolic extractions and saponification[90].

The antitumor effects of ergosterol may stem from its direct inhibition of angiogenesis induced by solid tumors. When included in the diet, ergosterol undergoes absorption in the alimentary tract, accumulates in organs such as the adrenals, and undergoes in vivo metabolism, producing bioactive byproducts like 17,24-dihydroxyergosterol. This specific derivative has demonstrated inhibitory effects on the proliferation of abnormal skin cells in culture, as evidenced in human keratinocytes and melanoma cell lines[90]. Ergosterol derivatives from *Ganoderma lucidum* also exhibit cytotoxicity in breast cancer cell lines (MDA-MD-231 and HepG2), with no effect on normal cells[92].

Ergosterol, abundant in mushrooms, plays a crucial role in suppressing inflammatory responses through various signaling pathways. For instance, it suppresses transcriptional activity in human chondrosarcoma cells and inhibits NF-κB luciferase activity in macrophages. Ergosterol and ergosterol peroxide directly bind to the active site of NF-κB p65, inhibiting phosphorylation and degradation of IκB-α and blocking NF-κB p65 phosphorylation[91].

1.3.4 Vitamin D_2

Vitamin D, commonly referred to as the "sunshine vitamin," was initially identified by Edward Mellanby during his studies on rickets in 1919. This essential vitamin plays a crucial role in calcium metabolism and the mineralization of bones. It stimulates the synthesis of the calcium transport proteins in the small intestine, enhancing the absorption of dietary calcium and thereby reducing the risk of osteomalacia in adults and rickets in children. Vitamin D is the general term for a closely related group of vitamins that share the biological activity of cholecalciferol, also known as vitamin D_3. There are various forms of this vitamin, but the most physiologically relevant ones are vitamin D_3 or cholecalciferol, which is the most biologically active form found in animals and humans produced after skin exposure to UVB radiation. Ergocalciferol, or vitamin D_2, represents the synthetic form of vitamin D. It can be produced from the plant or fungi steroid, ergosterol, through exposure to UV irradiation. Importantly, vitamin D_2 is believed to possess the same biological activity as cholecalciferol[93].

Vitamin D_2 is the commonly utilized form in food and pharmaceutical supplements. Wild mushrooms found in nature naturally contain minimal amounts of vitamin D_2. Despite the low levels in mushrooms, earlier studies, have identified them as a rich source of ergosterol that can be converted into vitamin D_2 by UV-radiation. The amount of vitamin D varies among mushroom species, and also within the same species. Mushrooms are highly regarded as a delicacy and are widely accepted by both vegetarians and non-vegetarians. Considering this, there is potential to use mushrooms as a dietary supplement for populations at risk of vitamin D deficiency symptoms, provided that their ergosterol content can be effectively converted into vitamin D[93].

During photolysis, ergosterol transforms into pre-vitamin $_{D2}$ (pre-ergocalciferol), which, upon thermal conversion, becomes vitamin D_2 (ergocalciferol). This form is crucial for human nutrition. Upon consumption, vitamin D_2 undergoes hepatic metabolism, converting it to 25-hydroxyvitamin D. Subsequently, this compound is transported to the kidney, where it undergoes further transformation into 1,25-dihydroxyvitamin D, known as calcitriol. Calcitriol is the active form in living organisms, playing essential roles in calcium homeostasis and bone health.[91]

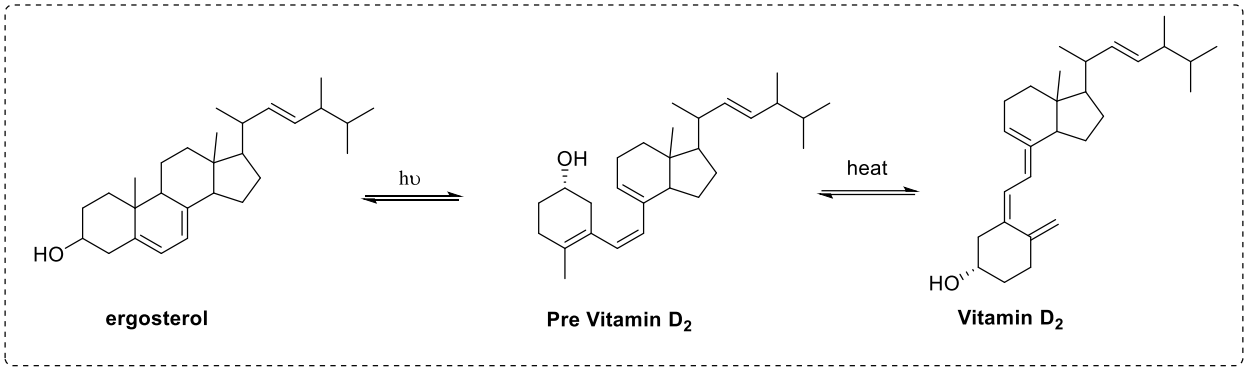

Figure *10: Production of vitamin D₂.*

Vitamin D2, or ergocalciferol, differs from D3 (cholecalciferol) due to a double bond between C22 and C23 and a methyl group at C24 in the side chain. It can be considered the first analog of vitamin D. These structural variations in the side chain reduce its affinity for vitamin D binding protein (DBP), leading to quicker clearance from the circulation. Additionally, these differences limit its conversion to 25-hydroxyvitamin D (25OHD). Consequently, unless administered daily, D2 supplementation does not result in as high a blood level of 25OHD as equivalent amounts of D3[94].

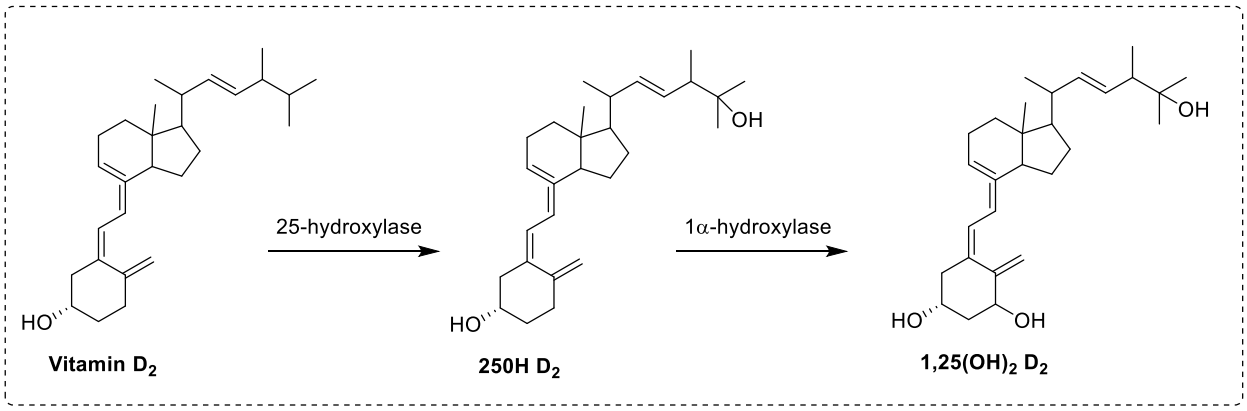

Figure 11: Activation of Vitamin D₂

Among others, vitamin D has impact on bone and muscle health, cancer disease, cardiovascular diseases, liver function, atopic dermatitis, obesity, depression and diabetes. It plays a crucial role in musculoskeletal health, and its deficiency is linked to conditions such as rickets in children and osteomalacia in adults, characterized by discomfort in muscles and bones. This vitamin is essential for bone formation and mineralization as it stimulates osteoblasts (cells responsible for bone formation) and inhibits osteoclasts (cells responsible for bone resorption). Numerous controlled

trials have demonstrated a positive correlation between vitamin D supplementation and fracture prevention, particularly in cases of falls, attributed to its role in maintaining adequate levels of calcium and phosphate.[95] The antitumor properties of vitamin D involve the activation of specific kinases that regulate the cell cycle, preventing DNA synthesis and inhibiting the growth of malignant cells. However, the effective use of vitamin D in cancer treatment is limited by the risk of calcemic toxicity. Despite efforts to counteract vitamin D-degrading enzymes in cells, there are unconfirmed reports suggesting that enhancing vitamin D levels through consumption of vitamin D-rich or fortified foods may improve chemotherapy outcomes and reduce the risk of malignancy.

Vitamin D has also gained attention for its cardioprotective functions in various cardiovascular diseases (CVD). One mechanism involves the promotion of vascular endothelial growth factor (VEGF) expression in heart valve endothelial cells, activating signaling pathways responsible for endothelial cell functions such as proliferation, survival, migration, and vascular permeability. Studies suggest that vitamin D supplementation can serve as adjuvant therapy to lower serum total cholesterol levels, contributing to improved cardiovascular function. It also plays a crucial role in preventing liver diseases by downregulating various signal transduction pathways, facilitating the expression of interferons, chemokines, and pro-inflammatory genes like TNF-α, IL-4, IL-6, and toll-like receptors. This downregulation contributes to a reduced risk and provides protection against conditions such as hepatitis B, liver inflammation, liver cirrhosis, and hepatocellular carcinoma (HCC)[95].

1.3.5 Polyphenols and Flavonoids

Polyphenols, naturally present in plant-based foods, exhibit a diverse range of complex structures. Compounds containing an aromatic ring with at least one hydroxyl group are termed "phenolics." When a compound has one or more aromatic rings with more than one hydroxyl group, it is specifically called polyphenols or polyphenolic compounds. In plant-derived foods, phenolics are broadly categorized into phenolic acids, flavonoids, and non-flavonoids. Phenolic acids, a subcategory, are characterized by hydroxyl derivatives of aromatic carboxylic acids that possess a single phenolic ring. These acids are often referred to as derivatives of benzoic acid or cinnamic acid, based on the C1-C6 or C3-C6 backbone. Flavonoids, which constitute the predominant class of plant polyphenols, are compounds with two phenolic rings connected by a three-carbon bridge, forming a common C6-C3-C6 structural framework[96]. Found abundantly in mushrooms,

polyphenols are considered bioactive compounds that play a role in safeguarding human health against chronic degenerative diseases. Due to their structural complexity, the study of polyphenols was initially delayed. However, these compounds, also known for their antioxidant properties, have emerged as crucial components in our diet. Polyphenols act as antioxidants by inhibiting oxidative changes, particularly in low-density lipoprotein, a fundamental mechanism in the development of endothelial lesions associated with conditions like atherosclerosis. Research has increasingly highlighted the preventive and therapeutic roles of polyphenols in various health conditions, including cardiovascular disease, osteoporosis, neurodegenerative diseases, cancer, and diabetes mellitus[97].

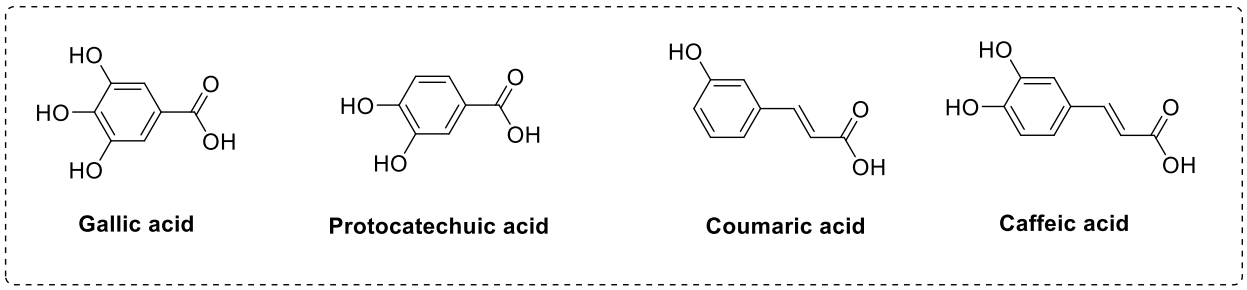

Figure *12: General structural formulae of phenolic acids.*

Proper characterization of polyphenols in mushrooms is still a developing science, however, according to a study carried out using Reishi mushroom, polyphenols including resveratrol, quercetin, caffeic acid and chlorogenic acid were identified using UHPLC-MS/MS analysis[98].

Oxidative stress (OS) plays a significant role in various cardiovascular diseases (CVDs), acting as both a primary and secondary contributor. Preclinical evidence has established a connection between OS and a range of CVDs, including atherosclerosis, ischemia, stroke, cardiomyopathy, cardiac hypertrophy, hypertension, and congestive heart failure[99]. Polyphenol-rich foods have been shown to mitigate the risk of CVDs, according to studies. Recent research indicates that polyphenols offer beneficial effects in vascular disorders by preventing platelet aggregation, inhibiting low-density lipoprotein (LDL) oxidation, improving endothelial function, lowering blood pressure, enhancing antioxidant defenses, and mitigating inflammatory responses. Polyphenols are particularly effective regulators of LDL oxidation, a key mechanism in atherosclerosis progression. They protect against CVDs through anti-inflammatory, antioxidant, and antiplatelet effects, while also increasing high-density lipoprotein (HDL) levels. Flavonoids in the diet may alleviate endothelial disorders associated with various atherosclerosis risk factors

before plaque formation[100]. Catechins have been shown to inhibit smooth muscle cell penetration and proliferation in the arterial wall. Resveratrol, another polyphenol, inhibits platelet aggregation by selectively targeting cyclooxygenase 1 (COX-1), which plays a role in the production of thromboxane A2, a contributor to platelet aggregation and vasoconstriction. Additionally, resveratrol enhances nitric oxide signaling in the endothelium, leading to vasodilation[96].

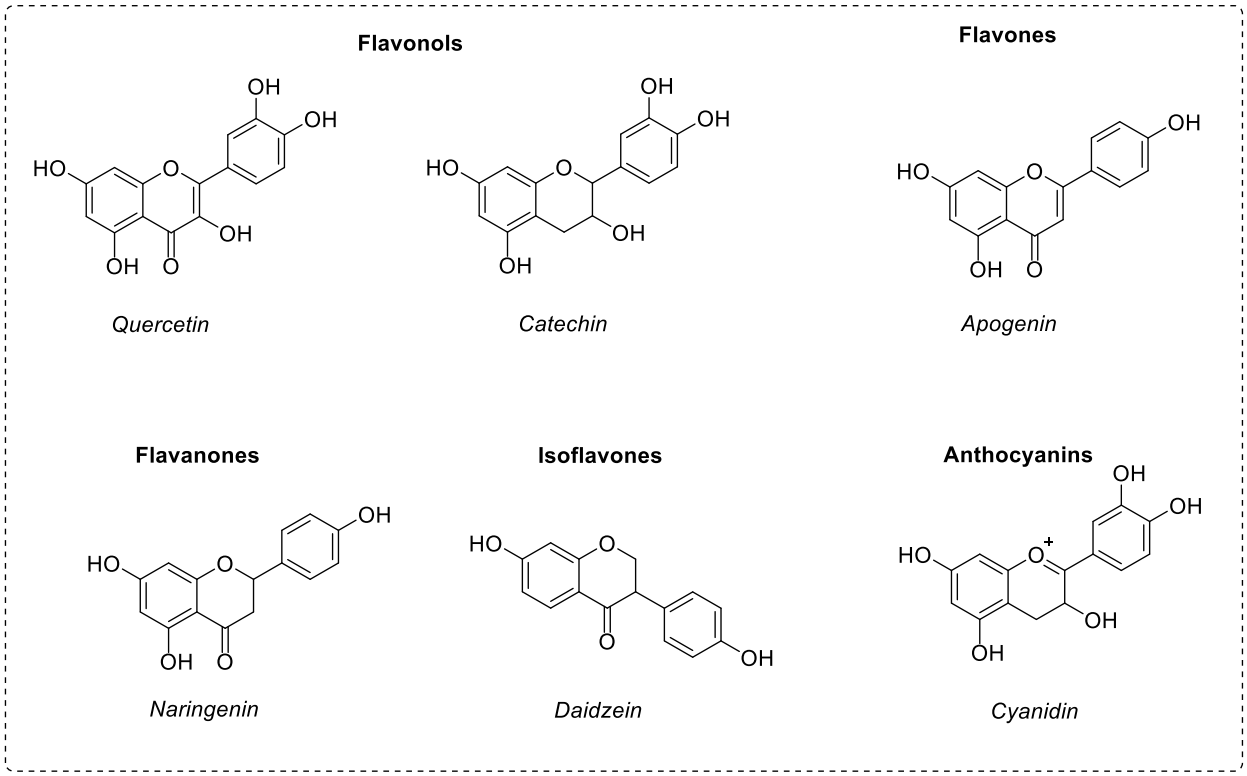

Figure 13: General structures of flavonoids.

The incidence of cancer is associated with oxidative stress (OS) and the elevated presence of free radicals, particularly reactive oxygen species (ROS), leading to biomolecular (DNA) and tissue damage. Research indicates that a diet rich in fruits and vegetables, containing polyphenols such as catechins, resveratrol, ellagic acid, naringenin, quercetin, etc., significantly reduces the risk of developing various cancers. The chemo preventive actions of polyphenols involve estrogenic and antiestrogenic effects, antiproliferation, cell cycle arrest or apoptosis activation, resistance to oxidation, induction of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity, and modulation of cellular signaling[101]. Resveratrol, another polyphenol, exerts anti-carcinogenic effects through its antioxidant function, inhibiting hydroperoxidase, the

Akt (PI3K-Akt) signaling pathway, matrix metalloprotease-9, NF-kB, protein kinase C, cyclooxygenase, focal adhesion kinase, and Bcl-2 biomarkers/enzymes[102].

Certain compounds derived from polyphenols demonstrate robust antidiabetic activities, providing protection against oxidative stress in diabetes. For instance, quercetin reduces lipid peroxidation and inhibits cellular oxidation in diabetes, while resveratrol prevents cytotoxicity and OS induced by excessive glucose levels. Resveratrol further mitigates diabetes-induced kidney alterations (diabetic nephropathy) and delays the onset of insulin resistance, potentially through the inhibition of K+ATP and K+ V channels in β cells[103].

1.3.6 Ganoderic Acid-A

Ganoderma lucidum **is rich in various naturally occurring bioactive compounds, including polysaccharides, terpenoids, glycopeptides, nucleotides, steroids, and unsaturated fatty acids.** The two major types of bioactive components are comprised of water-soluble polysaccharides, primarily glucan, galactan, and other heteropolysaccharides. The second category consists of triterpenes, with ganoderic acid being the predominant terpenoid in the reishi mushroom. Ganoderic acid has been found to engage with membrane receptor tyrosine kinases (RTKs), specifically impacting signaling pathways associated with insulin receptors (IR), insulin-like growth factor receptors 1 and 2 (IGFR-1, IGFR-2), vascular endothelial growth factor receptors 1 and 2 (VEGFR-1, VEFGR-2), and epidermal growth factor receptor (EGFR). This interaction influences cancer-related signaling pathways. Additionally, ganoderic acid has the ability to target nuclear factor-kappa B (NF-κB), RAS-MAPK, and the PI3K/Akt/mTOR pathways, leading to cell cycle arrest and ultimately inducing apoptosis[104].

The triterpenoids found in *Ganoderma lucidum* (GLTs) have chemical structures based on lanostane, derived from lanosterol through squalene cyclization in biosynthesis. After the development of *G. lucidum* fruiting bodies, they are harvested and processed to extract triterpenes. GLTs demonstrate diverse biological activities, including antitumor, anti-HIV-1, antihypertensive, antiangiogenic, immunomodulatory, antiandrogenic, antihepatitis B, antioxidant, anticomplement, and antimicrobial properties. Remarkably, all GLTs are tetracyclic triterpenes, with over 150 lanostane-type triterpenoids identified in *G. lucidum* . The chemical complexity of GLTs exceeds that of lanostanes from other species due to their highly oxidized states. These lanostane triterpenoids, categorized as ganoderma alcohols or ganoderic acids, are considered the key active

components responsible for the anticancer effects reported in preclinical studies. Ganoderic acids, featuring lanostane skeletons with trans configurations of rings A/B, B/C, and C/D, are particularly noteworthy among these compounds[104].

Ganoderic acid A features a tetracyclic ring with an internal double bond and a terminal carboxyl group on the branch. The -COOH group plays a crucial role in its bioactivity and binding affinities, while the -C=O or -OH group regulates signaling pathways through other receptors. Protein-ligand interaction analysis reveals that Gly583, Glu582, and Gln524 STAT3 residues are involved in binding with ganoderic acid A. The lanosterol moieties in ganoderic acid A interact with cysteine residues, leading to STAT3 activation and translocation from the cytoplasm to the nucleus. Ganoderic acid A, has demonstrated several pharmacological benefits in humans. It is known to inhibit histamine release, enhance digestive organ function, reduce blood fat, lower blood pressure, and regulate liver functions. Additionally, it shows significant potential as an apoptosis inducer[105].

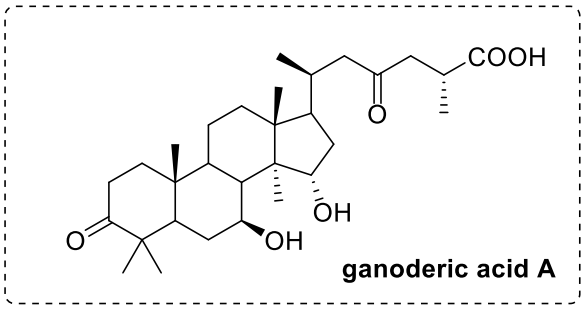

Figure 14: Structure of ganoderic acid A

Studies also suggest that ganoderic acid A could be an effective apoptosis-inducing *Ganoderma lucidum* triterpene (GLT). Specifically, it inhibits STAT-3 phosphorylation, which is crucial in responding to cytokines and growth factors, undergo activation, dimerization, nuclear translocation, and binding to target gene promoters. Ganoderic acid A's crystal structure bound to STAT protein DNA recognition sites and receptor-based crystal structures illustrate its interaction profile[105].

A study by Gill BS et al. evaluated antioxidant potentials and predicted ADMET properties of different ganoderic acid isoforms targeting the prostate cancer STAT3 pathway. Using Maestro 9.6, ganoderic acid A demonstrated the best docking performance, showing optimal binding interaction with the SH2 domain of STAT3. The calculated values for various parameters, including GScore,

Lipophilic Evdw, Electro, Glide emodel, H bond, and MM-GBSA, supported ganoderic acid A as the most favorable isoform for binding[106].

1.3.7 Fatty Acids

Edible mushrooms are known for their low lipid content, typically ranging from 1.1% to 8.3% (dry weight), with an average of approximately 4.0%[107]. Despite the modest lipid content, these mushrooms are rich in polyunsaturated fatty acids (PUFAs), making them a valuable addition to a healthy diet. This nutritional profile recommends them for individuals with elevated cholesterol levels (hypercholesterolemia). Various studies worldwide have investigated the fatty acid composition of wild edible mushrooms, aiming not only to understand their role in human nutrition but also to explore potential pharmacological benefits. These investigations typically involve hydrolyzing, methylating, and analyzing the lipid fraction using gas chromatography/mass spectrometry (GC/MS), presenting the results as total fatty acid content. However, the specific forms in which these fatty acids exist within the mushrooms, whether as free acids or associated with other molecules, have not been thoroughly explored in existing research[108].

Edible mushrooms are notably abundant in linoleic and oleic acids. Linoleic acid, a prominent component, has demonstrated anti-carcinogenic effects across various stages of tumorigenesis in animal models, showing efficacy in breast, prostate, and colon cancers. It achieves this by influencing the tumor cell growth process, specifically by modifying the 5-lipoxygenase metabolite, 5-hydroxyeicosatetraenoic acid (5-HETE), and facilitating the expression of the five-lipoxygenase activating protein (FLAP). Additionally, linoleic acid serves as a precursor to 1-octen-3-ol, an alcohol compound contributing significantly to the distinctive flavor of mushrooms. The lipid fraction of mushrooms also contains tocopherol, a noteworthy antioxidant component. Tocopherol plays a crucial role in neutralizing oxidative stress and contributes to the overall antioxidant profile of mushrooms[107]

Fatty acids, characterized by simple structures derived from hydrocarbon chains with carboxylic acids, are key components in the synthesis of energy-rich fats and oils. They typically have an even number of carbon atoms, ranging from four to thirty-six, as they result from the condensation of two-carbon (acetate) units in an unbranched chain of 12 to 24 carbons. Fatty acids can be categorized into saturated (containing only single bonds) and unsaturated (containing one or more double bonds) types. The nomenclature of unbranched fatty acids is denoted as [number of carbon

atoms in the chain: number of double bonds], and they can be named based on either the carboxyl group carbon or the methyl group carbon[109, 110]. Polyunsaturated fatty acids, crucial for human consumption, are commonly identified using the omega (ω) nomenclature, such as omega-3 (ω-3) and omega-6 (ω-6), indicating the position of double bonds relative to specific carbons in the chain. Mammals possess the ability to convert saturated stearic acid (C18:0) into monounsaturated oleic acid (C18:1, ω-9) through desaturase (Δ9) enzymes. Furthermore, they produce polyunsaturated ω-6 fatty acids like arachidonic acid (AA) and ω-9 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) through the action of other desaturases (Δ5, Δ6). Linoleic (C18:2, ω-6) and linolenic (C18:3, ω-3) fatty acids are metabolized to longer-chain fatty acids with 20 and 22 carbon atoms (AA, EPA, and DHA). Notably, linoleic and linolenic acids are considered essential fatty acids as humans lack the enzymes for ω-3 desaturation, making their presence in the diet essential[109].

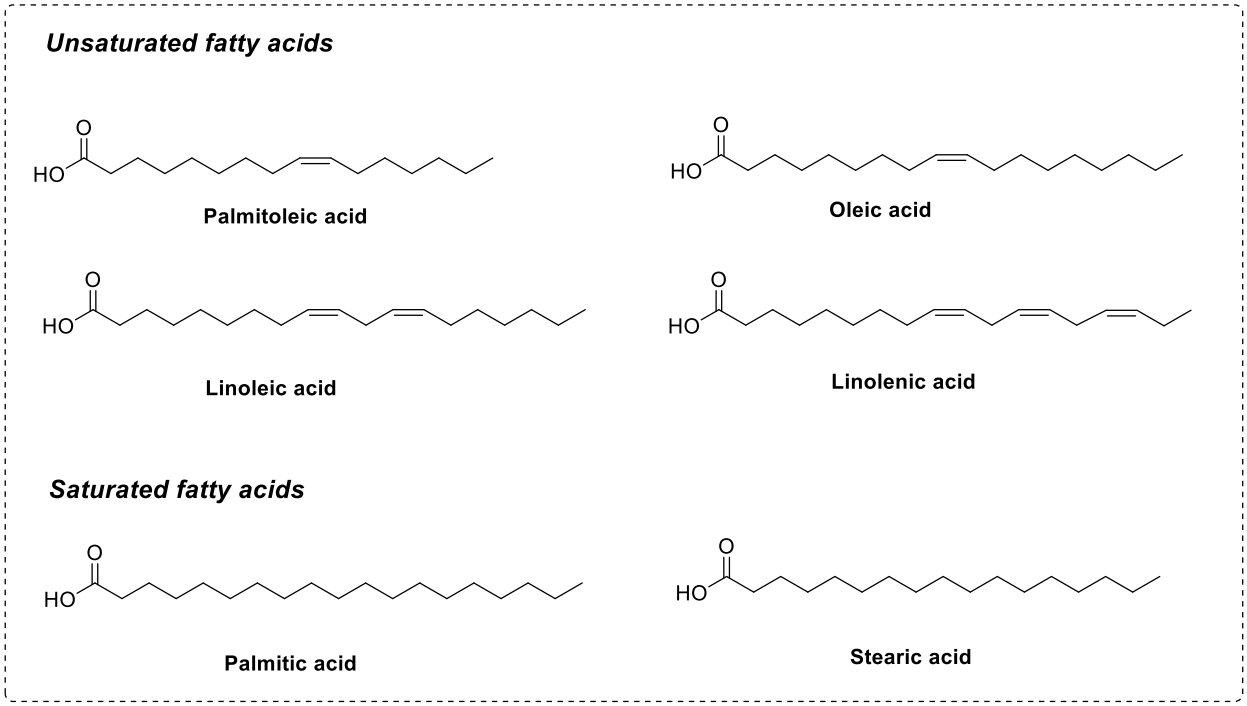

Figure 15: Common fatty acids found in mushrooms.

Consuming essential fatty acids in balanced proportions, such as a 1:1 or 2:1 ratio of ω-6 to ω-3, is associated with various health benefits. An unbalanced ratio has been linked to adipogenesis and obesity. Essential fatty acids also contribute to the formation of high-density lipoprotein (HDL), which transports fats from the blood to the liver, reducing the risk of cardiovascular disorders.

Additionally, ω-3 fatty acids like EPA and DHA can modify cell membrane structure, influence cell protein functions, impact the production of lipid mediators, and regulate gene expression, thereby contributing to overall health[109].

1.4 Extraction Techniques

The qualitative and quantitative analysis of bioactive compounds in mushrooms is heavily reliant on selecting appropriate extraction methods[111]. Extraction, the initial step in medicinal mushroom studies, plays a crucial role in determining the final outcomes. Often referred to as "sample preparation techniques," extraction methods are integral to the overall success of analytical studies[112]. While modern chromatographic and spectrometric techniques have simplified bioactive compound analysis, the effectiveness still hinges on the chosen extraction methods, input parameters, and the specific nature of mushroom parts[113]. Various factors influence extraction processes, including the matrix properties of mushroom parts, the choice of solvent, temperature, pressure, and time[114]. Understanding the dynamic chemical nature of diverse bioactive molecules has significantly advanced in the past decade, contributing to the progress of bioactive compound analysis[115]. Despite technological and technical advancements, bioactive compounds in mushrooms often coexist with other compounds. Identification and characterization of these bioactive compounds are feasible from different mushroom parts such as the fruiting bodies and mycelium[116]. The increasing awareness of the dynamic nature of bioactive molecules has attracted interest from pharmaceuticals, food additive producers, and the natural pesticides industry in utilizing bioactive compounds from natural sources[117].

Various methods can be utilized for extracting plant materials, and over the past 50 years, non-conventional approaches have been developed as more environmentally friendly alternatives. These methods, characterized by reduced reliance on synthetic and organic chemicals, shortened operational times, and improved extract yield and quality, have gained prominence. To enhance the overall yield and selectivity of bioactive components from plant materials, researchers have explored non-conventional methods, including ultrasound, pulsed electric field, enzyme digestion, extrusion, microwave heating, ohmic heating, supercritical fluids, and accelerated solvents[117].

Traditional extraction methods, known as conventional extraction techniques, have been in use for an extended period and continue to be employed today. These methods rely on the use of solvents

or mixing, often combined with heat. The maceration method and the Soxhletation method are the specific extraction techniques applied in this study[118].

1.4.1 Maceration (Traditional Method)

Various techniques can be utilized to extract primary and secondary metabolites from plants. For non-volatile compounds, the traditional method involves immersing the plant in a solvent (water, alcohol, oil), known as solid-liquid extraction or **maceration**. In recent years, modern extraction techniques have been developed, leveraging "assisted" technology like ultrasonic waves, pressure, and microwaves. These methods aim to enhance performance by reducing process duration and conserving energy and solvents through various intensification mechanisms. The physical treatment associated with these techniques can also influence the extraction mechanism, potentially boosting yields and resulting in different extraction selectivities compared to simple maceration[119].

Maceration has been a long-standing method for preparing homemade tonics, gaining popularity as an economical approach to obtain essential oils and bioactive compounds[117]. It represents a traditional extraction method known for its simplicity and cost-effectiveness, requiring only a basic container for the extraction process[120]. However, it has the drawback of being time-consuming. This technique is versatile and can be carried out in various settings, making it particularly common for extracting active compounds from mushrooms. Azmir et al 2013 and Tambun et al 2021 describes this extraction technique in great detail. In small-scale extraction using maceration, the process typically involves several steps. Firstly, mushroom materials are ground into small particles to enhance surface area, promoting effective mixing with the solvent[117]. Subsequently, an appropriate solvent, referred to as the menstruum, is added to a sealed vessel during the maceration process[117]. Thirdly, the liquid is separated, but the marc, which is the solid residue from extraction, undergoes pressing to recover a substantial amount of obstructed solutions. The strained liquid and the press-out liquid are combined and subjected to filtration to remove impurities. Periodic shaking in maceration serves two purposes: (a) enhancing diffusion and (b) removing concentrated solutions from the sample surface to introduce new solvent to the menstruum, thereby increasing extraction yield[117]. The effectiveness of this method is influenced by factors such as the quantity of raw materials, solvent selection, and the appropriate duration for extraction. commonly used solvents in the maceration extraction of mushroom include methanol, ethanol, ethyl acetate, and

distilled water[121]. Other solvents like hexane, chloroform, butanol, propanol, among others, may also be employed, taking into account considerations such as toxicity and cost[117, 118].

When determining the optimal operating parameters, process optimization often focuses on overall yield. However, in certain instances, it becomes necessary to prioritize the extraction of a specific family or compound. In such cases, selectivity is considered to identify the best conditions. This can involve enhancing an extract with target compounds or avoiding undesirable substances such as pollutants. In scenarios where avoiding unwanted compounds is crucial, purity becomes the optimized parameter[119].

1.4.2 Ultrasound Assisted Maceration

Ultrasound, a type of sound wave beyond human hearing, is commonly employed in chemistry within the frequency range of 20 kHz to 100 MHz. Similar to other waves, it generates compression and expansion as it passes through a medium, leading to a phenomenon known as cavitation— the formation, growth, and collapse of bubbles. This cavitation process results in the conversion of kinetic energy into heat within the bubble contents. Bubbles created during cavitation exhibit high temperatures (around 5000 K), pressure of 1000 atm, and rapid heating and cooling rates exceeding 1010 K/s, according to. Utilizing this principle, Ultrasound-Assisted Extraction (UAE) has been developed, with cavitation effects observed in liquid and solid-containing materials. UAE proves particularly advantageous for solid mushroom samples, as ultrasound energy aids in leaching organic and inorganic compounds from the mushroom matrix[117].

The underlying mechanism of UAE involves two main physical phenomena: (a) diffusion across the cell wall and (b) rinsing the cell contents after breaking the walls. Factors such as moisture content, milling degree, particle size, and solvent selection play crucial roles in achieving efficient extraction. Additionally, temperature, pressure, frequency, and sonication time are governing factors for ultrasound's effectiveness. UAE has been integrated with classical techniques such as maceration, to enhance conventional systems' efficiency, with an ultrasound device strategically positioned in a solvent extraction unit[117].

The advantages of UAE include reduced extraction time, energy consumption, and solvent usage. Ultrasound energy enables more effective mixing, faster energy transfer, reduced thermal gradients and extraction temperature, selective extraction, smaller equipment size, quicker response to

process extraction control, rapid start-up, increased production, and elimination of certain process steps[117].

In conclusion, ultrasonic waves enhance plant extraction by aiding in cell wall breakdown and compound release. However, other mechanisms induced by different intensities, temperatures, or devices can affect extraction and selectivity. Thermal degradation and hydrolysis, triggered by ultrasound, can chemically modify solutes, allowing for selective extraction. The studies discussed demonstrate how ultrasonic waves can alter the extraction process, resulting in different selectivities[119].

1.4.3 Soxhlet Extraction

The Soxhlet extractor, introduced by German chemist Franz Ritter Von Soxhlet in 1879, was initially devised for lipid extraction but has since evolved to accommodate a broader range of applications. This extraction method is extensively employed for extracting valuable bioactive compounds from diverse natural sources and serves as a benchmark for evaluating new extraction techniques[117].

The Soxhlet extraction process employs a specialized tool called a Soxhlet, which comprises essential components like a heat source, round bottom flask, Soxhlet extractor, and condenser, In laboratory-scale applications, these tools are assembled for the extraction of mushrooms. Finely ground raw material is positioned in a Soxhlet extractor enveloped in a thimble typically made of robust filter paper or cellulose, while the solvent is introduced into a distillation flask. The addition of condenser devices in this extraction method makes solvents recyclable. As the solvent is heated, the vapors condense in the condenser then drips into the thimble containing the raw material, extracting it upon contact. As the liquid level in Soxhlet reaches the top of the siphon tube, the liquid contents in it is siphoned into the distillation flask. This continuous process persists until a solvent drop from the siphon tube leaves no residue upon evaporation[118, 122].

Notably, this method offers advantages over previous techniques, enabling the extraction of substantial amounts of raw material using a significantly smaller quantity of solvent. This results in considerable savings in terms of time, energy, and overall financial resources. While at a small scale, it operates as a batch process, its efficiency becomes more pronounced when implemented as a continuous extraction method on a medium or large scale [96]. Solvents, such as methanol are

often utilized in mushroom extraction through the soxhlation method. Additional solvents, including hexane, chloroform, butanol, and propanol, could potentially be employed based on their boiling points[94].

1.5 Analytical Techniques for Quantification of Bio-actives

1.5.1 HPLC-DAD

High-Performance Liquid Chromatography (HPLC) is a pivotal tool in modern analytical chemistry, stemming from classical column chromatography. It plays a central role in various stages of drug discovery, development, and production within the pharmaceutical industry. HPLC is the preferred method for assessing peak purity of novel chemical entities, monitoring changes in synthetic procedures or scale-up, assessing new formulations, and conducting quality control and assurance for final drug products. The primary objective of HPLC is to separate and quantify the main drug, any reaction impurities, synthetic intermediates, and degradants[123].

HPLC has evolved into one of the most potent analytical tools, capable of separating, identifying, and quantifying compounds present in liquid-dissolved samples. Widely utilized for both quantitative and qualitative analyses of drug products, HPLC is particularly valuable for determining drug product stability. The HPLC process involves injecting a sample solution into a column of porous material (stationary phase), while a liquid phase (mobile phase) is pumped through the column at higher pressure. The separation principle relies on the adsorption of solute on the stationary phase based on its affinity towards the stationary phase. This method boasts high resolution, employs small-diameter stainless steel, facilitates rapid analysis, operates at relatively higher mobile phase pressure, and allows for controlled flow rates of the mobile phase.

Choosing a detector in chromatography involves considering various characteristics. No single detector possesses all these features, leading to the development of numerous detectors tailored to specific challenges. While ease of use, predictability, and reproducibility remain crucial, recent emphasis has shifted to factors like flow cell contribution to band broadening and faster detector responses. Additional considerations when choosing a detector, include factors like wavelength range, sensitivity, selectivity, and linear dynamic range. Complementary or orthogonal detectors used in combination (series or parallel) are gaining popularity, particularly in drug discovery or screening-type applications[124].

Photodiode array detectors (PDA) or Diode array detectors (DAD) in High-Performance Liquid Chromatography (HPLC) have an optical configuration akin to variable wavelength detectors, with the light passing through the flow cell before reaching the grating. This setup disperses the spectrum across an array of photodiodes. Commonly, Z-path or tapered detector cell designs are used in UV detectors for HPLC. The detector cell volumes are typically 8–10 µL for a 4.6 × 100 mm column (3 µm particle size), and the flow cell volume is crucial in preventing extra-column band broadening, which can be problematic with smaller column diameters and particle sizes[124].

Early PDA detectors were initially less sensitive than single-wavelength UV detectors, but advancements in detector cell technology have bridged this sensitivity gap. Modern PDA detectors utilize a light-guided flow cell resembling an optical fiber, allowing efficient light transfer in an internal reflectance mode. Despite maintaining a 10 mm flow cell path length, the volume is only 500 nL, reducing dispersion[124].

PDA detectors extend UV detection utility by providing spectra of eluting peaks, aiding in peak identification and monitoring co-elutions (peak homogeneity or purity). They can serve as a multi-wavelength UV/VIS detector. Spectra collected at the chromatographic peak apex can be used to create a library for subsequent identification. Spectral resolution, coupled with chromatography data system (CDS) software algorithms, allows for detailed spectral comparisons. CDS software can compare fine differences in spectra, either through direct point-to-point comparisons or complex vector analysis in multi-dimensional space[124].

1.5.2. GC-FID

Chromatographic separation methods are undeniably the most commonly employed analytical techniques for compositional analysis. Gas chromatography (GC) stands out as a distinctive and versatile technique initially applied to analyze gases and compounds with high volatility. GC serves as the analytical method for product identification under controlled conditions and coupling it with a mass spectrometer becomes essential for tasks beyond comparative fingerprinting, requiring positive identification of peaks on the chromatogram. The fundamental principle of gas chromatography lies in the compound's affinity for the stationary phase, determining retention time in the column before elution and detection. The column is a crucial component of the gas chromatograph, facilitating the separation of components. Factors like the source and control of carrier gas flow, sample introduction, and detection methods are integral to the process.

Temperature, influencing analyte volatility, is controlled by placing the column in a thermostatically controlled oven. The option of faster gas chromatographic separation is generally beneficial, reducing analysis time and increasing sample throughput. This reduction can be achieved by altering column parameters, such as length, inner diameter, and stationary phase thickness, or by adjusting operational parameters like temperature program rate and carrier gas flow. Gas chromatography has played a pivotal role for 50 years in determining food composition, understanding nutritional needs, ensuring food quality, and identifying organic contaminants at trace concentrations in complex samples. Widely employed in research, industry, environmental, and forensic labs, GC is favored for its ability to detect minimal quantities in a diverse range of samples, provided the compounds are thermally stable and reasonably volatile[125].

The separation process relies on retarding individual components as they traverse a lengthy column propelled by a carrier gas, typically helium or nitrogen. The column, often made of steel or glass, contains an inert packing material such as glass or ceramic beads, occasionally coated with an involatile liquid in gas-liquid chromatography (GLC). Alternatively, in gas-solid chromatography (GSC), the packing may be a solid substance without a liquid coating, although this is less common than GLC. The sample is introduced into the carrier gas stream, and as it progresses through the column, the molecules of each substance in the sample distribute between the gas and the liquid. Volatility determines the proportion of time a substance's molecules spend in the carrier gas, affecting its emergence from the column. This dynamic equilibrium results in the separation of substances within the column, and they emerge at different times. The time elapsed from injection to emergence is termed retention time (Rt), unique to each substance under specific conditions, influenced by factors such as volatility, column temperature, length, and diameter. Heating the column in an oven is often employed to reduce inconveniently long retention times at room temperature. Following separation, a detection and measurement method is required. Thermal conductivity detectors (TCDs) and flame ionization detection (FID) are common choices. TCDs rely on thermal conductivity changes in the gas leaving the column, whereas FID, preferred in food applications, is approximately a thousand times more sensitive for organic compounds, as it involves burning the emerging gas to form ions, conducting an electric current for amplification and recording. Despite a small number of ions formed, the constant proportion allows the recorded signal to be proportional to the amount of the chemical substance present[125].

The increase in usage of the Flame Ionization Detector (FID) has surpassed the Thermal Conductivity Detector (TCD) in current usage. Its popularity is also attributed to its reliable performance in ionization detection. The FID operates on the physical basis of an oxidative hydrogen flame burning organic molecules, producing ionized molecular fragments collected by an electrical field. The detector exhibits nearly uniform sensitivity to all pure organics composed of carbon and hydrogen. Optimized operating parameters yield a sample detection of about 20 pg or approximately 5 ppb of sample gas concentration. While the FID is nearly universal, it exhibits insensitivity to specific gases, such as fixed gases, oxides of nitrogen, H_2S, SO_2, COS, CS_2, CO, CO_2, IDO, NH_3, and HCOOH. The FID's linearity is affected by factors like operation at temperatures above 250°C, contamination on insulator surfaces, and concentrations greater than 0.1%, leading to partial grounding of the ion current. The FID is considered a destructive detector, requiring a sample splitter for preparative work. It also experiences sensitivity decreases in the presence of atoms of oxygen, nitrogen, phosphorus, sulfur, or halogen in the structure of organics, with high detectability requiring special precautions to maintain optimal performance[126].

1.5.3. Colorimetric/ Spectrophotometric Analysis

1.5.3.1. Bradford Assay

Various techniques exist for determining protein concentrations. Certain methods, like Kjeldahl or elemental analysis, are not specifically tailored for proteins. On the other hand, some methods exhibit higher specificity for proteins but were originally designed for animal tissues and physiological fluids, where protein concentrations are elevated, and interfering components are minimal. In ecological studies focusing on mushroom protein estimation, an ideal method should be both specific and sensitive to proteins, resistant to interference, cost-effective, quick, simple, and suitable for field sampling[127].

A swift and precise method for determining protein concentration is crucial in various biological and biochemical applications. The Bradford assay, initially introduced by Bradford, has become the preferred choice for protein quantification in many laboratories due to its simplicity, speed, and heightened sensitivity compared to the Lowry method. Additionally, it experiences less interference from common reagents and non-protein components in biological samples, making it more advantageous. Despite the emergence of alternative protein assays, the Bradford method continues to be a popular technique[128].

The Bradford assay relies on the interaction between the dye Coomassie Blue G250 and proteins. Extensive studies indicate that the free dye exists in four distinct ionic forms with pKa values of 1.15, 1.82, and 12.4. In the acidic assay reagent solution, the more cationic red and green forms have absorbance maxima at 470 nm and 650 nm, respectively, while the more anionic blue form, binding to proteins, exhibits an absorbance maximum at 590 nm. Consequently, protein quantity estimation involves determining the amount of dye in the blue ionic form, typically measured by the absorbance of the solution at 595 nm[128].

The dye predominantly binds to arginine and lysine residues of proteins, and to a lesser extent, histidine and aromatic residues (tryptophan, tyrosine, and phenylalanine). This specificity can introduce variation in the assay response to different proteins, constituting the primary limitation of the method. While modifications have been proposed to address this issue, the original Bradford method devised by Bradford remains the most convenient and widely used formulation, offering a balance of robustness and practicality.

1.5.2.1 Megazyme Assay

Mushroom and fungal β-glucans exhibit structural differences from cereal β-glucans, which are linear polysaccharides with varying 1,3-β- and 1,4-β-linkages, the ratio of which depends on the source (e.g., oats, barley, wheat). While specific enzymatic procedures exist for measuring cereal 1,3:1,4-β-d-glucans, such methods are less specific for measuring 1,3:1,6-β-d-glucans in commercial yeast products. No quantitative enzymatic procedure has been established for measuring β-glucan in mushroom fruiting bodies or mycelium until recently. Several studies employed complex extraction and acid hydrolysis processes to determine β-glucan content in mushrooms. In recent years some new methods were developed for the determination of β-glucans, among others, the enzymatic method (Megazyme, Ireland) for the determination of β-glucans in mushrooms and yeast and Congo red method, which enables to detect β-glucans with triple helical structure. The Yeast and Mushroom β-glucan kit by Synytsya et al. provides total glucan measurement through acid hydrolysis, while α-glucan is specifically measured by enzymatic hydrolysis. Glucose is determined enzymatically with glucose oxidase/peroxidase reagent, and β-glucan is calculated as the difference[129]. This is the method that is employed in this study.

Other non-quantitative methods, like ELISA and Congo red dye, have been described for measuring β-glucan in mushroom products. Due to the challenging enzymatic hydrolysis of β-

glucan in mushrooms, the preferred quantitative method involves complete acid hydrolysis to glucose, followed by glucose measurement to determine total glucan. α-Glucan can be either removed before acid hydrolysis or measured separately and accounted for. Dallies et al. used a similar acid hydrolysis method to measure β-glucan content in Saccharomyces cerevisiae[130]. The complexity of β-glucan linkages in mushrooms requires careful consideration when determining their quantitative composition[131].

1.5.2.1 DPPH Assay

The current methods employed for assessing antioxidant activity rely on spectrophotometric techniques, specifically utilizing mechanisms involving hydrogen atom transfer (HAT) and single electron transfer (SET). These assays encompass various techniques, including the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) radical scavenging activity assay, oxygen radical absorbance capacity (ORAC) assay, ferric reducing antioxidant potential (FRAP) assay, and cupric reducing antioxidant capacity (CUPRAC) assay. Among these, the DPPH• assay stands out for its simplicity and widespread use[132].

The DPPH radical dot assay is commonly employed to evaluate the free radical scavenging capabilities of antioxidant molecules, representing a standard and straightforward colorimetric method for assessing the antioxidant properties of pure compounds. DPPH•, existing as a stable radical in solution, exhibits a purple color and absorbs at 515 nm in methanol. The assay operates on the principle that when DPPH• accepts a hydrogen atom from an antioxidant molecule, it undergoes reduction to form DPPH2. This reduction results in a color change from purple to yellow, accompanied by a decrease in absorbance at 515 nm. Spectrophotometric monitoring of this color transition allows for the determination of antioxidant property parameters[133]. Originally conceptualized by Blois in 1958, the DPPH• assay gained attention for antioxidant property characterization about three decades later[134]. While the assay seems simple, its stability as a nitrogen radical introduces complexities. Different antioxidants may exhibit varying kinetics or even fail to react. Moreover, the reversibility of the reaction between DPPH• and antioxidants may lead to an underestimation of antioxidant capacity, as $DPPH_2$ can convert back to DPPH•. This reversibility factor has been highlighted in studies involving isoeugenol and other phenols with similar structures, emphasizing the potential for inaccuracies in estimating antioxidant capacity[135].

While there has been a considerable volume of research dedicated to exploring the medicinal properties of mushroom extracts, a notable gap exists in the integration of the extraction and analytical techniques mentioned in this study, for a comprehensive understanding. Despite the widespread use of Soxhlet extraction, there is a lack of a standardized protocol for its application in mushroom extraction. This highlights the need for a more cohesive and systematic approach that combines robust extraction methodologies with rigorous analytical techniques to unlock a holistic understanding of the medically beneficial properties inherent in mushroom extracts.

There are significant differences between water extracts and commercially available samples of mushrooms. Additionally, this implies that a detailed examination and isolation of individual compounds from these mushrooms have not been extensively explored or addressed in-depth. Comprehensive isolation and characterization of individual compounds are crucial for understanding the specific therapeutic or beneficial properties associated with each component. This study attempts to close this gap, revealing more refined extraction techniques using organic solvents and a more detailed examination of the individual bioactive compounds present in the mushrooms.

CHAPTER 2: Research Goals/ Objectives

The main objective of this study is to quantitatively and/or qualitatively analyze the nutraceutical value and fingerprint the bioactive components of medicinally valuable mushrooms. Mushrooms are known to contain a variety of bioactive compounds that contribute to their health benefits, resulting in high commercial value. This research aims to identify and analyze specific compounds such as protein, β-glucan, ganoderic Acid-A, vitamin D_2, ergosterol, polyphenols, fatty acids, and antioxidant capacity in mushrooms. The study will provide insights into the behavior of these compounds under different conditions, including degradation, reactivity, and polarity. Additionally, nutritional content will be determined to assess the overall nutritive value of the mushrooms. The findings of this research could contribute to the discovery and development of new drug therapies and enhance our understanding of the health-promoting properties of mushrooms. This will be done by:

2.1 Extracting Bio-active components

To analyze the target compounds present in mushrooms, various extraction methods will be utilized, namely maceration, ultrasound-assisted maceration, and Soxhlet extraction. Different solvents, including water, methanol, ethyl acetate, dichloromethane, and hexane, will be employed during maceration and ultrasound-assisted maceration processes. Soxhlet extraction will be performed using methanol, ethyl acetate, dichloromethane, and hexane. The choice of solvents aims to explore their differential extraction efficiencies for specific compounds. The study seeks to compare the effectiveness of these extraction techniques and solvents in isolating the target compounds from the mushroom samples, providing valuable insights into optimal extraction protocols for subsequent analyses.

2.2 Screening for Bio-active compounds

Quantification of the target bioactive compounds present in mushrooms was carried out using a combination of chromatography and various analytical assays. Protein content will be assessed through the Bradford assay, providing insights into the nutritional composition.

Specific bioactive compounds, including ganoderic acid-A, vitamin D_2, ergosterol, and polyphenols, will be quantified using HPLC analysis, thereby, contributing to a comprehensive

profile of mushroom constituents. Additionally, Fatty Acid analysis will be performed using GC-FID, to enhance the understanding of lipid composition of these mushrooms. A Megazyme assay will be employed for the analysis of β-glucan, a crucial polysaccharide. Furthermore, antioxidant activity of the mushroom extracts measured using the DPPH assay, will provide information on the mushrooms' capacity to neutralize free radicals. Through these analytical approaches, the research aims to elucidate the diverse array of bioactive components in mushrooms, contributing to a holistic comprehension of their potential health-promoting properties.

2.3 Determining Nutritional Value

The objective of this study is to assess the nutritional value of two species of mushrooms (lions mane and reishi) through proximate analysis. Various components, including moisture, ash, fat, fiber, protein, carbohydrate, and total energy content, will be systematically determined for both mushroom species. Proximate analysis involves the quantification of essential macronutrients and energy content, providing a detailed breakdown of the nutritional composition. The data obtained from these analyses will be compiled and reported as a percentage, offering valuable insights into the overall nutritional profile of the mushrooms under investigation. This objective aims to contribute to a thorough understanding of the mushrooms' dietary significance and potential health benefits, facilitating informed dietary recommendations and further exploration of their nutritional value.

CHAPTER 3: Materials and Methods

3.1 Materials

3.1.1 Sample Storage and Preparation

Fresh frozen mushroom samples are stored at -80°C in a freezer, ensuring preservation of their freshness. Conversely, dried mushroom samples are stored in a refrigerator at -4°C, maintaining their stability in a dehydrated state. Prior to extraction and analysis, the fresh frozen samples undergo preparation by being cut into small pieces to facilitate the subsequent processes. On the other hand, the dried samples are transformed into a powder form through grinding, enhancing the surface area for efficient extraction and analysis. Mushrooms were obtained from Collaborator Huxley Health and used as received in these processing steps. They obtained the mushrooms from a combination of Canadian-based growers, primarily in Southern Ontario.

3.2 Mushroom Extraction

3.2.1 Maceration

Please Note: Only dried lions mane mushroom was used for this extraction procedure.

A total of 1 g of mushroom samples were subjected to this extraction using various solvents, namely water, methanol, ethyl acetate, hexane, and dichloromethane, all of HPLC grade. The extraction was performed in 200ml of each solvent for three different time periods: 24 hours, 48 hours, and 72 hours at room temperature. To ensure reliability and consistency, all extractions were conducted in triplicate.

For the ultrasound-assisted macerations, a similar procedure was followed with some variations. Again, 1 g of mushroom sample was extracted in 200 ml of solvent, using water, methanol, ethyl acetate, hexane, and dichloromethane, all of which are HPLC grade. However, in this case, the extraction periods were altered to 30 minutes, 2 hours, and 4 hours, with the process carried out at a temperature of 30°C and a frequency of 40kHz. Similar to the conventional extractions, all ultrasound-assisted macerations were performed in triplicates.

All extracts were collected, filtered and dried using a rotary evaporator (BUCHI R-100) followed by a high vacuum, then weighed to determine their percent yields.

3.2.2 Soxhlet Extraction

Please Note: Both fresh and dried lion' s mane and reishi mushroom were used for this extraction procedure.

Initially, 5 g of mushroom sample was extracted using 300 ml of methanol at a temperature range of 95-100℃ for a duration of 24 hours. To monitor the extraction progress, aliquots of 10ml were withdrawn at regular intervals, specifically every 30 minutes during the initial 4 hours, and subsequently every 4 hours until the completion of the 24-hour period. Upon determining the optimal extraction time, which was found to be 8 hours, further extractions were carried out using different solvents and temperatures. Specifically, hexane was employed at 70℃, ethyl acetate at 75-80℃, and dichloromethane at 40℃. In these subsequent extractions, 10 ml aliquots were collected every 2 hours. All extraction procedures, including the initial and subsequent ones, were conducted in triplicates.

All collected samples underwent a drying process using a rotary evaporator, followed by high vacuum drying. The final step involved weighing the samples to determine their percentage yields.

3.3 Analysis

3.3.1 Proximate Analysis

Please Note: Only dried lions mane and reishi mushroom samples were used for these analyses. Equations for calculating the percent moisture, ash, fat, fiber, protein, carbohydrate and total energy in kcal/100g are presented in the results and discussion.

Moisture Test- Initially, 2 g of each mushroom sample was placed in an oven set at 110℃ for a duration of 3 hours. After the initial drying period, the samples were allowed to cool for an hour in a desiccator then weighed. Subsequently, the samples underwent an additional drying phase for 1 hour, followed by a cooling period of 30 minutes before weighing again. This cycle was repeated two more times or until no further reductions in mass were observed, indicating that the samples had reached a stable moisture content. This was done in triplicate.

Ash Test- Two grams of mushroom sample was incinerated in a muffle furnace for 6 hours at 550℃. The ash was weighed afterwards. This was done in triplicate.

Fat Test- Two grams of mushroom sample was extracted using 150 ml of petroleum ether in a Soxhlet extraction for 6 hours at a temperature of 50-60℃. The collected extract was dried in an oven at 100℃ for 40mins. Sample was cooled in a desiccator, then weighed. This was done in triplicate.

Fiber Test- Two grams of mushroom sample was boiled in 200 ml of 0.128M sulphuric acid for 30 mins at 310℃. The sample was filtered, then washed with hot distilled water 2-3 times and squeezed dry. The residue was then boiled in in 200 ml of 0.313M sodium hydroxide for 30 mins at 200℃. Again, the sample was filtered, then washed with hot distilled water 2-3 times and squeezed dry. The residue was then dried in the oven for 3 hours at 110℃, cooled in the desiccator, then weighed. Finally, sample was incinerated in the furnace at 550 ℃ for 4 hours, cooled, and then weighed again. This analysis was done in triplicate.

Protein Test- Using the Kjeldahl method, two grams of each mushroom sample was subjected to digestion. The digestion process involved combining the sample with 34 ml of concentrated sulfuric acid, 2 g of copper (II) sulfate, and 14 g of potassium sulfate. The mixture was digested until a clear green-blue solution was achieved. After cooling, the digested sample was collected and made up to volume with distilled water in a 100ml volumetric flask within an ice water bath. For distillation, a 10 ml aliquot of the digested sample was taken and treated with approximately 10 ml of 45% sodium hydroxide until a dark blue color was attained. This was also done in an ice water bath. Subsequently, 50 ml of distilled water was added, and the solution was distilled into a collecting solution containing 25 ml of 4% boric acid. Distillation was continued until the flask was empty, and the final volume was measured. Both digestion and distillation steps were performed in duplicate. Finally, a 1/10th portion of the distilled solution was titrated with 0.1N HCl solution using Tashiro indicator. This titration process was conducted three times for each sample. A blank analysis was also conducted, i.e., a digestion, distillation and titration was carried out on all the reagents without mushroom sample.

Carbohydrate and **Total energy (kcal/100g)** were determined by calculation.

3.3.2 Bradford assay- Protein Analysis

This assay was done using the Quick Start Bradford assay kit from Bio-Rad. A standard curve was made using Bovine Serum Albumin BSA standards in a range from 0.15625 – 5 mg/ml. Ten

microliters of standard and 990 µL of Bradford Reagent x1 was prepared in a 3ml cuvette and read at 595nm. This was done in triplicate and a standard curve of average absorbance against concentration was plotted. For measurement of the samples, 10 µL of mushroom extract with 990 µL of Bradford Reagent x1 was prepared in a 3 ml cuvette and read at 595nm.

3.3.3 Megazyme Assay- β-glucan Analysis

This assay was carried out using the Megazyme β-Glucan Assay Kit (Yeast and Mushroom).

Measurement of Total glucan- Ninety milligrams of mushroom extract was treated with 2ml of cold 12M sulphuric acid and placed in an ice bath for 2 hours (vigorously stir every 20 mins). After which, a total of 10ml of distilled water was added to each sample. They were then put in a boiling water bath and incubated for two hours. In a 100ml volumetric flask, the contents along with 6mL 8M NaOH were added, then the volume was made up with 200mM Sodium acetate buffer (pH 4.5). This was done for all samples and controls. An aliquot was then transferred to a microcentrifuge tube and centrifuged for 5 mins at 13,000 rpm. One hundred microlitre aliquots of the supernatant from centrifuged extract were transferred to a test tube, along with 100µL of prepared bottle 1 to hydrolyse any remaining glucan fragments to glucose, then incubated at 40°C for 60mins. While incubating, reagent blanks were prepared by adding 200µL of 200mM NaOAc buffer (pH 4.5) to tubes in duplicate. For the standards, 100µL of 200mM NaOAc buffer (pH 4.5), plus 100µL of D-glucose standard solution (bottle 5) were added to a test tube, also in duplicate. After incubation, 3ml of GOPOD reagent was added to all tubes (standards, blank and samples) then incubated for 20 mins at 40°C. Absorbance was measured at 510nm against reagent blank.

Measurement of α-Glucan- One hundred milligrams of sample was transferred to a tube and 2 ml of 1.7M NaOH was added. It was then placed in an ice water bath for 20 mins to dissolve the phytoglycogen/starch while stirring. After that, 8 ml of 1.2M NaOAc buffer (pH 3.8) was added to each tube. Immediately, 0.2mL of bottle 2 was added, then samples were incubated for 30 mins at 40°C. After this incubation period, the contents were transferred to a 100ml volumetric flask and adjusted with distilled water. Samples were then centrifuged for 10 mins at 13,000rpm. One hundred microlitres of the centrifuged extracts were added to test tubes with 100µL of 200mM NaOAc buffer (pH 4.5). Finally, 3ml of GOPOD reagent was added to all tubes, then incubated at 40°C for 20 mins. Absorbance was read at 510nm. The standards and reagents blanks prepared for the total glucan analysis were used for this analysis as well.

3.3.4 DPPH Assay- Antioxidant Analysis

DPPH solution was prepared by dissolving 7.89mg of DPPH solution in 100mL of methanol, then sonicated for 5-10mins. Initially, methanol, ethyl acetate, hexane and DCM mushroom extract samples were prepared by adding 500µL of DPPH reagent, 400µL of TRIS-HCL buffer and 100µL of sample. Blanks for each sample were also created to account for the colour of the extracts. This was done by adding 500µL of methanol and 400µL of TRIS-HCL buffer to 100µL of sample. Samples and blanks were incubated for 30mins at room temperature, then read at 517nm.

To determine the IC_{50} values, concentrations of 0.3mg/ml- 0.025mg/ml were prepared for only for hexane samples. These samples, of vary concentrations, were then prepared the same way as the samples before (500µL of DPPH reagent, 400µL of TRIS-HCL buffer and 100µL of sample), along with their respective blanks (500µL of methanol and 400µL of TRIS-HCL buffer to 100µL of sample). Again, they were allowed to incubate for 30mins at room temperature, then read at 517nm.

3.3.5 HPLC-DAD

All HPLC analyses were carried out using an Agilent Infinity 1260 fitted with a DAD detector.

Sample preparation: One hundred microlitres of hexane, ethyl acetate and DCM mushroom extracts were dried down under a stream of nitrogen and reconstituted in 2mL of ethanol for HPLC analysis. Methanol extracts on the other hand, were diluted by a factor of 10, centrifuged for 5 mins at 4500rpm, then analysed by HPLC.

3.3.5.1 Ergosterol and Vitamin D2

Analysis: HPLC analysis of extract was performed using an Agilent Zorbax SB-C-18 column (5um x 150mm x 4.6 mm) and Methanol/ 0.1% formic acid as mobile phase by isocratic elution at a flow rate of 1 ml/min. Column was conditioned at 35 degrees centigrade for the duration of the analysis. Nine standard dilutions of ergosterol were prepared in ethanol for a range of concentrations from 0.606 mg/ml to 0.0006 mg/ml. Injection of standard dilutions was in triplicates and data analysis was performed at end of HPLC analysis. Detection of ergosterol and vitamin D2 was at 282 nm and 254 nm respectively.

3.3.5.2 Ganoderic Acid A

Analysis: Mobile phase was a combination of 100% acetonitrile (mobile phase A) and 0.03% phosphoric acid in water (mobile phase B). A gradient run was applied with increasing acetonitrile proportion from 31% to 100% and a total run time of 21 min. Flow rate of mobile phase was 1 ml/min with sample injection volume of 5ul and detection of GAA at 252 nm. This analysis was conducted using an Agilent Zorbax SB-C-18 column (5um x 150mm x 4.6 mm) set at temperature of 35℃.

Data generated from HPLC analysis were processed and a calibration curve generated for eight GAA standard concentrations (0.5 mg/ml- 0.0005 mg/ml) to enable quantification of GAA in samples, as well as determine precision of method. Injection of standard dilutions was in triplicates and data analysis was performed at end of HPLC analysis.

3.3.5.3 Polyphenol Analysis

Analysis: A semi-quantitative HPLC analysis of all mushroom extracts were performed using an Infinity Lab Poroshell 120 EC-C18, 2.1 x 150mm, 1.9µm column set at 30℃. Mobile phase was a combination of 0.1% Trifluoracetic Acid (TFA) in water and 0.1% TFA in methanol. A gradient run was applied with increasing methanol proportion from 5% to 100%, then back to 5% to equilibrate. This analysis had a total run time of 50 mins, with a flow rate of 1mL/min and injection volume of 16µL. Polyphenols were detected at 220, 210, 252 and 280nm.

3.3.6 GC-FID – Fatty Acid Analysis

Sample preparation: Two hundred microlitres of mushroom extract was dried down under a stream of nitrogen, its dry mass recorded, and then 1mL of NaOH in MeOH (2:3 v/v) was added. Oxygen was removed under a stream of nitrogen prior to heating for 10 mins at 100℃. After cooling, 1mL of 17% boron trichloride in MeOH was added and oxygen was again removed using nitrogen. Samples were heated for 30 mins, cooled, after which 1mL of hexane and 5mL of concentrated sodium chloride was added. The sample was mixed thoroughly, and the hexane layer was collected, concentrated, then reconstituted in 200µL of hexane for GC analysis.

Analysis: A semi-quantitative FAMES (Fatty Acid Methyl Ester) analysis was conducted on the hexane mushroom extracts using a Perkin Elmer GC-FID instrument (Clarus 590) according to Zengin et al 2015. An Agilent DB-Wax column with dimensions 30m x 0.25mm x 0.25µm was used. The column temperature was set at 60°C for 1 min and increased up to 190°C at the rate of

20°C/min, held for 60 min then increased again at the rate of 1°C/min to 220°C and held for 10 min at 220°C. The injection and FID temperatures were set at 250 and 260°C, respectively. Injection volume of sample was 0.5µL and the split ratio was set at 60:1[136]. Fatty acids were identified by comparing the profile of an oleic acid standard to the profile of the samples, also using olive oil as a positive control in the analysis.

CHAPTER 4: Results and Discussion – Extraction and Proximate Analysis

4.1 Mass Recovery of Macerations (Percent Yield)

The conducted study focused on investigating the impact of extraction time and solvent type on the mass recovery of mushroom extracts using maceration extractions. With consideration to existing literature, it is well-established that parameters such as temperature, time, solid to liquid ratio, and the type of solvent, including potential interaction effects among solvents, play crucial roles in the extraction of bioactive compounds from mushrooms. The linearity of these parameters is not indefinite, as highlighted in the discussion. For instance, it is true that temperature and percent yield are directly proportional to each other, i.e., the higher the temperature, the greater the mass recovered. On the other hand, after "optimal temperature" is achieved, there will be no further increase in the percent yield. Moreover, surpassing this optimal temperature threshold can potentially result in the degradation of the target bioactive compounds, underscoring the necessity for careful optimization in mushroom extraction methods. As a result, optimization of mushroom extraction methods are fundamental[137].

It is crucial to note that the characteristics of the target bioactive compounds are to be considered when optimizing mushroom extractions. The polarity and interaction of these compounds with different solvents are the most significant factors to examine. This recognition underscores the nuanced nature of mushroom extraction, where understanding the specific properties of the compounds being targeted is essential for achieving optimal extraction efficiency.

This study explores the impact of extraction time and solvent type on mass recovery, recognizing the potential non-linear relationships involved. The selection of five different solvents (methanol, ethyl acetate, DCM, hexane, water) and three different extraction times (24, 48, and 72 hours) reflects a systematic approach to understanding the complex interplay of these variables. By holding temperature and solid to liquid ratio constant at room temperature and 1:200, respectively, the study maintains controlled conditions, allowing for a focused examination of the specific effects of extraction time and solvent type.

We expect that the percent yield will be increased as the time for extraction increases. The assumption that longer extraction times lead to increased mass recovery is grounded in the idea

that a more prolonged exposure allows for a more comprehensive extraction of bioactive compounds from the mushroom matrix. Extended maceration periods provide the solvent with additional time to penetrate the tissue, facilitating the dissolution of a wider range of compounds. This is especially relevant for bioactive compounds that may have slower diffusion rates or are present in lower concentrations[137]. Furthermore, using the following formula the extraction yield was calculated as seen in Figure 16.

$$\text{percent yield} = \frac{mass\ of\ extract(g)}{mass\ of\ sample\ (g)}\ x\ 100$$

Note: Maceration extractions were carried out using only dried *Hericium erinaceus* (lion's mane) mushroom. **A T-test was carried out to compare 24 to 48-hour, 24 to 72-hour and 48 to 72-hour extraction. The presence of a bar indicates that there is a significance difference between the two extraction times compared, with a P value that is <0.05.**

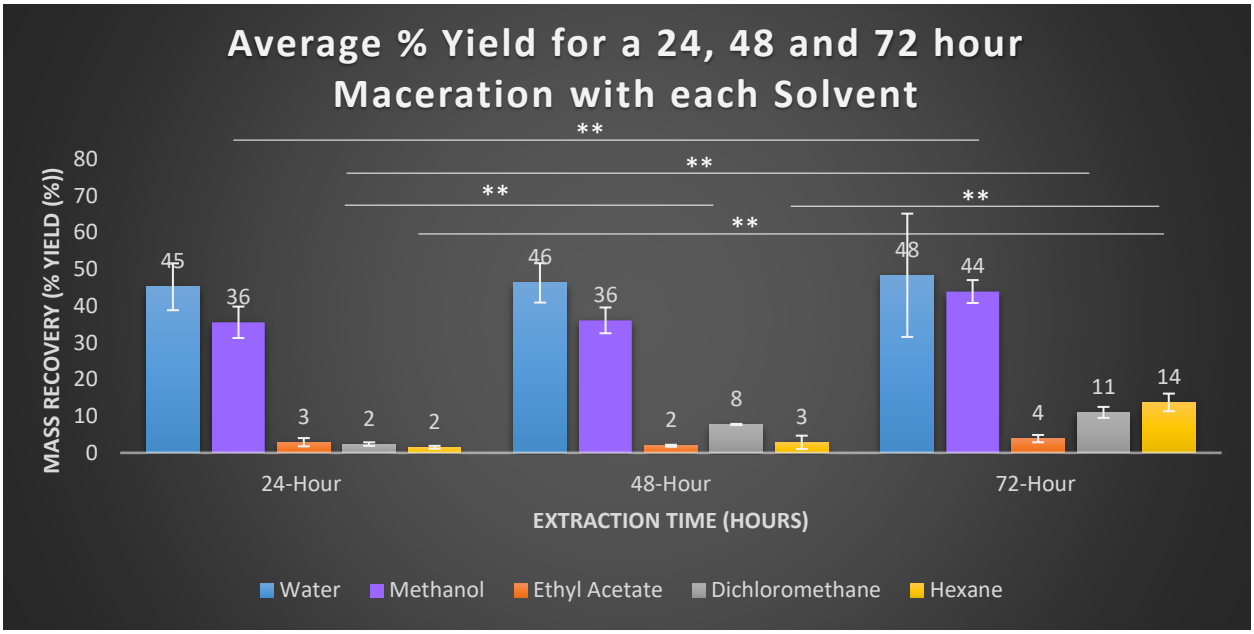

*Figure 16: Average Yield (%) produced for macerations carried out with methanol, ethyl acetate, dichloromethane, hexane, and water for 24-hours, 48-hours and 72-hours. Extractions were conducted using of 1 g of dried Hericium erinaceus (lion's mane) mushroom with 200ml of solvent. The extraction yields at 24-hour, 48-hour and 72-hours were methanol: 35.54, 36.06 and 43.92%; ethyl acetate: 2.90, 1.93 and 3.88%; DCM: 2.39, 7.72 and 11.01%; hexane: 1.51, 2.86 and 13.73%; water: 45.21, 43.27 and 48.34% respectively. Based on a two-tailed T-test, **=P<0.05.*

The data presented in Figure 16 reveal a consistent pattern in the extractions, where the percentage yield increases with longer extraction times. The 72-hour macerations stand out by producing the highest yield, while the 24-hour macerations consistently exhibit the lowest yield. This trend aligns with the general expectation that a more extended extraction period allows for a more thorough extraction of bioactive compounds from mushrooms[137].

While the 72-hour extractions generally yield slightly higher percentages, the difference in yield is very small**.** This raises a critical consideration regarding the necessity of conducting lengthy 72-hour extractions, especially given the associated time consumption. As a result, maceration for 24 or 48 hours may be sufficient as this may potentially streamline the extraction process without compromising overall yield. It's important to emphasize the balance between extraction time and yield, recognizing that the choice of extraction duration should align with the specific goals of the study and the characteristics of the target bioactive compounds.

The results presented in Figure 18 highlight a notable trend in the yields of mushroom extracts based on the type of solvent used. Water extracts consistently demonstrated the highest yields, with percentages of 52.17%, 55.09%, and 73.59% for 24, 48, and 72 hours, respectively. Following closely behind were methanol extracts. This outcome aligns with expectations, given that water and methanol are highly polar solvents. The high polarity of these solvents facilitates the extraction of a broad range of compounds, including both polar and some non-polar substances, as discussed in previous research[74].

Ethyl acetate and dichloromethane (DCM) are moderately polar solvents, while hexane is nonpolar. As anticipated, these solvents yielded significantly lower percentages, attributable to their limited ability to extract a diverse array of compounds. The lower yields from moderately polar and nonpolar solvents are often attributed to their reduced capacity to interact with and solubilize certain bioactive compounds present in the mushroom tissues. The assertion that ethyl acetate and DCM are moderately polar aligns with their performance in the extractions, where ethyl acetate, in particular, showed variability in recovery at different time points. The lower yields from hexane, emphasizing its nonpolar nature, is consistent with the expected behavior of nonpolar solvents in mushroom extractions. These solvents might be less effective in isolating certain compounds due to their inability to permeate the tissue. Mushroom tissues can be complex matrices with varying degrees of permeability, and solvent choice plays a vital role in ensuring the

extraction of a comprehensive profile of bioactive compounds. These observations further underscore the importance of solvent selection based on the nature of the compounds being targeted[138].

The ultrasound-assisted extractions were conducted in a similar manner to the regular macerations. The impact of extraction time and solvent type on mass recovery was investigated. Five different solvents (methanol, ethyl acetate, DCM, hexane, water) and three different extraction times (30mins, 2 and 4 hours) were utilized. Temperature, frequency and solid to liquid ratio was held constant at 30°C, 40kHz and 1:200, respectively.

Similar to the regular macerations, it is expected that as time increases, so does the mass recovery. Also, the %yields are expected to be higher than the regular maceration due to the use of ultrasound waves to further penetrate the cell.

Note: A T-test was carried out to compare 30 mins to 2-hour, 30 mins to 4-hour and 2 to 4-hour extraction. The presence of a bar indicates that there is a significance difference between the two extraction times compared, with a P value that is <0.05.

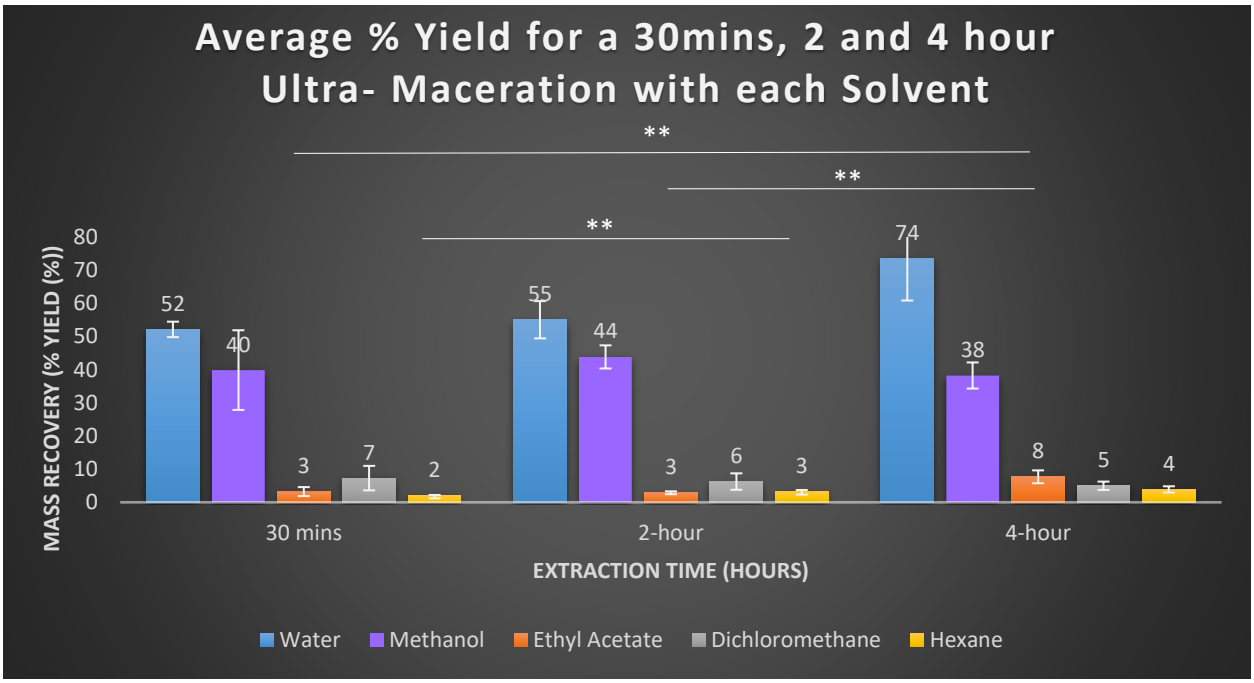

*Figure 17: Average Yield (%) produced for ultra-macerations carried out with methanol, ethyl acetate, dichloromethane, hexane and water for 24-hours, 48-hours and 72-hours. Extractions were conducted using of 1 g of dried Hericium erinaceus (lion's Mane) mushroom with 200ml of solvent. The extraction yields at 24-hour, 48-hour and 72-hours were methanol: 39.89, 43.87 and 38.26%; ethyl acetate: 3.23, 2.88 and 7.70%; DCM: 7.30, 6.26 and 4.99%; hexane: 2.15, 3.27 and 3.89%; water: 52.17, 55.09 and 73.59% respectively. Based on a two-tailed T-test, **=P<0.05.*

The utilization of ultrasound in extraction allows for an increase in mass recovery with less extraction time. This is especially demonstrated by the water extracts, where when the percent yield for normal maceration at 72 hours (M = 48.3, SD = 16.8) was compared with ultrasound assisted maceration for 30 mins (M = 52.2 SD = 2.3), showed no significant difference in mass recovery (percent yield), t(2)= 4.3, p = 0.7.

As seen in Figure 17, a general trend of greater percent yield was achieved with prolonged extraction time. This aligns with the general understanding that a more extended extraction period allows for a more thorough penetration of the solvent into the cellular matrix, facilitating the release and dissolution of bioactive compounds from the mushroom tissue. Again, water and methanol had higher percent yields, while DCM, ethyl acetate and hexane had lower recoveries due to their differing polarities.

The introduction of ultrasound waves as an additional extraction force is expected to enhance the extraction efficiency. Ultrasound waves can impart mechanical vibrations, creating microturbulences and promoting the disruption of cell walls. This physical action helps to release

intracellular components, including bioactive compounds, into the solvent more effectively than in regular macerations. Consequently, the use of ultrasound yielded higher percentage yields with less extraction times, compared to conventional maceration techniques. This is best demonstrated in the water and methanol extracts in Figure 18[139].

4.2 Mass Recovery of Soxhlet Extracts (Percent Yield)

The extraction strategy employed in this study, involving periodic sampling at various time intervals (every 30 mins for the first 4 hours, then every 4 hours till the end of extraction) during the 24-hour maceration with methanol, reflects a systematic effort to optimize the extraction process. The goal was to track the extraction kinetics and determine the optimal time for extraction by assessing the percentage yield at different time points. This approach allows for a more nuanced understanding of how the extraction process evolves over time.

The decision to sample at shorter intervals in the initial phase (every 30 minutes for the first 4 hours) followed by longer intervals was deliberate. The early frequent sampling captures rapid changes in the extraction process, while the subsequent less frequent sampling provides a broader overview of the later stages. This tiered sampling strategy helps to capture the dynamics of compound release during the critical phases of the extraction process[140]. This was done to determine the optimal time for extraction by calculating the percent yield for each aliquot taken using the following formula. The average values were used to plot a graph of average percent yield against time (hrs).

$$\text{percent yield} = \frac{mass\ of\ extract(g)}{mass\ of\ sample\ (g)}\ x\ 100$$

Peaks or plateaus in the graph may indicate points where the extraction efficiency reaches its maximum or stabilizes, suggesting the optimal time for terminating the extraction process. Conversely, a continuous increase may suggest that more time is needed for a more comprehensive extraction. It is expected that after the optimal time is approach, the percent yields will become constant. There will be no further significant increase in the yield after the optimal point and so the extraction can be stopped in order to shorten the extraction time. It is also expected that extraction with methanol will produce the highest yields.

Note: Maceration extractions were carried out using both fresh frozen and dried *Hericium erinaceus* (lion's Mane) and *Ganoderma lucidum* (reishi) mushroom.

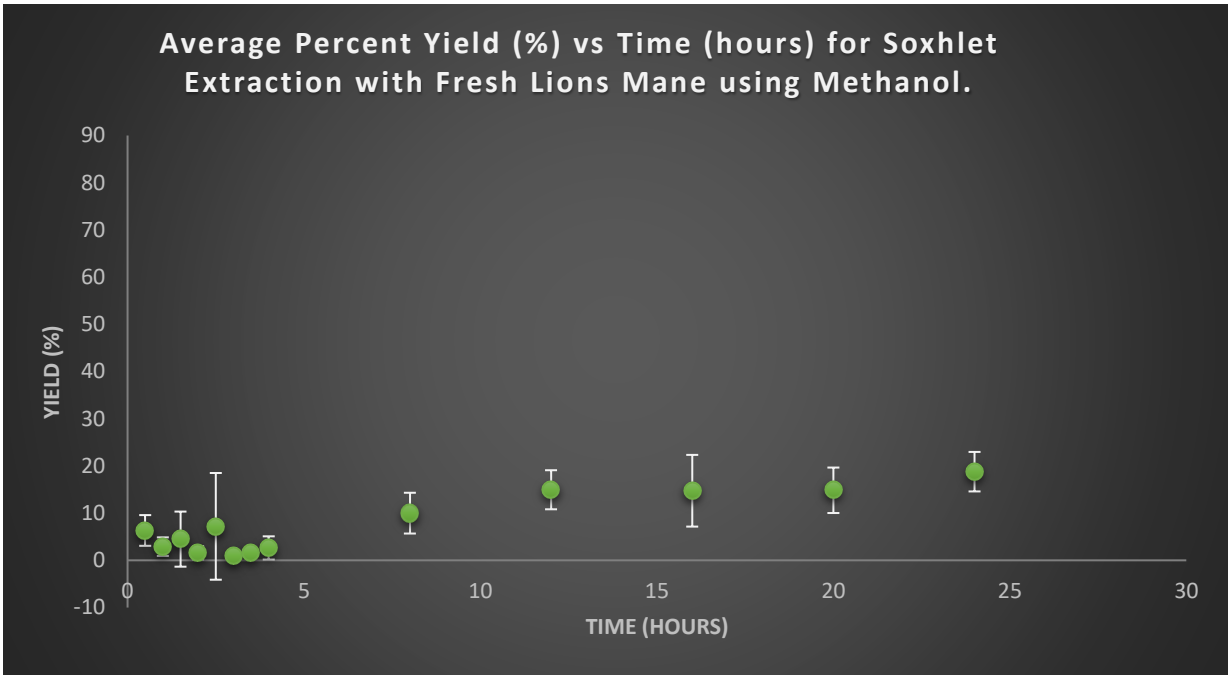

Figure 18: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh Hericium erinaceus (lion's Mane) mushroom with methanol as the solvent for 24 hours. Produced a total percent yield of 9.6%.

Based on the data presented in Figure 18, Soxhlet extraction using methanol for fresh *Hericium erinaceus* (Lion's Mane) produced an overall trend where, as the time increased, so did the yield. It also showed that extraction is largely complete after 8 hours. This conclusion aligns with the general principles of Soxhlet extraction kinetics, where there is an initial rapid phase of extraction followed by a slower, often asymptotic, phase[140]. The plateauing of the percentage yield after 8 hours suggests that the majority of the target compounds have been efficiently extracted from the mushroom matrix into the methanol solvent. This allows for the optimization of extraction time. By determining the point at which the extraction becomes largely complete, this study provides a guideline for the optimal duration of Soxhlet extraction for mushrooms.

The decision to end the extraction after 8 hours is not only based on maximizing efficiency but also takes into account the potential risks associated with extended extraction times. Prolonged exposure to solvents can lead to the degradation of sensitive compounds, and in the case of natural products like mushrooms, the risk of material decomposition is a valid concern. By ending the extraction at the point where the percentage yield becomes constant, this study mitigates the risk of undesired alterations in the extracted material[140].

After the optimal time for extraction was determined (8 hours), extractions with three more solvents were conducted at this "optimal time". This approach allows for a comparative analysis of the efficiency of these solvents in extracting bioactive compounds from mushrooms under similar conditions. The choice of solvents used (ethyl acetate, DCM and hexane) was as a result of their varying polarities. In these extractions, samples were taken every 2 hours to track the extraction.

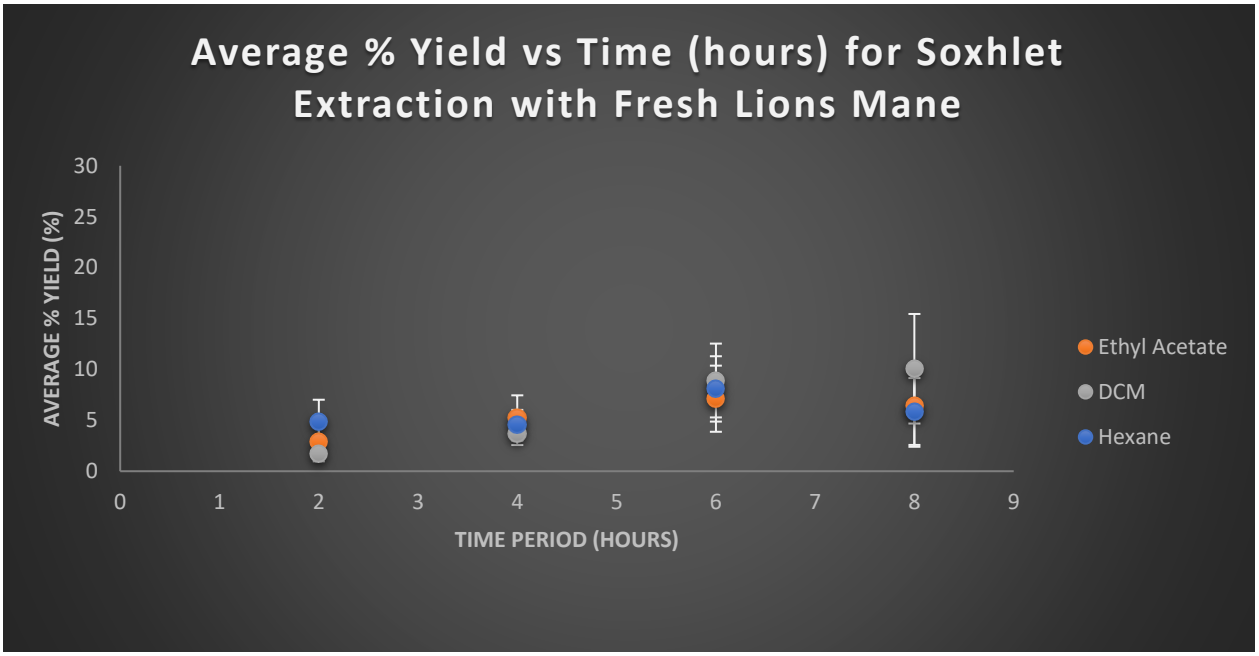

Figure 19: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh Hericium erinaceus (lion's Mane) mushroom with ethyl acetate, DCM and hexane as the solvent for 8 hours. This produced a total percent yield of 5.8, 3.1 and 4.8% respectively.

According to Figure 19, as expected and seen with the macerations, ethyl acetate, DCM and hexane produced lower yields than extractions using methanol. Also, there was overall increase yield as the time progressed.

As a general trend surrounding the percent yield starts to reveal itself, Soxhlet extractions dried lion's mane, as well as fresh and dried reishi mushroom were conducted. This provides a comparative framework to discern general trends in the extraction efficiency across different species and sample states. The same solvents, solvent to volume ratio and temperatures were used to see if the trends would remain the same across the two different species and samples (dried or fresh). It would be expected that, despite differences in the actual yields, similar trends in extraction efficiency would emerge if the solvent interactions with the mushroom matrices follow

consistent principles. If this does occur, the dried samples will produce higher yields compared to their fresh counterparts.

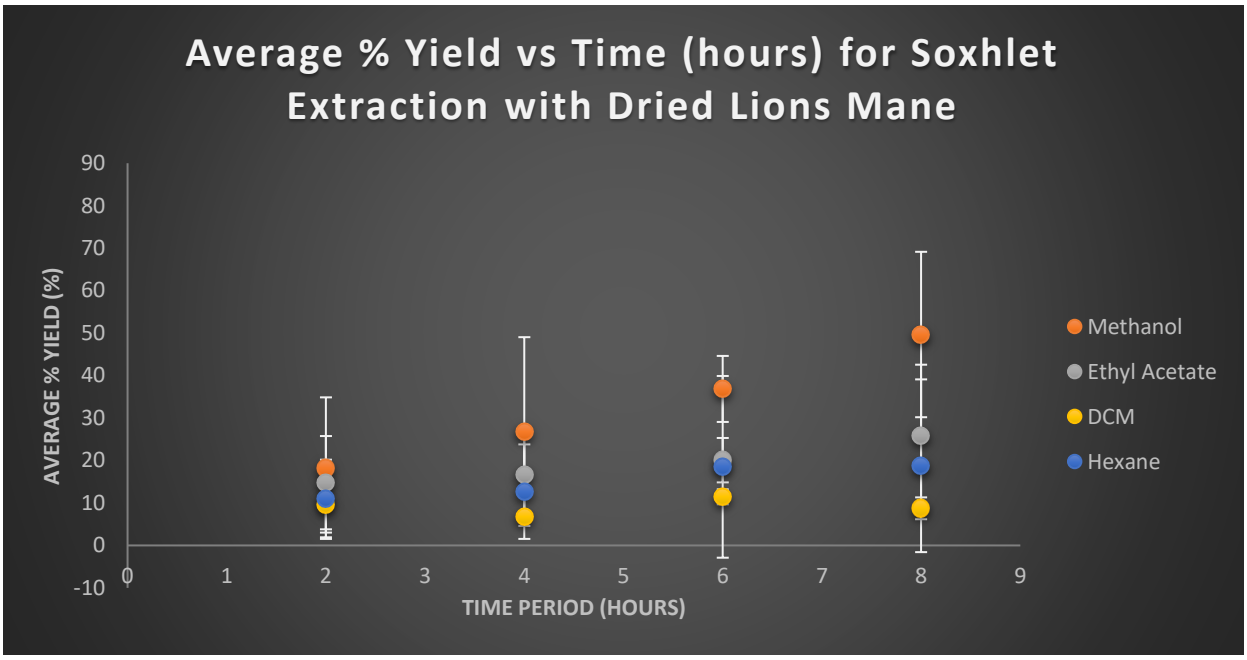

Figure 20: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried Hericium erinaceus (lion's mane) mushroom with methanol, ethyl acetate, DCM and hexane as the solvent for 8 hours. This produced a total yield of 31.3, 8.8, 11.7 and 9.2%, respectively.

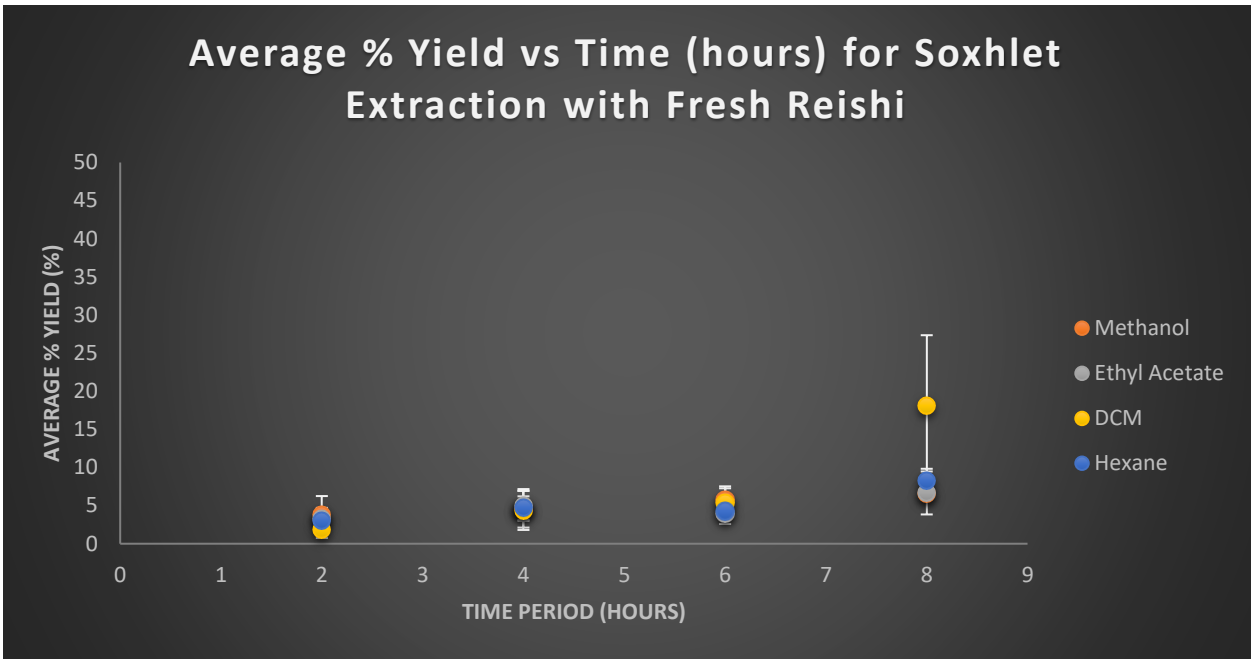

Figure 21: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh reishi (Ganoderma lucidum) mushroom with methanol, ethyl acetate, DCM and hexane as the solvent for 8 hours. This produced a total yield of 3.5, 3.7, 2.8 and 2%, respectively.

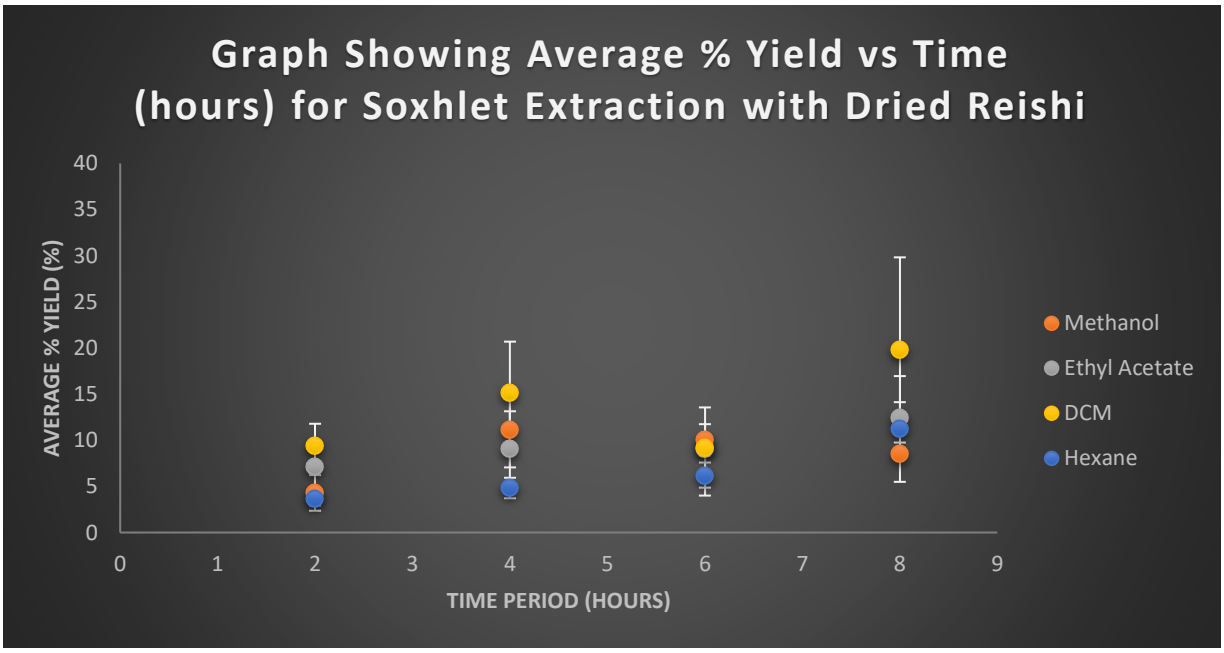

Figure 22: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried reishi (Ganoderma lucidum) mushroom with methanol, ethyl acetate, DCM and hexane as the solvent for 8 hours. This produced a total yield of 7.8, 3.9, 5.5 and 2.8%, respectively.

The mass recoveries of extracts obtained from soxhlet extractions with fresh lion's mane mushroom using methanol (M = 7.8, SD = 6.2), ethyl acetate (M = 5.4, SD = 1.9), DCM (M = 6.1, SD = 4), and hexane (M = 5.8, SD = 1.6) were compared with those from fresh reishi mushroom extractions using methanol (M = 5.2, SD = 1.2), ethyl acetate (M = 4.7, SD = 1.5), DCM (M = 7.4, SD = 7.3), and hexane (M = 5.1, SD = 2.3). The results of the t-tests indicate that there is no statistically significant difference in mass recoveries between the two species for all solvents: t(14) = 2.1, p = 0.2 (methanol); t(14) = 2.1, p = 0.2 (ethyl acetate); t(5) = 2.6, p = 0.8 (DCM); t(5) = 2.6, p = 0.6 (hexane).

The mass recoveries of extracts obtained from soxhlet extractions with dried lion's mane mushroom using methanol (M = 32.9, SD = 13.5), ethyl acetate (M = 19.3, SD = 4.9), DCM (M = 9.2, SD = 2), and hexane (M = 15.2 SD = 4) were compared with those from dried reishi mushroom extractions using methanol (M = 8.5, SD = 3), ethyl acetate (M = 9.4, SD = 2.2), DCM (M = 13.4, SD = 5.1), and hexane (M = 6.5, SD = 3.3). The results of the t-tests indicate that there is no statistically significant difference in mass recoveries between the two species for extraction with DCM, t(4) = 2.8, p = 0.2. However, extractions with methanol (t(3) = 3.2, p = 0.04), ethyl acetate (t(4) = 2.8, p = 0.02) and hexane (t(6) = 2.4, p = 0.01) demonstrated significant differences in mass recovery.

Based on Figure 20, extractions with dried lion's mane produced higher yields than the fresh extractions. This aligns with expectations, as the presence of water in fresh samples can hinder the extraction process. Water competes with solvents for available binding sites on the bioactive compounds, potentially limiting their solubility[141]. The lower yields from fresh samples emphasize the importance of considering the water content in optimizing extraction protocols. This was also seen within the reishi samples from Figures 21 and 22. Extractions done with fresh samples has less mass recovery than when the dried samples were utilized. Also, there is less mushroom in 5 grams of fresh sample compared to 5 grams of dried sample. The presence of water contributes to the overall mass of the sample. This can be corrected by conducting a moisture test on the fresh samples to determine what precent of it water. The mass recovery can then be corrected to account for the mass of water.

Also, methanol consistently produced the highest mass recoveries, while hexane consistently yielded the lowest. This trend holds true for both Lion's Mane and reishi mushrooms, whether they

are fresh or dried. The variations in solvent polarity play a pivotal role in determining the types of compounds that can be effectively extracted. The higher polar nature of methanol makes it more versatile in extracting a wide range of bioactive compounds, while the nonpolar nature of hexane limits its solubilization capabilities.

The consistent trends observed across both species (lion's mane and reishi) and sample states (fresh and dried) highlights the robustness of these findings. The generalizability of the trends suggests that the observed effects of sample moisture content and solvent polarity on mass recovery are not species-specific but are likely governed by fundamental principles applicable to different mushrooms.

4.3 Proximate Analysis

The analysis of major macromolecules in dried *Hericium erinaceus* (lion's mane) and *Ganoderma lucidum* (reishi) mushrooms provides valuable insights into their nutritional composition. The results, as presented in Table 1, indicate the content of ash, moisture, fiber, fat, and protein, with the calculated values for total carbohydrate and total energy. Ash content represents the inorganic residue left after complete combustion and is indicative of the mineral content in the mushrooms. The ash values obtained from the analysis contribute to understanding the overall mineral composition, which includes essential elements such as calcium, potassium, and magnesium[142]. Moisture content is a critical parameter as it influences the texture, shelf life, and overall quality of the mushrooms. The low moisture content in dried samples is expected, as moisture reduction is a common practice to enhance the stability and preservation of food products[142]. Fiber is an essential component for digestive health, and its presence in mushrooms contributes to their dietary fiber content. The fiber values offer insights into the potential nutritional benefits related to digestive processes and overall gut health[142]. The expectation of low-fat content aligns with general knowledge about the nutritional profile of mushrooms. Mushrooms are typically low in fat, making them a healthy and low-calorie food option[142]. In contrast, the anticipation of high protein content in mushrooms is consistent with their reputation as a valuable protein source, particularly for individuals following vegetarian or plant-based diets[143]. Calculating total carbohydrate and total energy content is essential for evaluating the overall nutritional contribution of the mushrooms. The determination of total energy in kcal/100g provides a quantitative measure of the caloric value

of the mushrooms. These values are critical for individuals managing their dietary intake and energy balance[144, 145]. The results are shown in Table 1 followed by the formulae for the calculations.

Table 1: Showing the values for ash, moisture, fiber, fat, protein, carbohydrate and total energy for lion's mane and reishi mushrooms.

Test	Hericium erinaceus (Lions Mane)	Ganoderma lucidum (Reishi)
Moisture (%)	9.54± 0.25	7.28± 1.19
Ash (Dry Mass) (%)	7.4 ± 1.89	0.76± 1.8
Ash (Wet mass) (%)	8.03± 0.26	0.77± 0.05
Fiber (%)	11.02± 0.33	4.38± 3.14
Fat (%)	1.04± 0.15	3.87± 2.82
Protein (%)	23.94± 7.15	18.03± 7.41
Carbohydrate (%)	57.45± 7.15	65.67± 7.41
Energy (kcal/100g)	312.72± 11.29	355.44± 11.71

Ash Content (Wet mass) (%)[142]:

$$= \frac{weight\ of\ ash\ (g)}{weight\ of\ sample\ (g)}\ x\ 100$$

Ash Content (Dry mass) (%)[142]:

$$= \frac{weight\ of\ ash\ (g)}{weight\ of\ sample\ (g)}\ x\ 100$$

Ash content was determined on a dry and wet basis. The dry weight was established by simply carrying out the protocol on the samples from the moisture test, while the dry weight was

determined by doing the analysis as is. The dry weight can also be calculated using the following formula:

Converting wet basis to dry basis:

% Ash on dry basis = ash on wet basis x 100/ (100- %moisture content)[146]

Moisture Content (%)[142]:

$$= \frac{W2-W3}{W2-W1}\ x\ 100$$

Where:

W1 = mass of dish

W2 = mass of dish + sample before drying

W3 = mass of dish + sample after drying

Fiber Content (%)[142]:

$$= \frac{Loss\ of\ weight\ after\ ignition\ (g)}{Weight\ of\ sample\ (g)}\ x\ 100$$

Fat Content (%)[142]:

$$= \frac{W2-W1}{Weight\ of\ sample}\ x\ 100$$

Where:

W1 = weight of flask

W2 = weight of flask with fat

Protein Content[143]:

$$\%\ protein = \frac{(b-a)\ x\ 0.1\ x\ 14.00}{Ws}\ x\ 100\ x\ \frac{4.38}{1000}\ x\ 10$$

where Ws = weight (g) or volume (ml) of sample

a = volume (ml) of 0.1N HCL used in blank titration

b = volume (ml) of 0.1N HCL used in sample titration

14.00 = atomic weight of nitrogen

1000 = the conversion of mgN/100 g to gN/100 g sample

4.38 =the protein-nitrogen conversion of mushroom

10 = ratio as only a portion of the digested sample was used.

Note: 4.38 was used instead of 6.25 as the conversion factor for calculating protein content because mushrooms contain significant amounts of non-protein nitrogen[72].

Total Carbohydrate Content[144]:

$= 100 - (total\ moisture + total\ ash + total\ fat + total\ fiber + total\ protein)$

Total Energy Content (Kjeldahl) (kcal/100g)[145]:

= (2.62 x %protein) + (8.37 x %fat) + (4.2 x % carbohydrate)

As seen in Table 1, the moisture, ash, fiber, fat and protein values for both lions mane and reishi were found to be similar to the literature[63, 142, 143, 147]. The carbohydrate and total energy content were then calculated based on those values and also compared to the literature, to which our values are analogous to[142, 147].

The alignment of these values for both lion's mane and reishi mushrooms with existing literature establishes the reliability and consistency of the analytical results. This concordance between experimental findings and literature values is crucial for validating the accuracy of the analytical methods employed and ensuring the representativeness of the samples under study. The consistency of the measured values with literature data has broader implications for the nutritional assessment of lion's mane and reishi mushrooms. These mushrooms are recognized for their bioactive compounds and potential health benefits, and an accurate understanding of their nutritional composition is foundational for evaluating their overall nutritional profile.

CHAPTER 5: Results and Discussion – Analysis of Soxhlet Extracts

5.3 Protein Quantification

The determination of protein concentration in both fresh and dried lion's mane and reishi mushrooms using a Bradford assay provides valuable insights into the dynamics of protein extraction under different conditions. We expect that protein content will increase with prolonged extraction time. The extended exposure to the solvent allows for a more thorough penetration into the mushroom tissues, facilitating the release of intracellular proteins. However, it's important to note that this increase may not be linear, and there may be a point of diminishing returns where further extraction time does not significantly enhance protein yield. This will be demonstrated in the 24-hour extraction with fresh lions mane[137].

It is also expected that methanol extracts will have the highest protein content is grounded in the solvent's polarity. Methanol is known for its ability to solubilize a wide range of compounds, including proteins. This expectation is particularly relevant when dealing with soluble proteins, as the solvent's affinity for polar compounds may result in a more efficient extraction of proteins compared to less polar solvents like hexane, ethyl acetate, and DCM[74, 138].

The analysis of both fresh and dried samples allows for a nuanced understanding of how the drying process may impact protein extraction. Drying can influence the structure and composition of the mushrooms, potentially affecting the accessibility of proteins for extraction. Comparing fresh and dried samples provides insights into whether the drying process enhances or diminishes protein yield[148].

It's important to acknowledge that the Bradford assay specifically quantifies soluble proteins. Proteins that are tightly bound within the cell walls or are less soluble may not be fully captured by this analysis. Therefore, while the assay provides valuable information about the soluble protein fraction, it may not reflect the total protein content in the mushrooms[149]. The Kjeldahl analysis is a better representation of the total protein content.

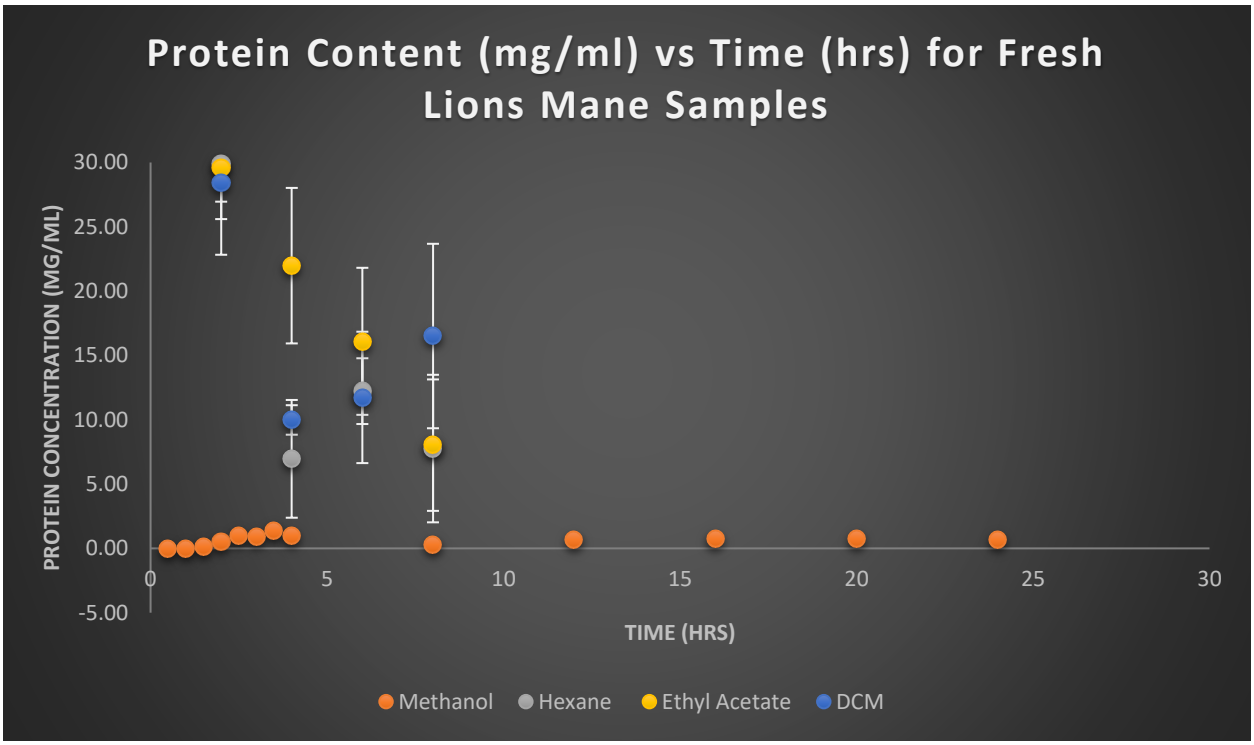

Figure 23: Protein Content (mg/ml) plotted against Time Period (hrs) for extraction with methanol, hexane, ethyl acetate and DCM. The data obtained from the extraction of 5.0 g of fresh lion's mane mushroom. The protein concentration was found to be 9.28, 64.52, 75.68 and 77.82% for methanol, hexane, ethyl acetate and DCM, respectively.

According to Figure 23, the protein content increased as the extraction time progressed for the methanol extraction of fresh lion's mane. This was expected as more protein in being released from the cells as the extraction time increases. However, it started to become constant towards the end of the extraction. This was expected as after a while, no more protein can be extracted. This shows that an 8-hour extraction is sufficient for protein extraction.

The analysis of the extractions done with hexane, DCM and ethyl acetate were also done. This resulted in more protein in these extracts than the methanol samples. This was unexpected as methanol, being more polar, is better at extracting proteins. The low protein content was as a result of degradation. Since the methanol extractions were done first, the time between extraction and analysis is greater than the other extracts. This analysis will be repeated, and the data will be updated if needed.

Protein analysis was then conducted using the dried lion's mane extracts as well as on the fresh and dried reishi extracts. This was done to see how the protein content behaves as extraction progresses between species and sample type (fresh and dried).

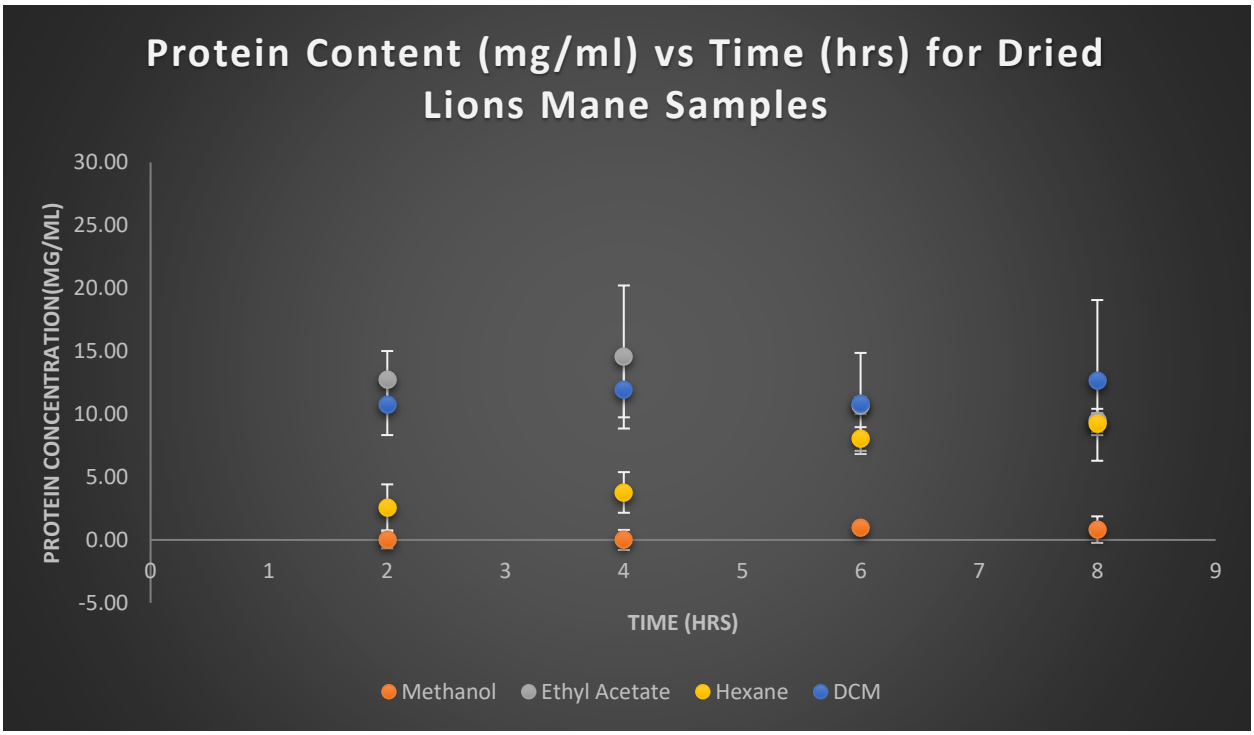

Figure 24: Protein Content (mg/ml) plotted against Time Period (hrs) for extraction with methanol, hexane, ethyl acetate and DCM. The data obtained from the extraction of 5.0 g of dried lion's mane mushroom. The protein concentration was found to be 3.46, 29.95, 54.73 and 52.23% for methanol, hexane, ethyl acetate and DCM, respectively.

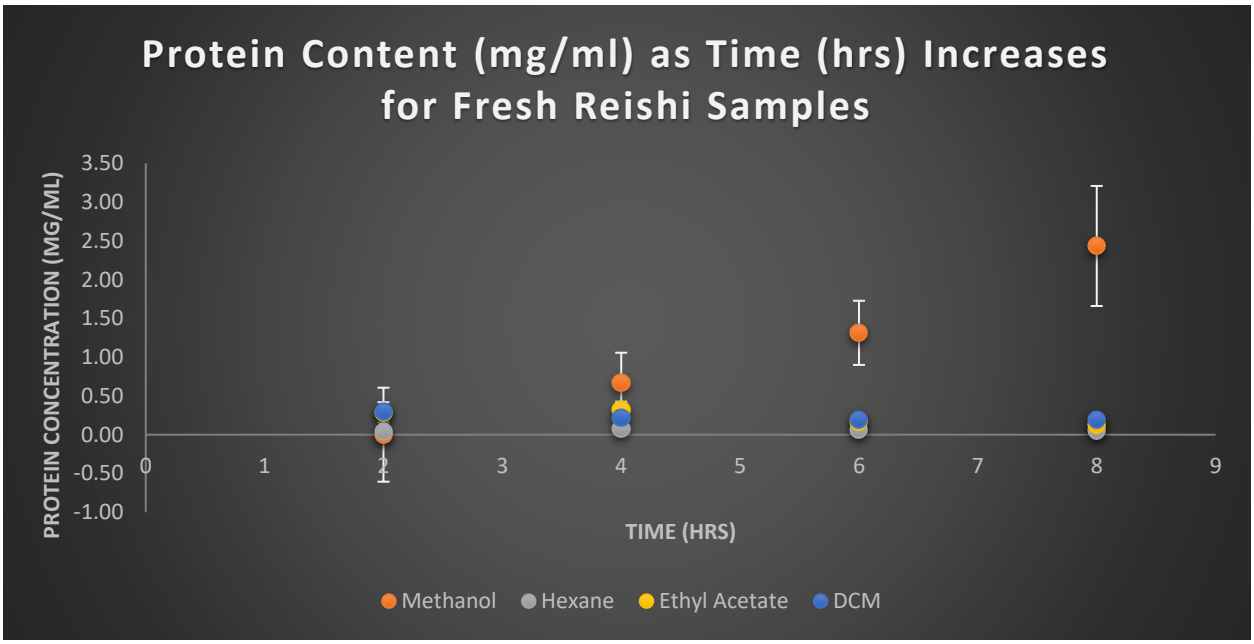

Figure 25: Protein Content (mg/ml) plotted against Time Period (hrs) for extraction with methanol, hexane, ethyl acetate and DCM. The data obtained from the extraction of 5.0 g of fresh reishi mushroom. The protein concentration was found to be 12.73, 0.35, 0.98 and 0.98% for methanol, hexane, ethyl acetate and DCM, respectively.

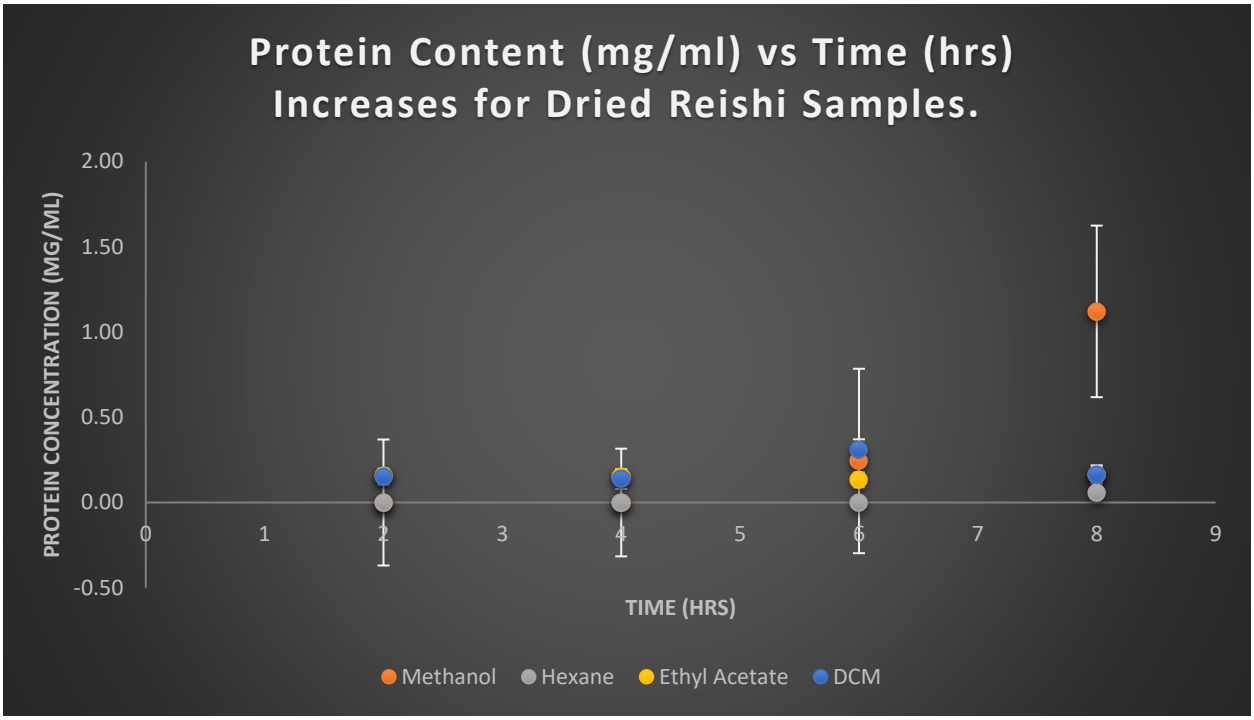

Figure 26: Protein Content (mg/ml) plotted against Time Period (hrs) for extraction with methanol, hexane, ethyl acetate and DCM. The data obtained from the extraction of 5.0 g of fresh reishi mushroom. The protein concentration was found to be 5.23, 0.10, 0.72 and 0.89% for methanol, hexane, ethyl acetate and DCM, respectively.

According to Figure 24, the protein content of the dried lion's mane extracts was lower than the fresh samples. We suspect that this was because some of the protein was degraded/ denatured by the heat in the drying process. This trend was seen in Figure 25 and 26 for the reishi samples as well, where the dried samples had less soluble protein than the fresh samples.

Similar to the fresh lion's mane samples, methanol extracts for dried lion's mane had less protein content than hexane, DCM and ethyl acetate. This was again due to a greater degradation of protein in the methanol extracts. However, in the reishi samples, the methanol extracts had the greatest protein concentration of all the solvents. This trend was seen in both the fresh and dried samples. This was expected as the higher polarity of methanol makes it better at extracting protein.

Finally, lion's mane mushroom has a higher soluble protein content than reishi mushroom. This trend was also seen on in proximate analysis where according to the Kjeldahl analysis for total protein (soluble and insoluble) lion's mane had greater values.

5.3 β-Glucan Analysis

The analysis of β-glucan content in the final time points of the Soxhlet extracts, conducted using the Megazyme assay, provides valuable insights into the extraction efficiency of this polysaccharide from both fresh and dried Lion's Mane and reishi mushrooms. It is expected that there will be low β-glucan content in all extracts. Being polysaccharides, they are generally poorly extracted by organic solvents[81]. However, the methanol extracts should have higher concentrations of this polysaccharide when compared to the others. Since it is a polar solvent, is theoretically more capable of extracting polar compounds like β-glucans. The dried samples should also have more β-glucan than the fresh samples as there is less water present. β-glucans are typically more concentrated in dried samples due to the reduction in water content. Water, being a polar solvent, may interfere with the extraction of β-glucans in certain solvents, and a lower water content is expected to enhance the extraction efficiency[82].

Note: ANOVA was used to compare the β-glucan content for methanol, hexane, ethyl acetate and DCM for both fresh and dried extracts. The presence of a broken line indicates that there is a significance difference between that solvent and the others, with a P value that is <0.05. A solid line shows there is no significant difference.

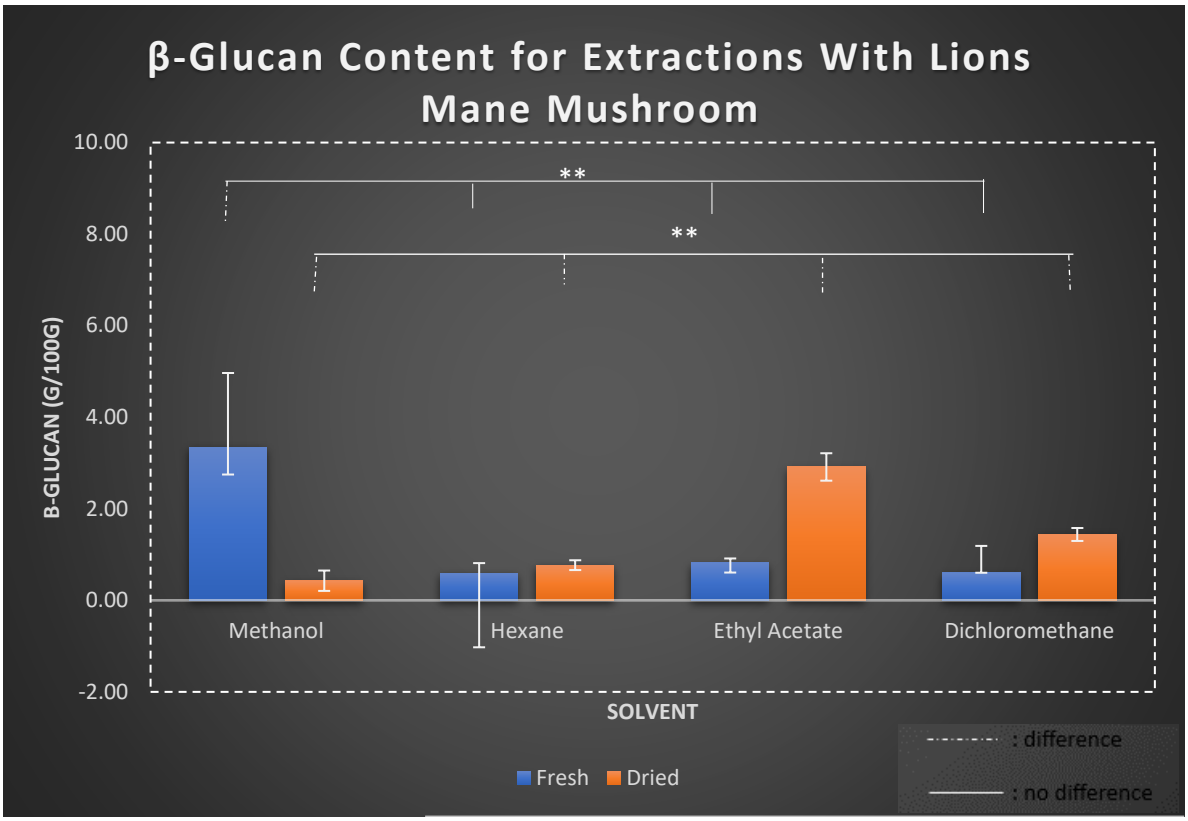

Figure *27: Shows the β-Glucan analysis of lion's mane mushroom extracted with methanol, hexane, ethyl acetate and DCM. Dried samples were found to be 3.34, 0.60, 0.82 and 0.60g/100g, while the fresh samples were found to be 0.43, 0.77, 2.91 and 1.44g/100g, respectively. Based on a single factor ANOVA, **=P<0.05.*

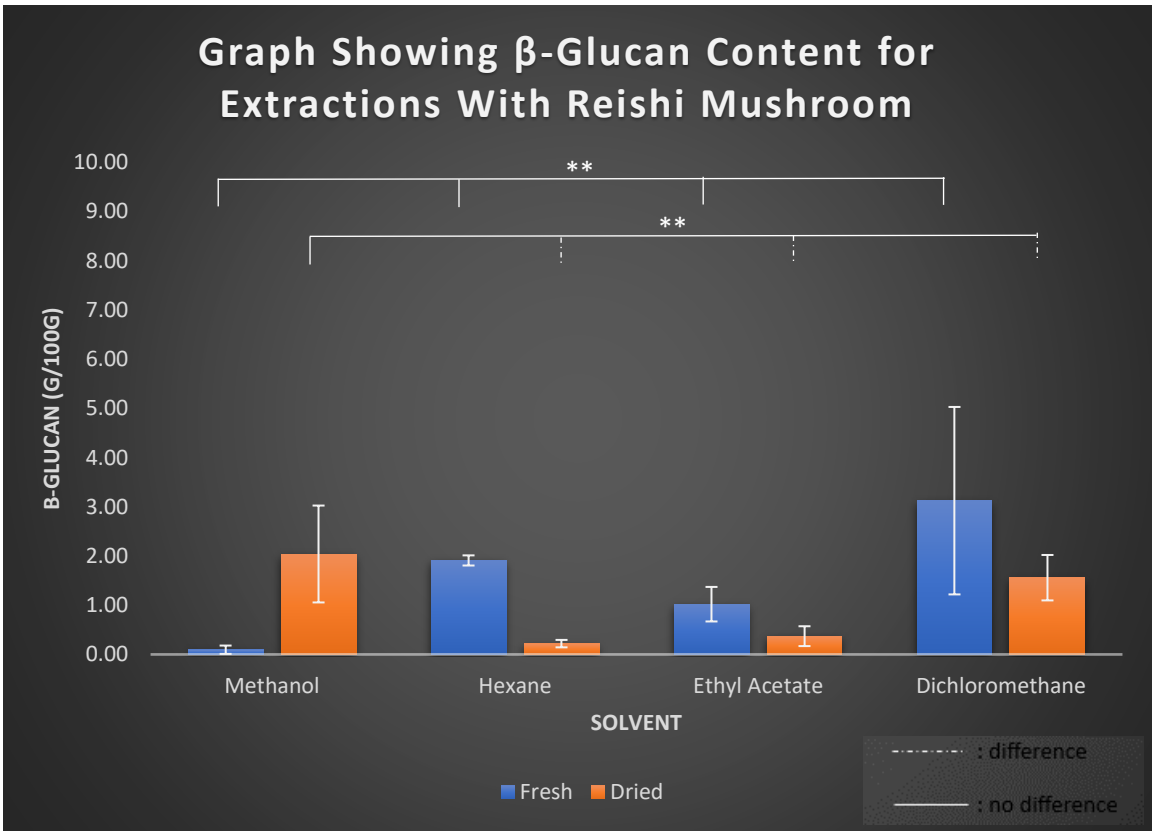

*Figure 28: β-Glucan analysis of reishi mushroom extracted with methanol, hexane, ethyl acetate and DCM. fresh samples were found to be 0.1, 1.91, 1.02 and 3.13g/100g, while the dried samples were found to be 2.04, 0.22, 0.37 and 1.56g/100g, respectively. Based on a single factor ANOVA, **=P<0.05.*

According to Figure 27, when analysis was done on the lion's mane extracts, β-glucan content was found to be higher in the dried samples, except for methanol (3.34g/100g). The was because the methanol extractions using fresh lion's mane were conducted for 24-hours, as opposed to the other extractions, which we carried out for 8 hours. The longer duration allows for a more comprehensive release of β-glucans from the cellular matrix, resulting in higher values[137].

The divergent trends in β-glucan content between fresh and dried reishi samples indicate that factors beyond extraction time play a role. The higher β-glucan content in fresh reishi samples may be attributed to inherent variations in β-glucan composition between different batches of mushrooms. Therefore, even though the extraction was done the same conditions, the fresh mushroom samples used for extraction had higher beta-glucan content in general and this is clearly reflected in the values obtained. Factors such as nutrient availability in growth substrate, cultivation methods, differing growth conditions and the use of fertilizers can contribute to these variations[150]. The observed discrepancies highlight the need for consistency in sample selection to

ensure accurate comparisons. The methanol extracts, however, did not follow this trend as the beta glucan was higher in the dried reishi samples (2.04g/100g). This introduces another layer of complexity. The heterogeneous nature of reishi mushrooms, with varying textures, may contribute to this variation. It is plausible that the different parts of the mushroom, whether fluffy or hard, have distinct β-glucan concentrations[151]. Lastly, consistent with existing literature, the reported values indicate that Lion's Mane mushrooms possess a higher β-glucan content compared to reishi mushrooms. This observation aligns with prior research findings and supports the established understanding that the two mushroom species exhibit variability in their β-glucan compositions[152].

5.5 DPPH Assay for Antioxidant Capacity

Initially, the antioxidant ability of each type of extract was investigated by conducting the DPPH assay on the final time points for the Soxhlet extracts. The extracts with the highest ability will have a greater average % inhibition. It is expected that the methanol extracts will have the greatest radical scavenging ability as they contained compounds that play active role as antioxidants. Since methanol has more polarity and solubility, in addition to getting higher percent yields of the extracts, extracts of active antioxidants are also obtained (e.g. Polysaccharides)[153].

Note: ANOVA was used to compare the antioxidant capacity of hexane to methanol, ethyl acetate and DCM for both fresh and dried extracts. The presence of a broken line indicates that there is a significance difference between that solvent and the others, with a P value that is <0.05. A solid line shows there is no significant difference.

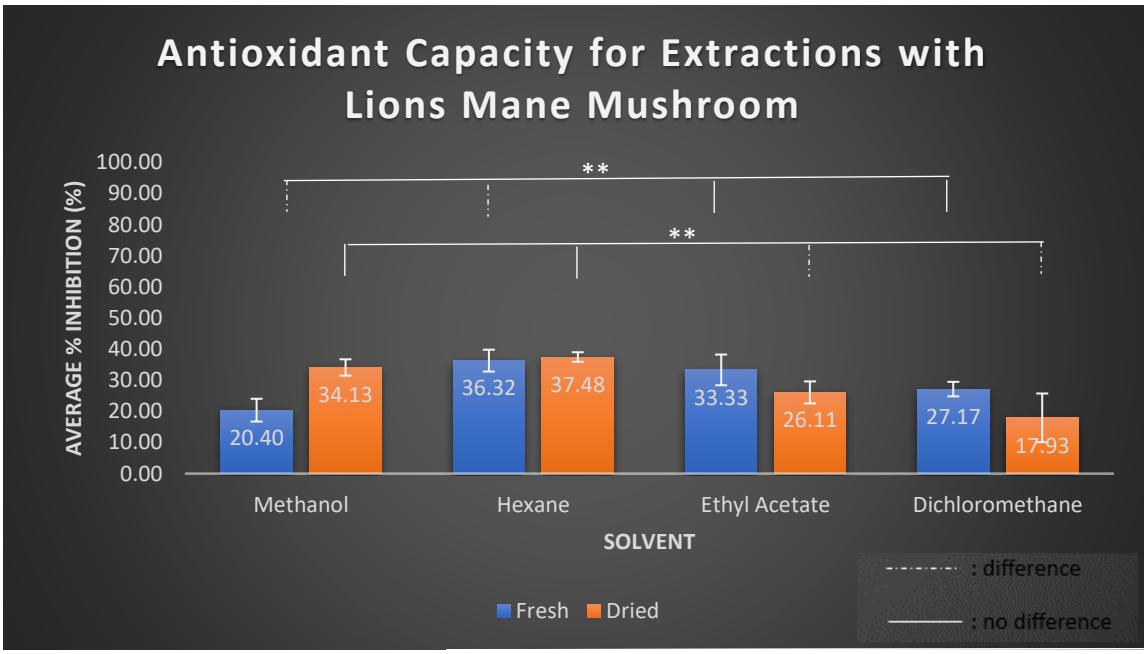

*Figure 29: Antioxidant ability of lion's Mane extracts. The Average % Inhibition of each type of extract (methanol, hexane, ethyl acetate and DCM) were determined. They were found to be 20.40, 36.32, 33.33 and 27.17% for the fresh samples and 34.13, 37.48, 26.11 and 17.93% for the dried samples, respectively. Based on a single factor ANOVA to compare the hexane samples to the other solvents, **=P<0.05.*

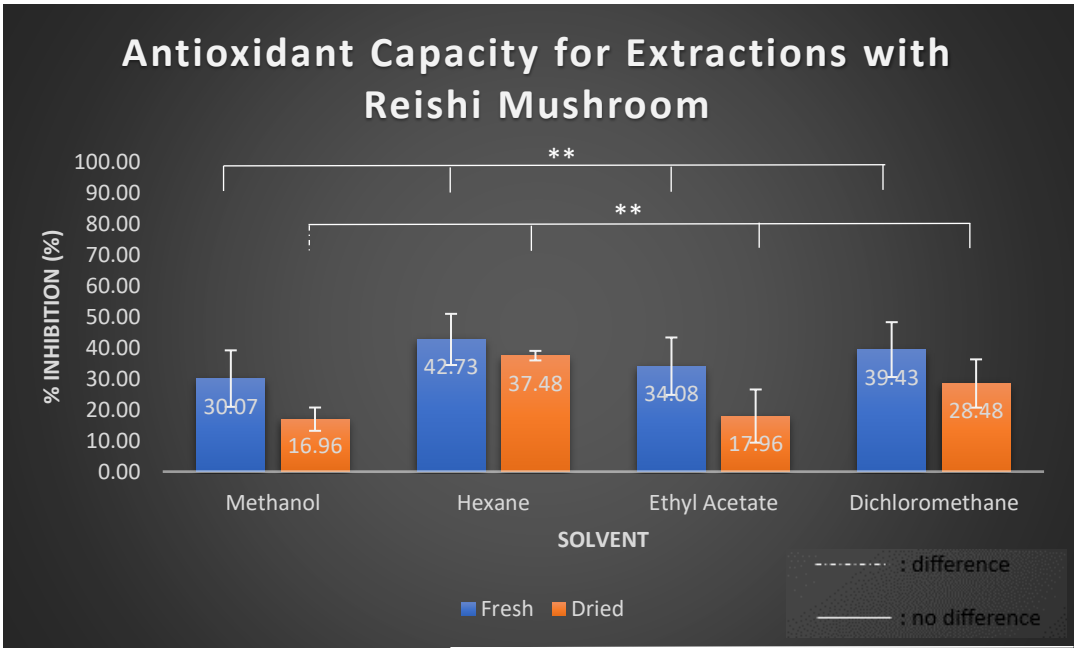

*Figure 30: Antioxidant ability of reishi extracts. The Average % Inhibition of each type of extract (methanol, hexane, ethyl acetate and DCM) were determined. They were found to be 30.07, 42.73, 34.08 and 39.43% for the fresh samples and 16.96, 37.48, 17.96 and 28.48%, respectively. Based on a single factor ANOVA to compare the hexane samples to the other solvents, **=P<0.05*

According to Figures 29 and 30, an unexpected trend was revealed where the hexane samples had the highest % inhibition. This was true for both the lion's mane and reishi samples. In a similar study with where the same type of solvent to extract mushrooms were used, then analysed using a DDPH assay for antioxidant capacity, methanol was able to produce the most potent antioxidant extracts and hexane were the weakest. We think the difference is due to the different extraction techniques that were employed. That study macerated their samples for 24 hours at room at room temperature, while this study utilized Soxhlet extraction. The higher temperatures that are needed for soxhlation could have allowed different compounds (higher molecular weight compounds) to be extracted that also contribute to the mushroom's overall antioxidant capabilities. These compounds might not necessarily be extracted with regular maceration techniques as the extra energy associated with higher temperatures, that may be needed for their release from the cells, is absent. It is suspected that the triterpenes in reishi (ganoderic Acid-A, B, etc.) and the diterpenes in lion's mane (Erinacine A-K, etc.) are present in these hexane extracts and are largely contributing to the antioxidant activity. It is also possible that the fatty acids that are soluble in the hexane extracts contribute to the scavenging properties. These synergistic effects, resulted in higher %inhibition of the hexane samples, surpassing the abilities of methanol extracts[153].

It was also found that fresh samples had a greater antioxidizing effect than the dried samples. It is possible that the more volatile terpenes are responsible for the significant difference in values. These compounds would be lost or at least significantly reduced in the drying process of the mushrooms[154]. Lastly, reishi mushroom was found to have more antioxidant ability than lions mane. This coincides with the literature[155]. The EC_{50} values were then determined for the hexane samples as they had the highest activity.

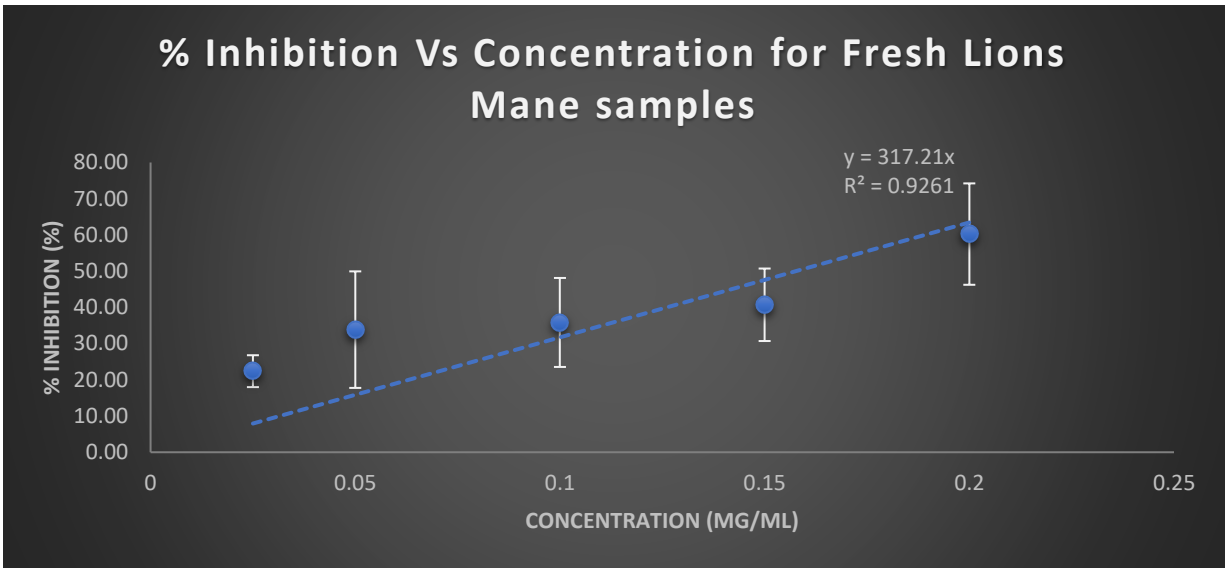

Figure *31: % Inhibition vs Concentration for hexane extractions conducted with fresh lion's mane Samples. The EC_{50} value was found to be 0.17mg/ml.*

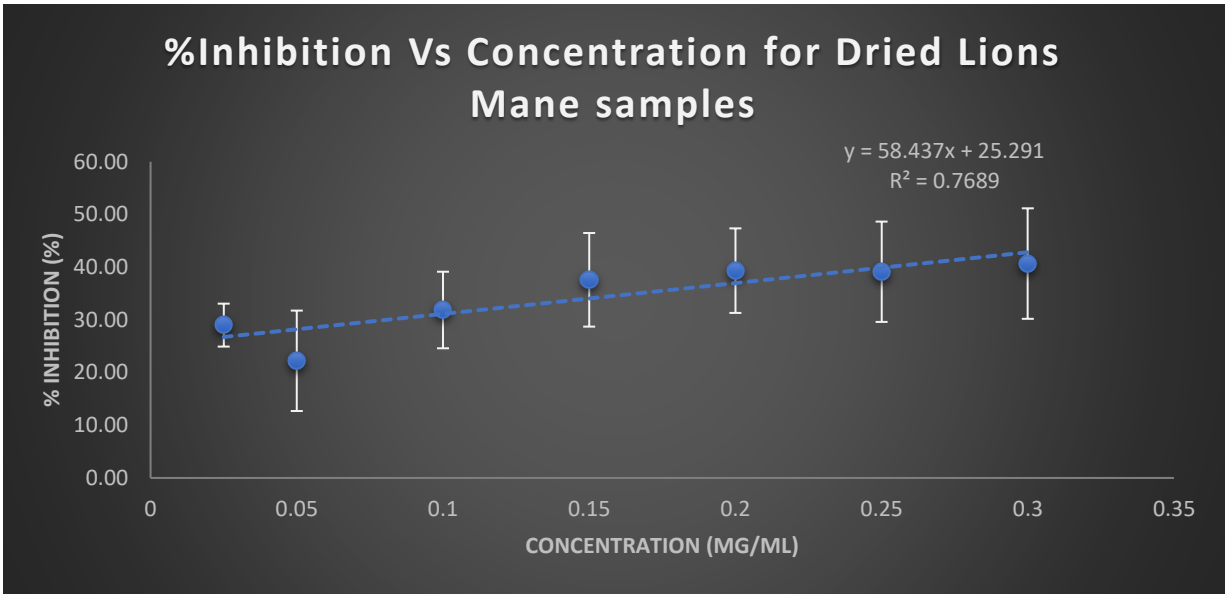

Figure 32: % Inhibition vs Concentration for hexane extractions conducted with dried lion's mane Samples. The EC50 value was found to be 0.42mg/ml.

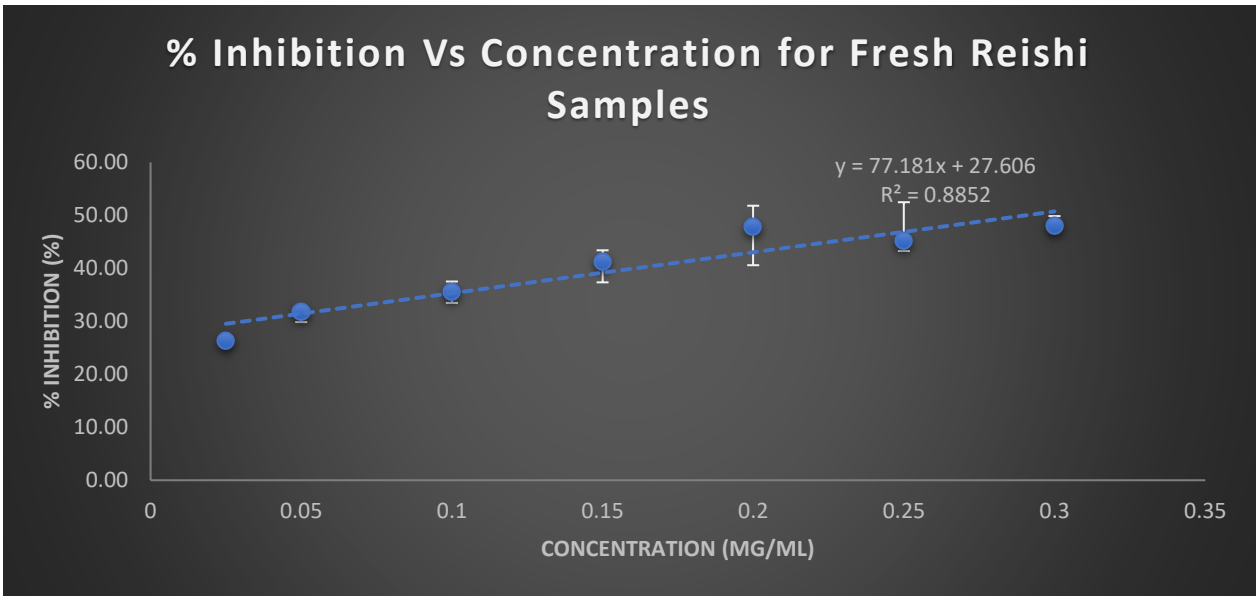

Figure 33: % Inhibition vs Concentration for hexane extractions conducted with fresh reishi Samples. The EC50 value was found to be 0.29mg/ml.

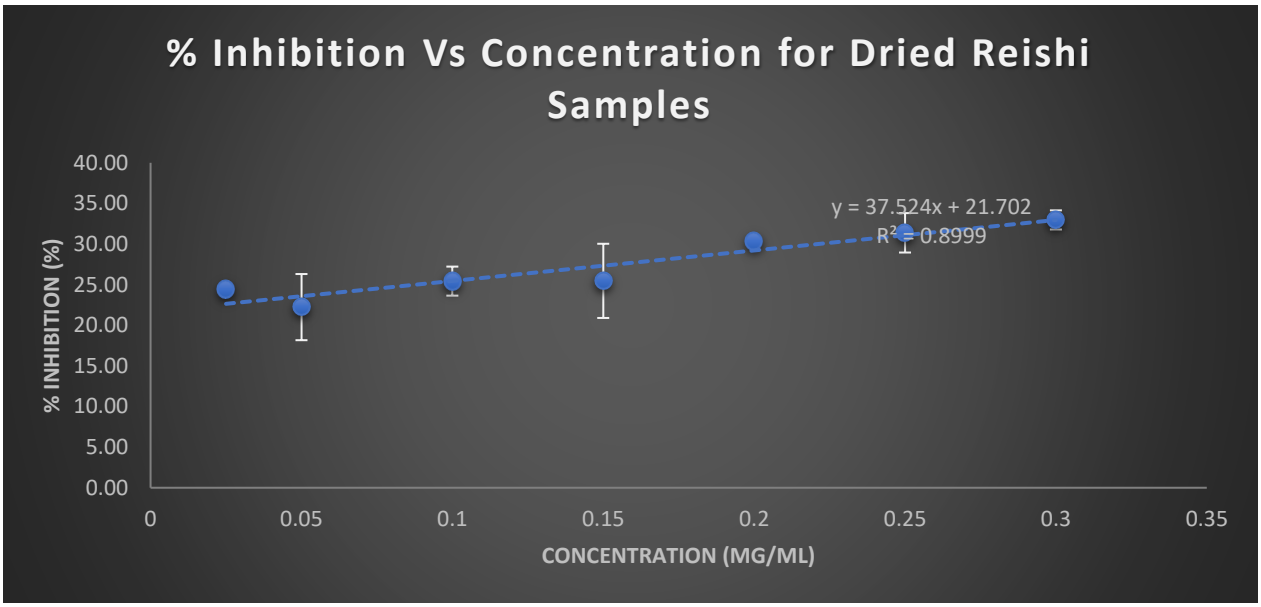

Figure 34: % Inhibition vs Concentration for hexane extractions conducted with dried reishi Samples. The EC50 value was found to be 0.75mg/ml.

The EC_{50} values, as extracted from Figures 31-34, underscore a notable trend in the antioxidant efficacy of hexane extracts from both fresh and dried Lion's Mane and reishi mushrooms. Specifically, a lower concentration of the hexane extract was required for 50% inhibition in Lion's Mane (0.17mg/ml for fresh, 0.42mg/ml for dried) compared to reishi (0.29mg/ml for fresh,

0.75mg/ml for dried). These findings align closely with established literature sources, providing further validation to the observed antioxidant potential in these mushroom extracts.

Furthermore, the consistent pattern emerges that fresh samples exhibit heightened scavenging activity in comparison to their dried counterparts. This recurrent observation reinforces the notion that the antioxidant capabilities of these mushrooms are more pronounced in their fresh state, substantiating the importance of considering sample condition in antioxidant assessments.

5.6 Ergosterol and Vitamin D_2 Quantification

The HPLC-DAD analysis employed for the quantification of ergosterol and vitamin D_2 has yielded insightful observations regarding the dynamic interplay between these two compounds over time. Initially, ergosterol and vitamin D_2 were quantified independently, emphasizing their distinct presence in the samples. As highlighted in the introduction, the transformation of ergosterol into vitamin D_2 is a known process induced by heat and UV radiation[93].

Upon closer examination during subsequent analyses, a compelling trend emerged: a reduction in ergosterol content as time progressed. Apart from the fact that ergosterol is a precursor to vitamin D_2, this dynamic shift indicates the in-situ degradation of ergosterol within the samples over the substantial duration between extraction and analysis. This intriguing transformation is depicted by a decrease in ergosterol from 357.34mg/g to 272.55mg/g for the hexane samples extracted from dried lion's mane.

The observed temporal changes in ergosterol and vitamin D_2 concentrations provide compelling evidence for the ongoing degradation process within the samples. This supports the notion that the degradation of ergosterol into vitamin D2 is a dynamic process influenced by the environmental factors acting on the samples during the time lapse.

As a result, the values for both compounds were added and reported as ergosterol in the sample. These results are presented in a plot of total ergosterol (mg/g) against type of solvent below.

Note: ANOVA was used to compare the total erogosterol content for methanol, hexane, ethyl acetate and DCM for both fresh and dried extracts. The presence of a broken line indicates that there is a significance difference between that solvent and the others, with a P value that is <0.05. A solid line shows there is no significant difference.

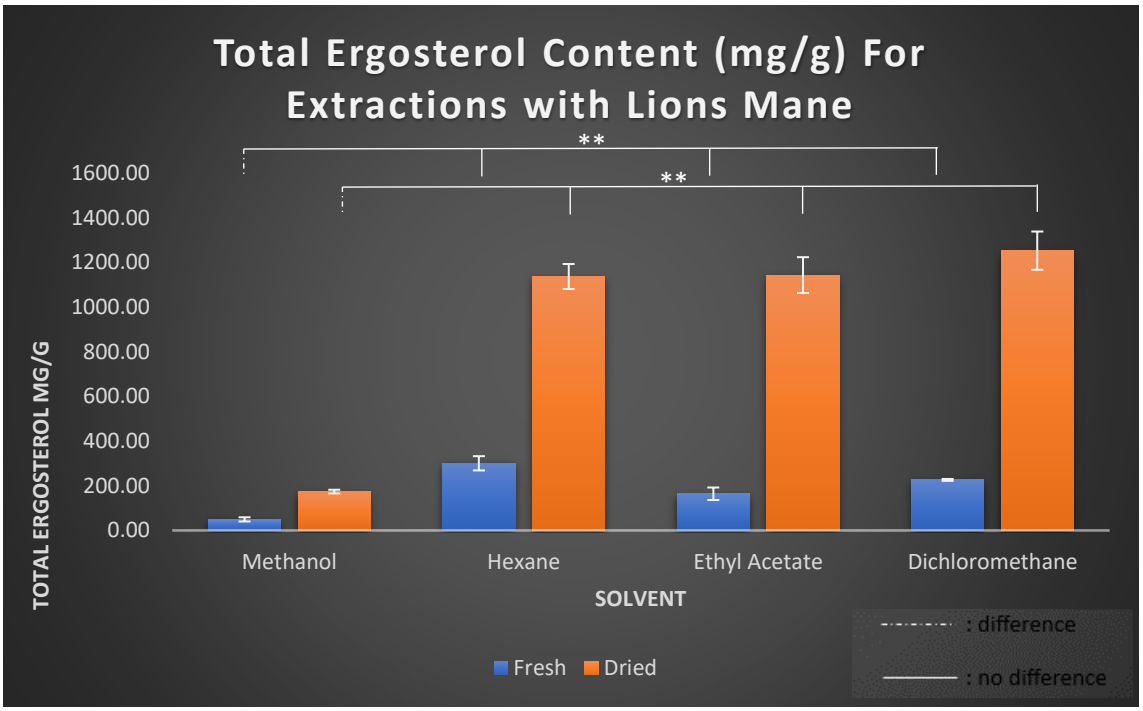

*Figure 35: Total Ergosterol analysis of lion's mane mushroom extracted with methanol, hexane, ethyl acetate and DCM. It was found to be 49.57, 300.70, 164.27 and 226.24mg/g for the fresh 173.92, 1137.19, 1143.42 and 1252.95mg/g for the dried samples, respectively. Based on a single factor ANOVA, **=P<0.05.*

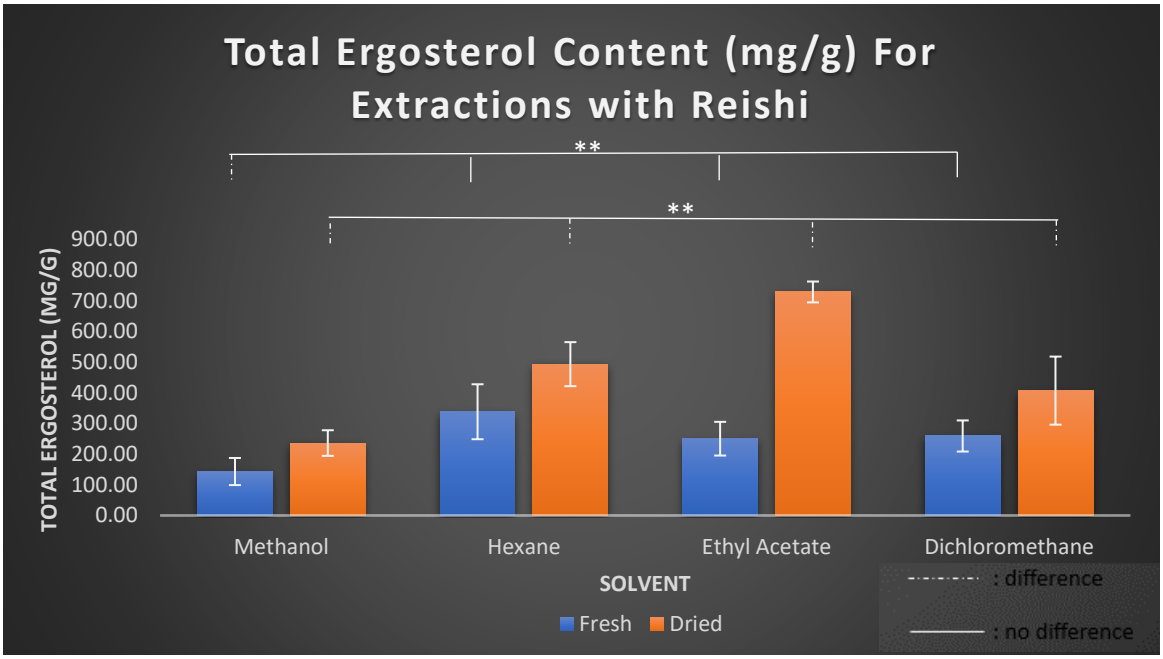

*Figure 36: Total Ergosterol analysis of reishi mushroom extracted with methanol, hexane, ethyl acetate and DCM. It was found to be 143.30, 338.31, 250.39 and 259.25mg/g for the fresh 236.19, 493.49, 728.98 and 407.16mg/g for the dried samples, respectively. Based on a single factor ANOVA, **=P<0.05.*

The data presented in Figures 35 and 36 reveal a notable disparity in ergosterol content among different solvent extracts, with ethyl acetate, DCM, and hexane extracts exhibiting significantly higher concentrations compared to methanol extracts. This observation aligns with existing literature, which establishes ergosterol as a non-polar sterol lipid molecule[91]. Nonpolar solvents, such as ethyl acetate, DCM, and hexane, are demonstrated to be highly efficient in extracting ergosterol. The enhanced solubility of ergosterol in these nonpolar solvents results in their ability to yield extracts with markedly higher ergosterol content.

Furthermore, lions mane samples consistently demonstrate higher ergosterol content compared to reishi samples. This discrepancy may be attributed to inherent variations in the composition of these mushrooms, underscoring the species-specific differences in ergosterol content. The distinct metabolic profiles and biochemical compositions of lion's mane and reishi mushrooms likely contribute to the observed variations in ergosterol extraction efficiency.

5.7 Ganoderic Acid Quantification

HPLC analysis was employed to quantify ganoderic Acid A in samples obtained from the final time points of the Soxhlet extractions. Given that ganoderic Acids are specific to Ganoderma mushrooms, it is anticipated that no ganoderic Acid A would be present in lions mane mushroom samples. The moderately polar nature of ganoderic Acid A suggests that ethyl acetate and dichloromethane (DCM) extracts are likely to yield the highest concentrations, as these solvents are known for their efficacy in extracting this compound[104].

Note: ANOVA was used to compare the ganoderic acid -A content for methanol, hexane, ethyl acetate and DCM for both fresh and dried extracts. The presence of a broken line indicates that there is a significance difference between that solvent and the others, with a P value that is <0.05. A solid line shows there is no significant difference.

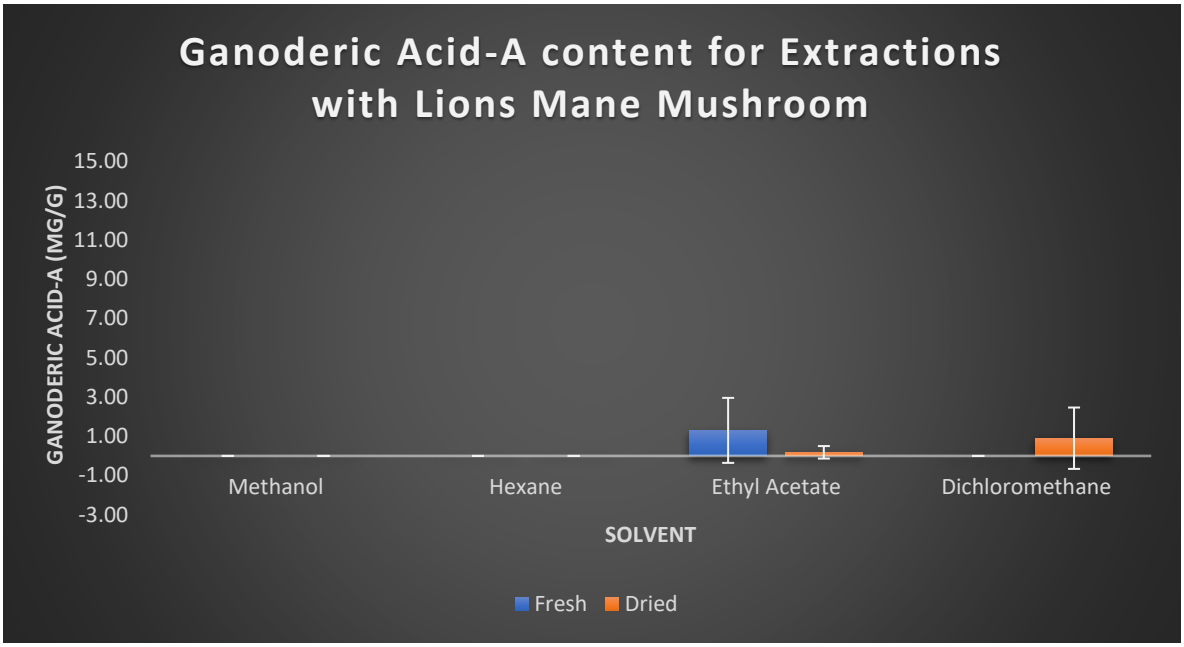

Figure 37: Ganoderic acid-A analysis of Lions mane mushroom extracted with methanol, hexane, ethyl acetate and DCM. The data revealed that there is no ganoderic acid A in lion's mane mushroom.

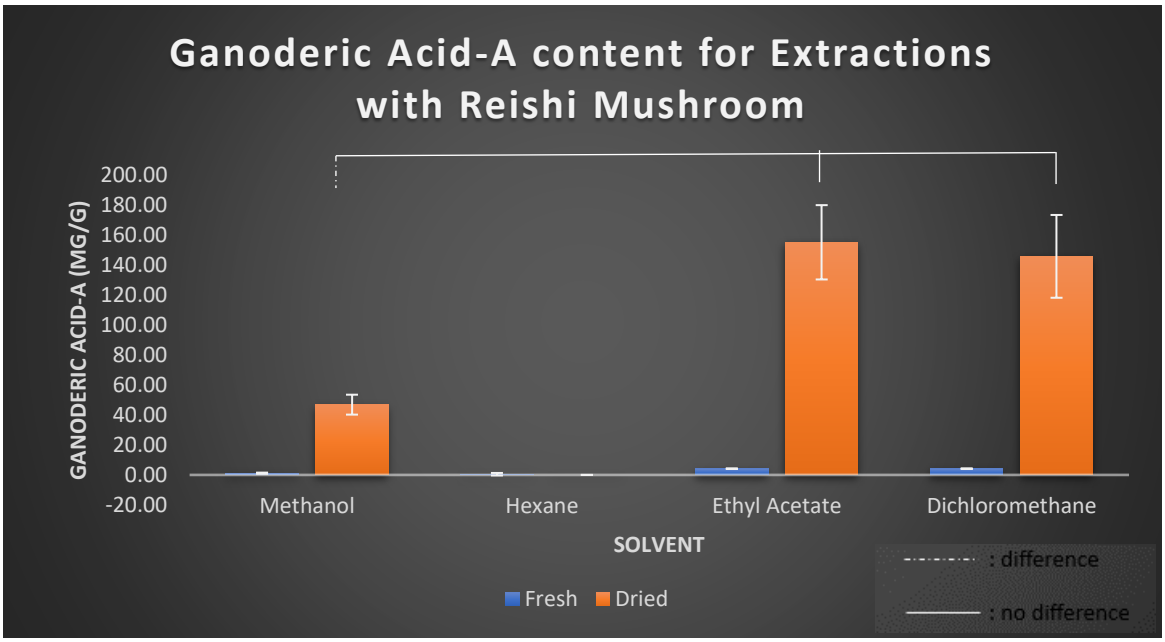

*Figure 38: Ganoderic acid-A analysis of reishi mushroom extracted with methanol, hexane, ethyl acetate and DCM. It was found to be 1.14, 0.48, 4.18 and 4.18 mg/g for fresh and 46.80, 0, 154.94 and 145.58 mg/g for dried, respectively. Based on a single factor ANOVA comparing the solvents extracted with dried samples, **=P<0.05.*

Examining Figures 37 and 38 reveals the absence of ganoderic acid A (GAA) in lions mane samples. However, for reishi, the order of GAA extraction efficiency from highest to lowest is observed as follows: ethyl acetate, followed by DCM, then methanol. Hexane extracts from reishi showed no detectable presence of GAA. Additionally, GAA was not quantified in the fresh reishi samples, while the dried samples exhibited higher GAA content compared to their fresh counterparts.

5.8 Polyphenol Analysis

Polyphenol quantification was conducted through HPLC analysis at the final time points, utilizing a polyphenol standard mix as a reference for compound identification. The anticipation is that the methanol extracts and dried samples will demonstrate the highest polyphenol content.

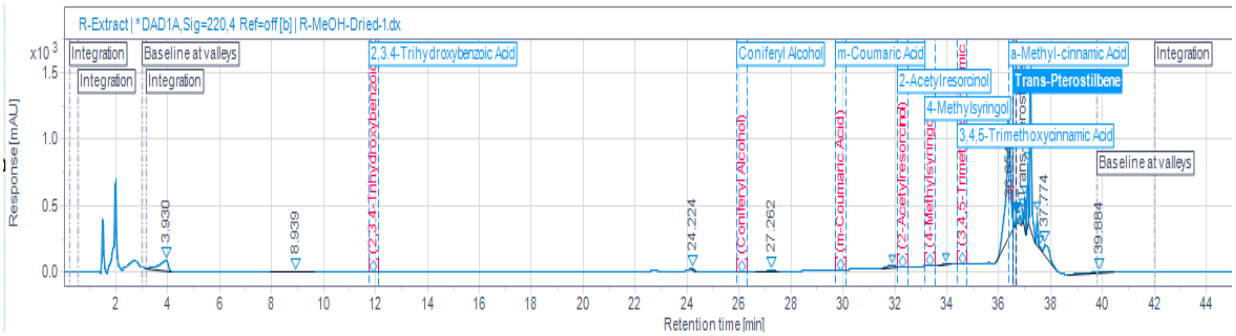

Figure 39: The liquid chromatogram of target polyphenols in mushroom samples. They could not be properly identified.

As illustrated in Figure 39, the unequivocal identification of the target polyphenols within any of the extracts was not evident. Although some suspected polyphenols were detected, their precise characterization remains uncertain. To enhance compound identification, a subsequent analysis will be undertaken using HPLC-MS/MS, allowing for a more definitive and accurate identification of the polyphenolic compounds present.

5.9 Fatty Acid Analysis

The fatty acid composition of the hexane extracts was analyzed using GC-FID, driven by the suspicion that these compounds play a role in the demonstrated antioxidant capabilities observed in the DPPH assay. Our anticipation is that the fresh samples will exhibit a higher quantity of fatty acids, a hypothesis supported by the trends observed in the DPPH assay results. The comparison of the samples to the profile of an oleic standard, run concurrently, will enable the identification and quantification of oleic acid, along with other suspected fatty acids.

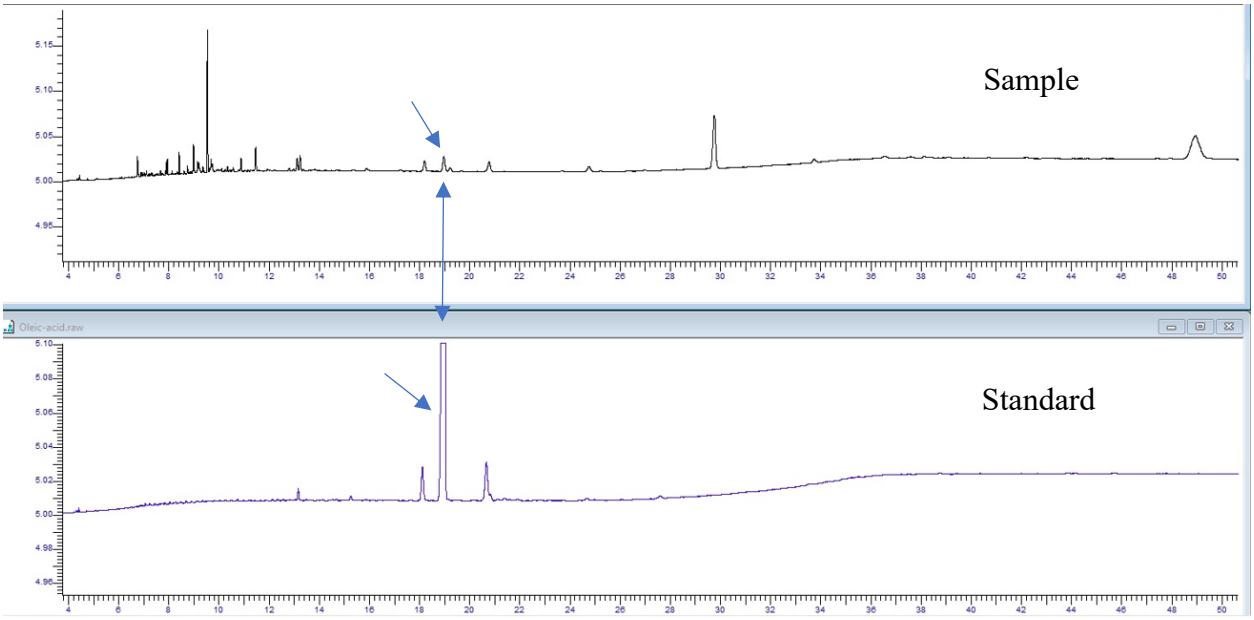

Figure 40: The gas chromatogram profile of Oleic Acid in the fresh reishi hexane samples and the profile of Oleic acid standard.

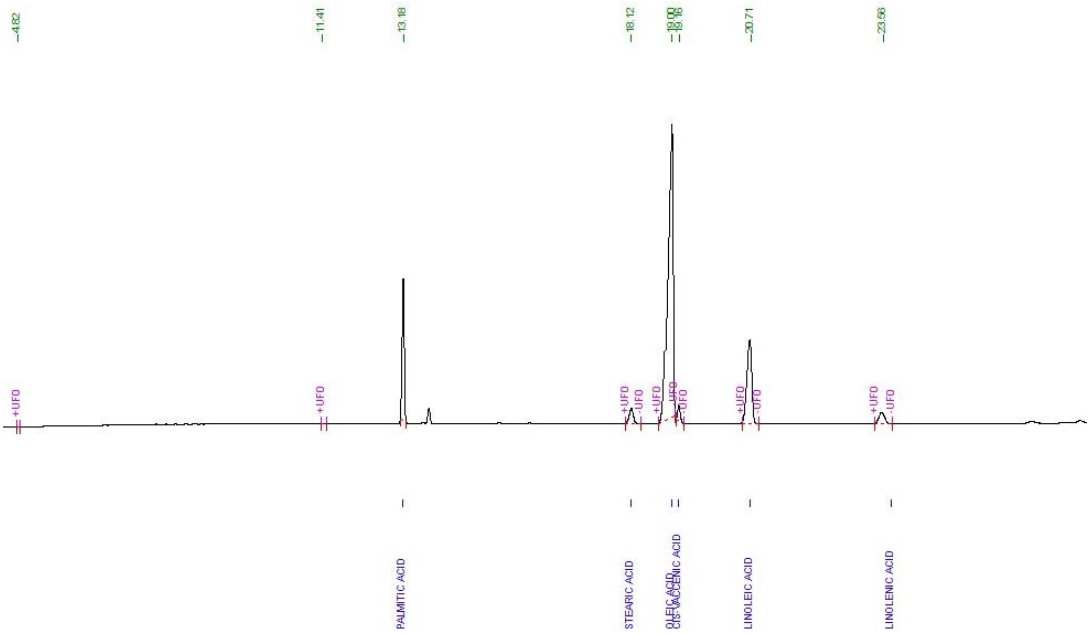

Figure *41: The fatty acid profile of olive oil (positive control).*

The analysis showed the absence of fatty acids in the dried samples, suggesting that these compounds may have been eliminated during the drying process. Additionally, no fatty acids were detected in the lion's mane samples. Figure 41 shows the fatty acid profile of olive oil. It was used as positive control to confirm the efficacy of the FAMES extraction procedure. Peak identification of fatty acid methyl esters in olive oil indicated in Figure 41 were generated from published literature of fatty acid profile for olive oil using the appropriate GC settings and column[156]. A look at the fatty acid profile (Figure 40) of reishi mushroom extracts shows that it is positive for fatty acids. This study shows the presence of not just oleic acid (which was confirmed using a standard), but other suspected unsaturated fatty acids such as linoleic and stearic acid. Further GC-FID studies need to be done to fully quantify the different fatty acids in mushrooms.

CHAPTER 6: Conclusion and Future Perspective

Exploring the myriad bioactive compounds within mushrooms is integral to unraveling their health benefits. In this pursuit, efficient extraction and meticulous analysis are paramount. The optimization of mushroom extraction underscores the significance of extraction time and solvent selection, particularly when targeting specific bioactive compounds. Our findings indicate an optimal extraction time of 8 hours for Soxhlet extraction and 72 hours for macerations, with methanol emerging as the superior solvent for both techniques.

Delving into the nutritional profiles, lion's mane exhibited 9.54% moisture, 7.4% ash, 11.02% fiber, 1.04% fat, 23.94% protein, 57.45% carbohydrate, and 312.72 kcal/100g total energy. On the other hand, reishi showcased 7.28% moisture, 0.76% ash, 4.38% fiber, 3.87% fat, 18.03% protein, 65.67% carbohydrate, and 355.44 kcal/100g total energy.

Observations on soluble protein concentrations revealed an upward trajectory with prolonged extraction time, demanding careful scrutiny due to potential protein degradation. Future investigations should balance extraction time to mitigate degradation risks. The Megazyme assay highlighted methanol's efficacy in extracting β-glucans, particularly in dried samples.

Antioxidant capacity, prominently seen in fresh samples extracted with hexane, showcased reishi's superior overall scavenging ability. However, lion's mane exhibited a lower EC_{50} value, particularly in fresh samples. The synergistic effects of various compounds contribute to these antioxidizing capabilities, prompting further exploration using advanced techniques like GC-MS, LC-MS/MS.

Vitamin D_2 analysis unveiled its conversion to ergosterol over time, most notably in dried hexane, ethyl acetate, and DCM extracts. Ganoderic acid-A was absent in lion's mane, but in reishi, ethyl acetate extracts displayed the highest levels. Comprehensive identification and quantification of target polyphenols were challenging, prompting the commitment to a more extensive study using HPLC-MS/MS in the future.

The final frontier entails a thorough quantification of fatty acids in both mushroom species, promising deeper insights into their antioxidant capacities. This comprehensive exploration

underscores the multifaceted nature of mushroom bioactive compounds and sets the stage for further nuanced investigations.

References

1. Chang, S.; Miles, P., Mushroom biology—a new discipline. *Mycologist* **1992,** *6* (2), 64-65.

2. Valverde, M. E.; Hernández-Pérez, T.; Paredes-López, O., Edible mushrooms: improving human health and promoting quality life. *International journal of microbiology* **2015,** *2015*.

3. Guillamón, E.; García-Lafuente, A.; Lozano, M.; Rostagno, M. A.; Villares, A.; Martínez, J. A., Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia* **2010,** *81* (7), 715-723.

4. Wasser, S. P., Medicinal mushroom science: history, current status, future trends, and unsolved problems. *International journal of medicinal mushrooms* **2010,** *12* (1).

5. Stamets, P.; Zwickey, H., Medicinal mushrooms: Ancient remedies meet modern science. *Integrative Medicine: A Clinician's Journal* **2014,** *13* (1), 46.

6. Calvatia gigantea, Giant Puffball, identification. https://www.first-nature.com/fungi/calvatia-gigantea.php (accessed December 19,).

7. Wikipedia Fomes fomentarius. https://en.wikipedia.org/wiki/Fomes_fomentarius (accessed October).

8. De volta as Raízes. https://www.devoltaasraizes.com.br/kitcultivoreishi/ (accessed December 19,).

9. Wikipedia Polyporus umbellatus. https://en.wikipedia.org/wiki/Polyporus_umbellatus (accessed December 19, 2023).

10. Lowe, H.; Toyang, N.; Steele, B.; Valentine, H.; Grant, J.; Ali, A.; Ngwa, W.; Gordon, L., The therapeutic potential of psilocybin. *Molecules* **2021,** *26* (10), 2948.

11. Chang, S.; Buswell, J., Mushroom nutriceuticals. *World Journal of Microbiology and biotechnology* **1996,** *12*, 473-476.

12. Thongbai, B.; Rapior, S.; Hyde, K. D.; Wittstein, K.; Stadler, M., Hericium erinaceus, an amazing medicinal mushroom. *Mycological Progress* **2015,** *14*, 1-23.

13. Health benefits of Lion's mane. https://www.forbes.com/health/body/health-benefits-of-lions-mane/ (accessed December 19).

14. Sokol, S.; Golak-Siwulska, I.; Sobieralski, K.; Siwulski, M.; Górka, K., Biology, cultivation, and medicinal functions of the mushroom Hericium erinaceum. *Acta Mycologica* **2015,** *50* (2).

15. Jang, J.-H.; Noh, K.-H.; Choi, J.-N.; Jin, K.-S.; Shin, J.-H.; On, J.-H.; Cho, C.-W.; Jeong, W.-S.; Kim, M.-J.; Song, Y.-S., Effect of Hericium erinaceus mycelia supplementation on the oxidative stress and inflammation processes stimulated by LPS and their mechanisms in BALB/C mice. *Journal of the Korean Society of Food Science and Nutrition* **2010,** *39* (2), 227-236.

16. Han, Z.-H.; Ye, J.-M.; Wang, G.-F., Evaluation of in vivo antioxidant activity of Hericium erinaceus polysaccharides. *International journal of biological macromolecules* **2013,** *52*, 66-71.

17. Xu, H.; Wu, P.-r.; Shen, Z.-y.; Chen, X.-d., Chemical analysis of Hericium erinaceum polysaccharides and effect of the polysaccharides on derma antioxidant enzymes, MMP-1 and TIMP-1 activities. *International Journal of Biological Macromolecules* **2010,** *47* (1), 33-36.

18. Mizuno, T., The extraction and development of antitumor-active polysaccharides from medicinal mushrooms in Japan. *International Journal of medicinal mushrooms* **1999,** *1* (1).

19. Mori, K.; Inatomi, S.; Ouchi, K.; Azumi, Y.; Tuchida, T., Improving effects of the mushroom Yamabushitake (Hericium erinaceus) on mild cognitive impairment: A double-blind placebo-controlled clinical trial. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* **2009,** *23* (3), 367-372.

20. Lee, J. S.; Wee, J. W.; Lee, H. Y.; An, H. S.; Hong, E. K., Effects of ascorbic acid and uracil on exo-polysaccharide production with Hericium erinaceus in liquid culture. *Biotechnology and Bioprocess Engineering* **2010,** *15*, 453-459.

21. Liu, C.; Gao, P.; Qian, J.; Yan, W., Immunological study on the antitumor effects of fungus polysaccharides compounds. *Wei Sheng yan jiu= Journal of Hygiene Research* **2000,** *29* (3), 178-180.

22. Wang, J. C.; Hu, S. H.; Wang, J. T.; Chen, K. S.; Chia, Y. C., Hypoglycemic effect of extract of Hericium erinaceus. *Journal of the Science of Food and Agriculture* **2005,** *85* (4), 641-646.

23. Yang, B.-K.; Park, J.-B.; Song, C.-H., Hypolipidemic effect of an exo-biopolymer produced from a submerged mycelial culture of Hericium erinaceus. *Bioscience, biotechnology, and biochemistry* **2003,** *67* (6), 1292-1298.

24. Choi, B.-S.; Sapkota, K.; Choi, J.-H.; Shin, C.-h.; Kim, S.; Kim, S.-J., Herinase: a novel bi-functional fibrinolytic protease from the monkey head mushroom, Hericium erinaceum. *Applied biochemistry and biotechnology* **2013,** *170*, 609-622.

25. Nagai, K.; Chiba, A.; Nishino, T.; Kubota, T.; Kawagishi, H., Dilinoleoyl-phosphatidylethanolamine from Hericium erinaceum protects against ER stress-dependent Neuro2a cell death via protein kinase C pathway. *The Journal of nutritional biochemistry* **2006,** *17* (8), 525-530.

26. Kolotushkina, E.; Moldavan, M.; Voronin, K. Y.; Skibo, G., The influence of Hericium erinaceus extract on myelination process in vitro. *Fiziol Zh* **2003,** *49* (1), 38-45.

27. Okamoto, K.; Sakai, T.; Shimada, A.; Shirai, R.; Sakamoto, H.; Yoshida, S.; Ojima, F.; Ishiguro, Y.; Kawagishi, H., Antimicrobial chlorinated orcinol derivatives from mycelia of Hericium erinaceum. *Phytochemistry* **1993,** *34* (5), 1445-1446.

28. Abdullah, N.; Ismail, S. M.; Aminudin, N.; Shuib, A. S.; Lau, B. F., Evaluation of selected culinary-medicinal mushrooms for antioxidant and ACE inhibitory activities. *Evidence-Based Complementary and Alternative Medicine* **2012,** *2012*.

29. Ferreira, I. C.; Barros, L.; Abreu, R., Antioxidants in wild mushrooms. *Current Medicinal Chemistry* **2009,** *16* (12), 1543-1560.

30. Kim, D.-M.; Pyun, C.-W.; Ko, H.-G.; Park, W.-M., Isolation of antimicrobial substances from Hericium erinaceum. *Mycobiology* **2000,** *28* (1), 33-38.

31. Kim, S. P.; Moon, E.; Nam, S. H.; Friedman, M., Hericium erinaceus mushroom extracts protect infected mice against Salmonella Typhimurium-induced liver damage and mortality by stimulation of innate immune cells. *Journal of agricultural and food chemistry* **2012,** *60* (22), 5590-5596.

32. Shang, X.; Tan, Q.; Liu, R.; Yu, K.; Li, P.; Zhao, G.-P., In vitro anti-Helicobacter pylori effects of medicinal mushroom extracts, with special emphasis on the Lion's Mane mushroom, Hericium erinaceus (higher Basidiomycetes). *International Journal of Medicinal Mushrooms* **2013,** *15* (2).

33. Wasser, S. P., Reishi or ling zhi (Ganoderma lucidum). *Encyclopedia of dietary supplements* **2005,** *1*, 603-622.

34. Rise, N. Reishi mushroom benefits: Plus how to use and side effects. https://www.naturesrise.com/blogs/brainfood/reishi-mushroom-benefits (accessed December 19).

35. Rai, M.; Tidke, G.; Wasser, S. P., Therapeutic potential of mushrooms. **2005**.

36. Battle, J.; Ha, T.; Li, C.; Della Beffa, V.; Rice, P.; Kalbfleisch, J.; Browder, W.; Williams, D., Ligand binding to the (1→ 3)-β-D-glucan receptor stimulates NFκB activation, but not apoptosis in U937 cells. *Biochemical and biophysical research communications* **1998,** *249* (2), 499-504.

37. Smith, J. E.; Rowan, N. J.; Sullivan, R., Medicinal mushrooms: a rapidly developing area of biotechnology for cancer therapy and other bioactivities. *Biotechnology Letters* **2002,** *24*, 1839-1845.

38. Babu, P. D.; Subhasree, R., The sacred mushroom "Reishi"-a review. *American-Eurasian Journal of Botany* **2008,** *1* (3), 107-110.

39. Dried red reishi mushroom. (accessed December 19).

40. Black Reishi. https://www.shroomstop.ca/black-reishi

(accessed December 19).

41. Species Spotlight: Reishi Mushrooms. https://northspore.com/blogs/the-black-trumpet/species-spotlight-reishi (accessed December 19).

42. Emberger, G. Ganoderma lobatum. https://www.messiah.edu/Oakes/fungi_on_wood/poroid%20fungi/species%20pages/Ganoderma%20lobatum%20.html (accessed December 19).

43. Travel photography and digital pictures: 09/1906 - Large purple-brown reishi mushrooms (Ganoderma lucidum) on a fallen tree in Lisiy Nos, west from Saint Petersburg. Russia, September 12, 2016. https://www.asergeev.com/pictures/archives/compress/2016/1906/09.htm (accessed December 19).

44. lingzhi mushroom ganoderma lucidum plant. https://www.123rf.com/photo_39177292_lingzhi-mushroom-ganoderma-lucidum-plant.html (accessed December 19).

45. Adaskaveg, J. E.; Gilbertson, R. L., Cultural studies and genetics of sexuality of Ganoderma lucidum and G. tsugae in relation to the taxonomy of the G. lucidum complex. *Mycologia* **1986,** *78* (5), 694-705.

46. Hapuarachchi, K.; Wen, T.; Deng, C.; Kang, J.; Hyde, K., Mycosphere essays 1: Taxonomic confusion in the Ganoderma lucidum species complex. **2015**.

47. Chang, S. T.; Wasser, S. P., The cultivation and environmental impact of mushrooms. In *Oxford research encyclopedia of environmental science*, 2017.

48. Stamets, P., Growth Parameters for Gourmet and Medicinal Mushroom Species, Growing Gourmet and Medicinal Mushrooms. **1993**.

49. Paterson, R. R. M., Ganoderma–a therapeutic fungal biofactory. *Phytochemistry* **2006,** *67* (18), 1985-2001.

50. Kim, H. W.; Kim, B. K., Biomedicinal triterpenoids of Ganoderma lucidum (Curt.: Fr.) P. Karst.(Aphyllophoromycetideae). *International journal of medicinal mushrooms* **1999,** *1* (2).

51. Kawagishi, H.; Mitsunaga, S.-I.; Yamawaki, M.; Ido, M.; Shimada, A.; Kinoshita, T.; Murata, T.; Usui, T.; Kimura, A.; Chiba, S., A lectin from mycelia of the fungus Ganoderma lucidum. *Phytochemistry* **1997,** *44* (1), 7-10.

52. Zhong, L.; Jiang, D.; Wang, Q., Effects of Ganoderma lucidum (Leyss ex Fr) Karst compound on the proliferation and differentiation of K562 leukemic cells. *Hunan yi ke da xue xue bao= Hunan yike daxue xuebao= Bulletin of Hunan Medical University* **1999,** *24* (6), 521-524.

53. Gao, Y.; Zhou, S.; Huang, M.; Xu, A., Antibacterial and antiviral value of the genus Ganoderma P. Karst. species (Aphyllophoromycetideae): a review. *International Journal of Medicinal Mushrooms* **2003,** *5* (3).

54. He, X.; Wang, X.; Fang, J.; Chang, Y.; Ning, N.; Guo, H.; Huang, L.; Huang, X.; Zhao, Z., Structures, biological activities, and industrial applications of the polysaccharides from Hericium erinaceus (Lion's Mane) mushroom: A review. *International journal of biological macromolecules* **2017,** *97*, 228-237.

55. Huong, L. M.; Thu, H. P.; Thuy, N. T. B.; Ha, T. T. H.; Thi, H. T. M.; Trang, M. T.; Hang, T. T. N.; Nghi, D. H.; Phuc, N. X.; Quang, D. T., Preparation and antitumor-promoting activity of curcumin encapsulated by 1, 3-β-glucan isolated from vietnam medicinal mushroom Hericium erinaceum. *Chemistry Letters* **2011,** *40* (8), 846-848.

56. El Sheikha, A. F., Nutritional profile and health benefits of Ganoderma lucidum "Lingzhi, Reishi, or Mannentake" as functional foods: Current scenario and future perspectives. *Foods* **2022,** *11* (7), 1030.

57. Wachtel-Galor, S., Herbal medicine: biomolecular and clinical aspects. **2011**.

58. Wachtel-Galor, S.; Yuen, J.; Buswell, J. A.; Benzie, I. F., Ganoderma lucidum (Lingzhi or Reishi): a medicinal mushroom. **2012**.

59. Mau, J.-L.; Lin, H.-C.; Ma, J.-T.; Song, S.-F., Non-volatile taste components of several speciality mushrooms. *Food chemistry* **2001,** *73* (4), 461-466.

60. Yew Keong, C.; Amini Abdul Rashid, B.; Swee Ing, Y.; Ismail, Z., Quantification and identification of polysaccharide contents in Hericium erinaceus. *Nutrition & Food Science* **2007,** *37* (4), 260-271.

61. Wu, D.-T.; Li, W.-Z.; Chen, J.; Zhong, Q.-X.; Ju, Y.-J.; Zhao, J.; Bzhelyansky, A.; Li, S.-P., An evaluation system for characterization of polysaccharides from the fruiting body of Hericium erinaceus and identification of its commercial product. *Carbohydrate polymers* **2015,** *124*, 201-207.

62. Miyazawa, M.; Matsuda, N.; Tamura, N.; Ishikawa, R., Characteristic flavor of volatile oil from dried fruiting bodies of Hericium erinaceus (Bull.: Fr.) Pers. *Journal of Essential Oil Research* **2008,** *20* (5), 420-423.

63. Mau, J.-L.; Lin, H.-C.; Chen, C.-C., Non-volatile components of several medicinal mushrooms. *Food Research International* **2001,** *34* (6), 521-526.

64. Gharib, M. A.-A.; Elhassaneen, Y. A. E. E.; Radwan, H., Nutrients and Nutraceuticals Content and In Vitro Biological Activities of Reishi Mushroom (Ganoderma lucidum) Fruiting Bodies. *Alexandria Science Exchange Journal* **2022,** *43* (2), 301-316.

65. Ahmad, R.; Riaz, M.; Khan, A.; Aljamea, A.; Algheryafi, M.; Sewaket, D.; Alqathama, A., Ganoderma lucidum (Reishi) an edible mushroom; a comprehensive and critical review of its nutritional, cosmeceutical, mycochemical, pharmacological, clinical, and toxicological properties. *Phytotherapy Research* **2021,** *35* (11), 6030-6062.

66. Sharif, S.; Mustafa, G.; Munir, H.; Weaver, C. M.; Jamil, Y.; Shahid, M., Proximate composition and micronutrient mineral profile of wild Ganoderma lucidum and four commercial exotic mushrooms by ICP-OES and LIBS. *Journal of Food and nutrition Research* **2016,** *4* (11), 703-708.

67. Chen, Y.; Zhu, S.-B.; Xie, M.-Y.; Nie, S.-P.; Liu, W.; Li, C.; Gong, X.-F.; Wang, Y.-X., Quality control and original discrimination of Ganoderma lucidum based on high-performance liquid chromatographic fingerprints and combined chemometrics methods. *Analytica Chimica Acta* **2008,** *623* (2), 146-156.

68. Kolesnikova, O.; Tuzova, M.; Kozlov, V., Screening of immunoactive properties of alkancarbonic acids derivatives and germanium-organic compounds in vivo. *Иммунология* **1997,** *18* (2), 36-38.

69. Jiménez-Colmenero, F.; Pintado, T.; Cofrades, S.; Ruiz-Capillas, C.; Bastida, S., Production variations of nutritional composition of commercial meat products. *Food Research International* **2010,** *43* (10), 2378-2384.

70. Parimelazhagan, T.; Thangaraj, P., Proximate composition analysis. *Pharmacological assays of plant-based natural products* **2016**, 21-31.

71. Lim, P. Y., Determination of chemical properties of meat: Protein determination by Kjeldahl method. In *Laboratory Manual on Analytical Methods and Procedures for Fish and Fish Products*, Marine Fisheries Research Department, Southeast Asian Fisheries Development …: 1992; pp B-1.1-B-1.3.

72. Srikram, A.; Supapvanich, S., Proximate compositions and bioactive compounds of edible wild and cultivated mushrooms from Northeast Thailand. *Agriculture and Natural Resources* **2016,** *50* (6), 432-436.

73. Iqbal, B.; Khan, H.; Saifullah, I. K.; Shah, B.; Naeem, A.; Ullah, W.; Ahmed, N., Substrates evaluation for the quality, production and growth of oyster mushroom (Pleurotus florida Cetto). *Journal of Entomology and Zoology Studies* **2016,** *4* (3), 98-107.

74. Fogarasi, M.; Socaci, S. A.; Dulf, F. V.; Diaconeasa, Z. M.; Fărcaș, A. C.; Tofană, M.; Semeniuc, C. A., Bioactive compounds and volatile profiles of five Transylvanian wild edible mushrooms. *Molecules* **2018,** *23* (12), 3272.

75. González, A.; Cruz, M.; Losoya, C.; Nobre, C.; Loredo, A.; Rodríguez, R.; Contreras, J.; Belmares, R., Edible mushrooms as a novel protein source for functional foods. *Food & function* **2020,** *11* (9), 7400-7414.

76. Xu, X.; Yan, H.; Chen, J.; Zhang, X., Bioactive proteins from mushrooms. *Biotechnology advances* **2011,** *29* (6), 667-674.

77. Liu, Q.; Wang, H.; Ng, T., First report of a xylose-specific lectin with potent hemagglutinating, antiproliferative and anti-mitogenic activities from a wild ascomycete mushroom. *Biochimica et Biophysica Acta (BBA)-General Subjects* **2006,** *1760* (12), 1914-1919.
78. Pohleven, J.; Obermajer, N.; Sabotič, J.; Anžlovar, S.; Sepčić, K.; Kos, J.; Kralj, B.; Štrukelj, B.; Brzin, J., Purification, characterization and cloning of a ricin B-like lectin from mushroom Clitocybe nebularis with antiproliferative activity against human leukemic T cells. *Biochimica et Biophysica Acta (BBA)-General Subjects* **2009,** *1790* (3), 173-181.
79. Kino, K.; Yamashita, A.; Yamaoka, K.; Watanabe, J.; Tanaka, S.; Ko, K.; Shimizu, K.; Tsunoo, H., Isolation and characterization of a new immunomodulatory protein, ling zhi-8 (LZ-8), from Ganoderma lucidium. *Journal of Biological Chemistry* **1989,** *264* (1), 472-478.
80. Lin, Y.-L.; Liang, Y.-C.; Tseng, Y.-S.; Huang, H.-Y.; Chou, S.-Y.; Hseu, R.-S.; Huang, C.-T.; Chiang, B.-L., An immunomodulatory protein, Ling Zhi-8, induced activation and maturation of human monocyte-derived dendritic cells by the NF-κB and MAPK pathways. *Journal of leukocyte biology* **2009,** *86* (4), 877-889.
81. Zhu, F.; Du, B.; Bian, Z.; Xu, B., Beta-glucans from edible and medicinal mushrooms: Characteristics, physicochemical and biological activities. *Journal of Food Composition and Analysis* **2015,** *41*, 165-173.
82. Ahmad, A.; Anjum, F. M.; Zahoor, T.; Nawaz, H.; Dilshad, S. M. R., Beta glucan: a valuable functional ingredient in foods. *Critical reviews in food science and nutrition* **2012,** *52* (3), 201-212.
83. Dong, Q.; Wang, Y.; Shi, L.; Yao, J.; Li, J.; Ma, F.; Ding, K., A novel water-soluble β-D-glucan isolated from the spores of Ganoderma lucidum. *Carbohydrate Research* **2012,** *353*, 100-105.
84. MORI, H.; AIZAWA, K.; INAKUMA, T.; ICHII, A.; YAMAUCHII, R.; KATO, K., Structural analysis of the β-D-glucan from the fruit-body of Hericium erinaceum. *Journal of Applied Glycoscience* **1998,** *45* (4), 361-365.
85. Tao, Y.; Zhang, L.; Cheung, P. C., Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. *Carbohydrate Research* **2006,** *341* (13), 2261-2269.
86. Brown, G. D.; Gordon, S., Immune recognition of fungal β-glucans. *Cellular microbiology* **2005,** *7* (4), 471-479.

87. Chen, J.; Seviour, R., Medicinal importance of fungal β-(1→ 3),(1→ 6)-glucans. *Mycological research* **2007,** *111* (6), 635-652.

88. Chanput, W.; Reitsma, M.; Kleinjans, L.; Mes, J. J.; Savelkoul, H. F.; Wichers, H. J., β-Glucans are involved in immune-modulation of THP-1 macrophages. *Molecular nutrition & food research* **2012,** *56* (5), 822-833.

89. Rop, O.; Mlcek, J.; Jurikova, T., Beta-glucans in higher fungi and their health effects. *Nutrition reviews* **2009,** *67* (11), 624-631.

90. Shao, S.; Hernandez, M.; Kramer, J. K.; Rinker, D. L.; Tsao, R., Ergosterol profiles, fatty acid composition, and antioxidant activities of button mushrooms as affected by tissue part and developmental stage. *Journal of agricultural and food chemistry* **2010,** *58* (22), 11616-11625.

91. Rangsinth, P.; Sharika, R.; Pattarachotanant, N.; Duangjan, C.; Wongwan, C.; Sillapachaiyaporn, C.; Nilkhet, S.; Wongsirojkul, N.; Prasansuklab, A.; Tencomnao, T., Potential beneficial effects and pharmacological properties of ergosterol, a common bioactive compound in edible mushrooms. *Foods* **2023,** *12* (13), 2529.

92. Chen, S.; Yong, T.; Zhang, Y.; Su, J.; Jiao, C.; Xie, Y., Anti-tumor and anti-angiogenic ergosterols from Ganoderma lucidum. *Frontiers in chemistry* **2017,** *5*, 85.

93. Jasinghe, V. J.; Perera, C. O., Distribution of ergosterol in different tissues of mushrooms and its effect on the conversion of ergosterol to vitamin D2 by UV irradiation. *Food chemistry* **2005,** *92* (3), 541-546.

94. Bikle, D. D., Vitamin D metabolism, mechanism of action, and clinical applications. *Chemistry & biology* **2014,** *21* (3), 319-329.

95. Taofiq, O.; Fernandes, A.; Barros, L.; Barreiro, M. F.; Ferreira, I. C., UV-irradiated mushrooms as a source of vitamin D2: A review. *Trends in Food Science & Technology* **2017,** *70*, 82-94.

96. Rudrapal, M.; Khairnar, S. J.; Khan, J.; Dukhyil, A. B.; Ansari, M. A.; Alomary, M. N.; Alshabrmi, F. M.; Palai, S.; Deb, P. K.; Devi, R., Dietary polyphenols and their role in oxidative stress-induced human diseases: Insights into protective effects, antioxidant potentials and mechanism (s) of action. *Frontiers in pharmacology* **2022,** *13*, 283.

97. Abbas, M.; Saeed, F.; Anjum, F. M.; Afzaal, M.; Tufail, T.; Bashir, M. S.; Ishtiaq, A.; Hussain, S.; Suleria, H. A. R., Natural polyphenols: An overview. *International Journal of Food Properties* **2017,** *20* (8), 1689-1699.

98. Kolniak-Ostek, J.; Oszmiański, J.; Szyjka, A.; Moreira, H.; Barg, E., Anticancer and antioxidant activities in Ganoderma lucidum wild mushrooms in Poland, as well as their phenolic and triterpenoid compounds. *International Journal of Molecular Sciences* **2022,** *23* (16), 9359.

99. Ceriello, A., Possible role of oxidative stress in the pathogenesis of hypertension. *Diabetes care* **2008,** *31*, S181.

100. Khan, J.; Deb, P. K.; Priya, S.; Medina, K. D.; Devi, R.; Walode, S. G.; Rudrapal, M., Dietary flavonoids: Cardioprotective potential with antioxidant effects and their pharmacokinetic, toxicological and therapeutic concerns. *Molecules* **2021,** *26* (13), 4021.

101. García-Lafuente, A.; Guillamón, E.; Villares, A.; Rostagno, M. A.; Martínez, J. A., Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflammation research* **2009,** *58* (9), 537-552.

102. Athar, M.; Back, J. H.; Tang, X.; Kim, K. H.; Kopelovich, L.; Bickers, D. R.; Kim, A. L., Resveratrol: a review of preclinical studies for human cancer prevention. *Toxicology and applied pharmacology* **2007,** *224* (3), 274-283.

103. Chen, W.-P.; Chi, T.-C.; Chuang, L.-M.; Su, M.-J., Resveratrol enhances insulin secretion by blocking KATP and KV channels of beta cells. *European journal of pharmacology* **2007,** *568* (1-3), 269-277.

104. Liang, C.; Tian, D.; Liu, Y.; Li, H.; Zhu, J.; Li, M.; Xin, M.; Xia, J., Review of the molecular mechanisms of Ganoderma lucidum triterpenoids: Ganoderic acids A, C2, D, F, DM, X and Y. *European Journal of Medicinal Chemistry* **2019,** *174*, 130-141.

105. Yao, X.; Li, G.; Xu, H.; Lü, C., Inhibition of the JAK-STAT3 signaling pathway by ganoderic acid A enhances chemosensitivity of HepG2 cells to cisplatin. *Planta medica* **2012**, 1740-1748.

106. Gill, B. S.; Kumar, S.; Navgeet, Evaluating anti-oxidant potential of ganoderic acid A in STAT 3 pathway in prostate cancer. *Molecular biology reports* **2016,** *43*, 1411-1422.

107. Rathore, H.; Prasad, S.; Sharma, S., Mushroom nutraceuticals for improved nutrition and better human health: A review. *PharmaNutrition* **2017,** *5* (2), 35-46.

108. Hou, C. T.; Lin, J.-T.; Dulay, R. M. R.; Ray, K., Identification of molecular species of acylglycerols of Philippine wild edible mushroom, Ganoderma lucidum. *Biocatalysis and Agricultural Biotechnology* **2017,** *9*, 19-27.
109. Sande, D.; de Oliveira, G. P.; e Moura, M. A. F.; de Almeida Martins, B.; Lima, M. T. N. S.; Takahashi, J. A., Edible mushrooms as a ubiquitous source of essential fatty acids. *Food Research International* **2019,** *125*, 108524.
110. Heleno, S. A.; Barros, L.; Martins, A.; Queiroz, M. J. R.; Santos-Buelga, C.; Ferreira, I. C., Phenolic, polysaccharidic, and lipidic fractions of mushrooms from Northeastern Portugal: chemical compounds with antioxidant properties. *Journal of Agricultural and Food Chemistry* **2012,** *60* (18), 4634-4640.
111. Smith, R. M., Before the injection—modern methods of sample preparation for separation techniques. *Journal of chromatography A* **2003,** *1000* (1-2), 3-27.
112. Hennion, M.-C.; Cau-Dit-Coumes, C.; Pichon, V., Trace analysis of polar organic pollutants in aqueous samples: Tools for the rapid prediction and optimisation of the solid-phase extraction parameters. *Journal of Chromatography A* **1998,** *823* (1-2), 147-161.
113. Poole, S. K.; Dean, T. A.; Oudsema, J. W.; Poole, C. F., Sample preparation for chromatographic separations: an overview. *Analytica Chimica Acta* **1990,** *236*, 3-42.
114. Hernández, Y.; Lobo, M. G.; González, M., Factors affecting sample extraction in the liquid chromatographic determination of organic acids in papaya and pineapple. *Food Chemistry* **2009,** *114* (2), 734-741.
115. Kurt, B. G. T., *Natural product chemistry: a mechanistic, biosynthetic and ecological approach*. Swedish Pharmaceutical Press: 1997.
116. Anklam, E.; Berg, H.; Mathiasson, L.; Sharman, M.; Ulberth, F., Supercritical fluid extraction (SFE) in food analysis: a review. *Food Additives & Contaminants* **1998,** *15* (6), 729-750.
117. Azmir, J.; Zaidul, I. S. M.; Rahman, M. M.; Sharif, K.; Mohamed, A.; Sahena, F.; Jahurul, M.; Ghafoor, K.; Norulaini, N.; Omar, A., Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of food engineering* **2013,** *117* (4), 426-436.
118. Tambun, R.; Alexander, V.; Ginting, Y. In *Performance comparison of maceration method, soxhletation method, and microwave-assisted extraction in extracting active compounds*

from soursop leaves (Annona muricata): A review, IOP Conference Series: Materials Science and Engineering, IOP Publishing: 2021; p 012095.

119. Lefebvre, T.; Destandau, E.; Lesellier, E., Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. *Journal of Chromatography A* **2021,** *1635*, 461770.

120. Naviglio, D.; Scarano, P.; Ciaravolo, M.; Gallo, M., Rapid Solid-Liquid Dynamic Extraction (RSLDE): A powerful and greener alternative to the latest solid-liquid extraction techniques. *Foods* **2019,** *8* (7), 245.

121. Patel, K.; Panchal, N.; Ingle, P., Techniques Adopted for Extraction of Natural Products.

122. Organization, U. N. I. D.; Handa, S. S.; Khanuja, S. P. S.; Longo, G.; Rakesh, D. D., *Extraction technologies for medicinal and aromatic plants*. Earth, Environmental and Marine Sciences and Technologies: 2008.

123. Bhardwaj, S. K.; Dwivedia, K.; Agarwala, D., A review: HPLC method development and validation. *International Journal of Analytical and Bioanalytical Chemistry* **2015,** *5* (4), 76-81.

124. Swartz, M., HPLC detectors: a brief review. *Journal of Liquid Chromatography & Related Technologies* **2010,** *33* (9-12), 1130-1150.

125. Al-Bukhaiti, W. Q.; Noman, A.; Qasim, A. S.; Al-Farga, A., Gas chromatography: Principles, advantages and applications in food analysis. *International Journal of Agriculture Innovations and Research* **2017,** *6* (1), 2319-1473.

126. Hartmann, C. H., Gas chromatography detectors. *Analytical Chemistry* **1971,** *43* (2), 113A-125a.

127. Jones, C. G.; Daniel Hare, J.; Compton, S. J., Measuring plant protein with the Bradford assay: 1. Evaluation and standard method. *Journal of chemical ecology* **1989,** *15*, 979-992.

128. Kruger, N. J., The Bradford method for protein quantitation. *The protein protocols handbook* **2009**, 17-24.

129. SyNytSyA, A.; Mickova, K.; Jablonsky, I.; SlUKoVá, M.; Copikova, J., Mushrooms of genus Pleurotus as a source of dietary fibres and glucans for food supplements. *Czech J. Food Sci* **2008,** *26* (6), 441-446.

130. De Antoni, A.; D'ANGELO, M.; DAL PERO, F.; SARTORELLO, F.; PANDOLFO, D.; PALLAVICINI, A.; Lanfranchi, G.; Valle, G., The DNA sequence of cosmid 14–13b from

chromosome XIV of Saccharomyces cerevisiae reveals an unusually high number of overlapping open reading frames. *Yeast* **1997,** *13* (3), 261-266.

131. McCleary, B. V.; Draga, A., Measurement of β-glucan in mushrooms and mycelial products. *Journal of AOAC International* **2016,** *99* (2), 364-373.

132. Sirivibulkovit, K.; Nouanthavong, S.; Sameenoi, Y., based DPPH assay for antioxidant activity analysis. *Analytical sciences* **2018,** *34* (7), 795-800.

133. Mishra, K.; Ojha, H.; Chaudhury, N. K., Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food chemistry* **2012,** *130* (4), 1036-1043.

134. Blois, M. S., Antioxidant determinations by the use of a stable free radical. *Nature* **1958,** *181* (4617), 1199-1200.

135. Bondet, V.; Brand-Williams, W.; Berset, C., Kinetics and mechanisms of antioxidant activity using the DPPH. free radical method. *LWT-Food Science and Technology* **1997,** *30* (6), 609-615.

136. Zengin, G.; Sarikurkcu, C.; Aktumsek, A.; Ceylan, R.; Anwar, F.; Solak, M. H., A Comparative fatty acid compositional analysis of different wild species of mushrooms from Turkey. *Emirates Journal of Food and Agriculture* **2015**, 532-536.

137. Mousavi, R. S.; Nateghi, L.; Soltani, M.; Asgarpanah, J., Optimization of the phenolic and antioxidants extraction from Ganoderma Lucidum using maceration method. *Journal of Food Research* **2022,** *32* (3), 1-16.

138. Abugri, D. A.; McElhenney, W. H., Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal mushrooms as affected by different solvents. *J Nat Prod Plant Resour* **2013,** *3* (3), 37-42.

139. Zheng, S.; Zhang, W.; Liu, S., Optimization of ultrasonic-assisted extraction of polysaccharides and triterpenoids from the medicinal mushroom Ganoderma lucidum and evaluation of their in vitro antioxidant capacities. *PLoS One* **2020,** *15* (12), e0244749.

140. Leal, P. F.; Almeida, T. S.; Prado, G. H.; Prado, J. M.; Meireles, M. A. A., Extraction kinetics and anethole content of fennel (Foeniculum vulgare) and anise seed (Pimpinella anisum) extracts obtained by soxhlet, ultrasound, percolation, centrifugation, and steam distillation. *Separation Science and Technology* **2011,** *46* (11), 1848-1856.

141. Agustini, T. W.; Suzery, M.; Sutrisnanto, D.; Ma'ruf, W. F., Comparative study of bioactive substances extracted from fresh and dried Spirulina sp. *Procedia Environmental Sciences* **2015,** *23*, 282-289.
142. Salamat, S.; Shahid, M.; Najeeb, J., Proximate analysis and simultaneous mineral profiling of five selected wild commercial mushroom as a potential nutraceutical. *Int J Chem Stud* **2017,** *5* (3), 297-303.
143. Shahid, M.; Fatima, H.; Anjum, F.; Riaz, M., Proximate composition, antioxidant activities and fatty acid profiling of selected mushrooms collected from Azad Jammu and Kashmir. *Acta Poloniae Pharmaceutica-Drug Research* **2020,** *77* (1), 145-153.
144. Sunday, E. A.; Israel, A. U.; Magu, T. O., Proximate analysis and mineral element composition of false yam (Icacina trichantha) tuber and oyster mushroom (Pleurotus ostreatus). *International Journal of Chemical Sciences* **2016,** *1* (1).
145. Crisan, E.; Sands, A., Nutritional value//The biology and cultivation of edible mushrooms. *Acad. Ress. Inc. Ltd., London* **1978**, 37-168.
146. Ismail, B. P., Ash content determination. In *Food analysis laboratory manual*, Springer: 2017; pp 117-119.
147. Bacha, S. A. S.; Shujaat, A.; Li, Y.; Rehman, H.; Farooq, S.; Mushtaq, A.; Wahocho, S., Lion's mane mushroom; new addition to food and natural bounty for human wellness: A review. *International Journal of Biosciences* **2018,** *13* (4), 396-402.
148. Denavi, G.; Tapia-Blacido, D. R.; Añon, M. C.; Sobral, P. J. d. A.; Mauri, A. N.; Menegalli, F., Effects of drying conditions on some physical properties of soy protein films. *Journal of Food Engineering* **2009,** *90* (3), 341-349.
149. Braaksma, A.; Schaap, D., Protein analysis of the common mushroom Agaricus bisporus. *Postharvest Biology and Technology* **1996,** *7* (1-2), 119-127.
150. Kiss, A.; Grünvald, P.; Ladányi, M.; Papp, V.; Papp, I.; Némedi, E.; Mirmazloum, I., Heat treatment of Reishi medicinal mushroom (Ganoderma lingzhi) basidiocarp enhanced its β-glucan solubility, antioxidant capacity and lactogenic properties. *Foods* **2021,** *10* (9), 2015.
151. Mushroom, R.; Mushroom, C.; Mushroom, H.; Mushroom, O.; Polypore, U.; Ear, W., Ganoderma lucidum. *Herbal Medicine: Biomolecular and Clinical Aspects* **2011**, 175.

152. Łysakowska, P.; Sobota, A.; Wirkijowska, A., Medicinal Mushrooms: Their Bioactive Components, Nutritional Value and Application in Functional Food Production—A Review. *Molecules* **2023,** *28* (14), 5393.
153. Hidayat, F.; Fatmawati, S. In *Antioxidant Evaluation of Ganoderma lucidum Extracts*, IOP Conference Series: Materials Science and Engineering, IOP Publishing: 2019; p 012042.
154. Tibuhwa, D. D., A comparative study of antioxidant activities between fresh and dry mushrooms in the genera Cantharellus and Afrocantharellus from Tanzania. *Food and nutrition sciences* **2014,** *2014*.
155. Szydłowska-Tutaj, M.; Szymanowska, U.; Tutaj, K.; Domagała, D.; Złotek, U., The Addition of Reishi and Lion's Mane Mushroom Powder to Pasta Influences the Content of Bioactive Compounds and the Antioxidant, Potential Anti-Inflammatory, and Anticancer Properties of Pasta. *Antioxidants* **2023,** *12* (3), 738.
156. Siano, F.; Vasca, E., GC-FID Analysis to Evaluate the Possible Adulteration of Extra Virgin Olive Oil with Different Vegetable Oils. *Journal of Chemical Education* **2020,** *97* (11), 4108-4116.

Appendices

Extraction Supporting Data

I. Mass Recovery of Macerations

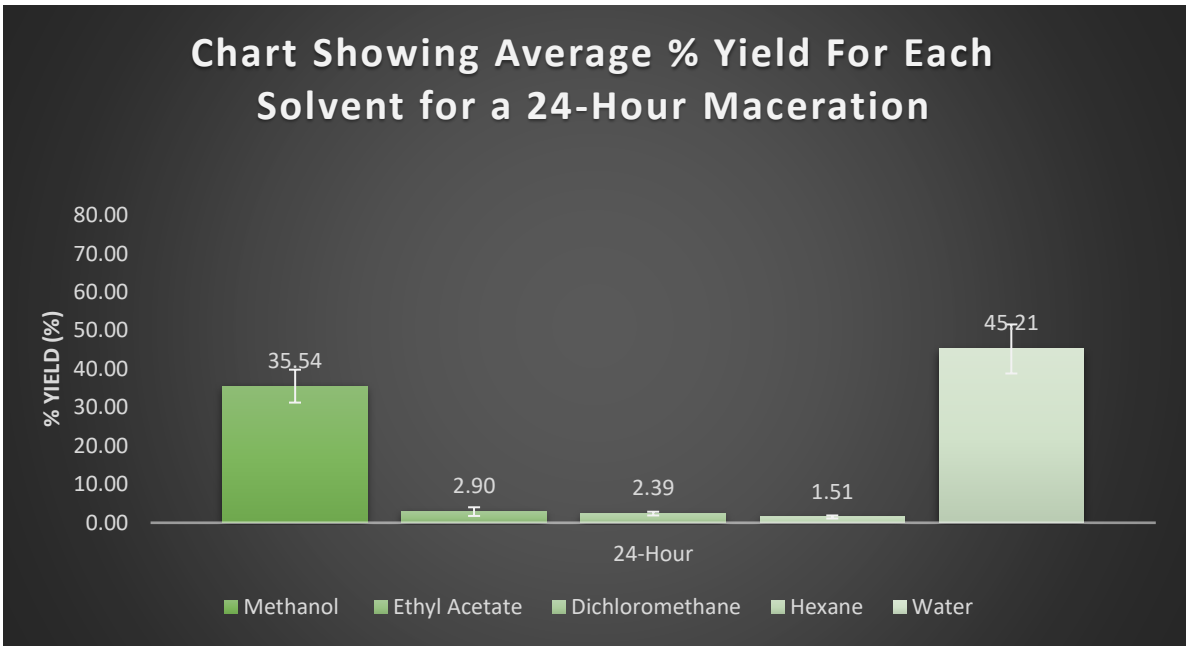

Figure 42: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 24 hours. The data is obtained from the maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

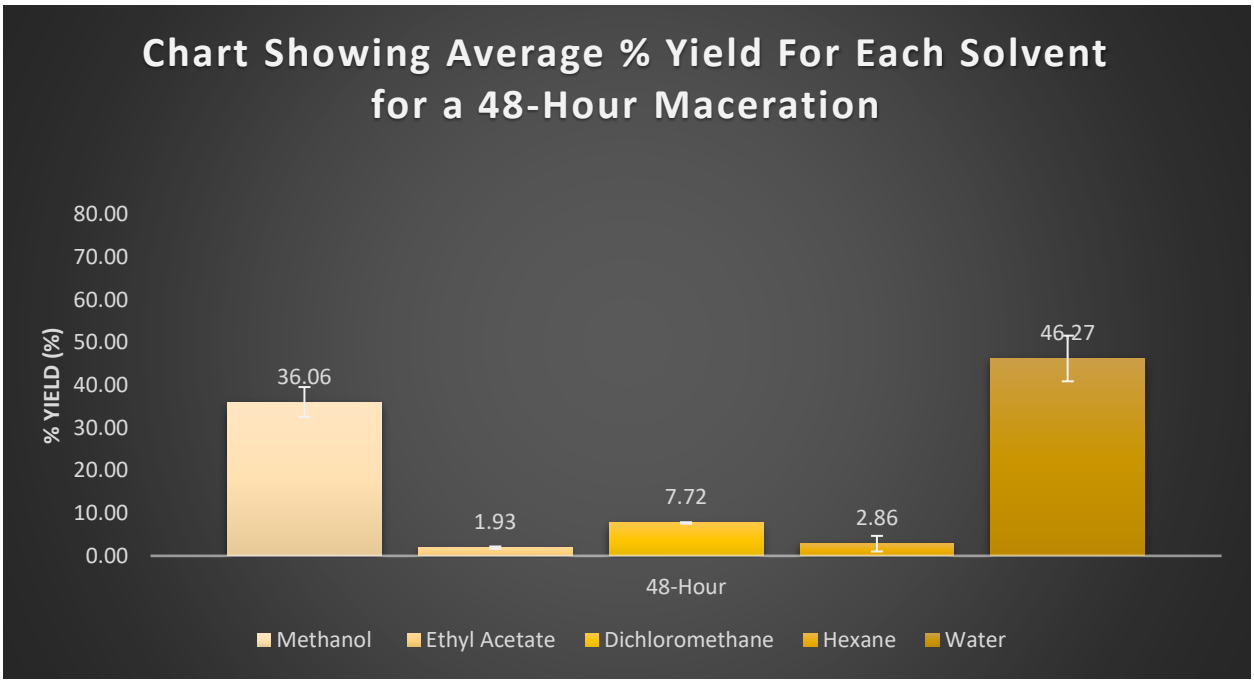

Figure 43: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 48 hours. The data obtained from the maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

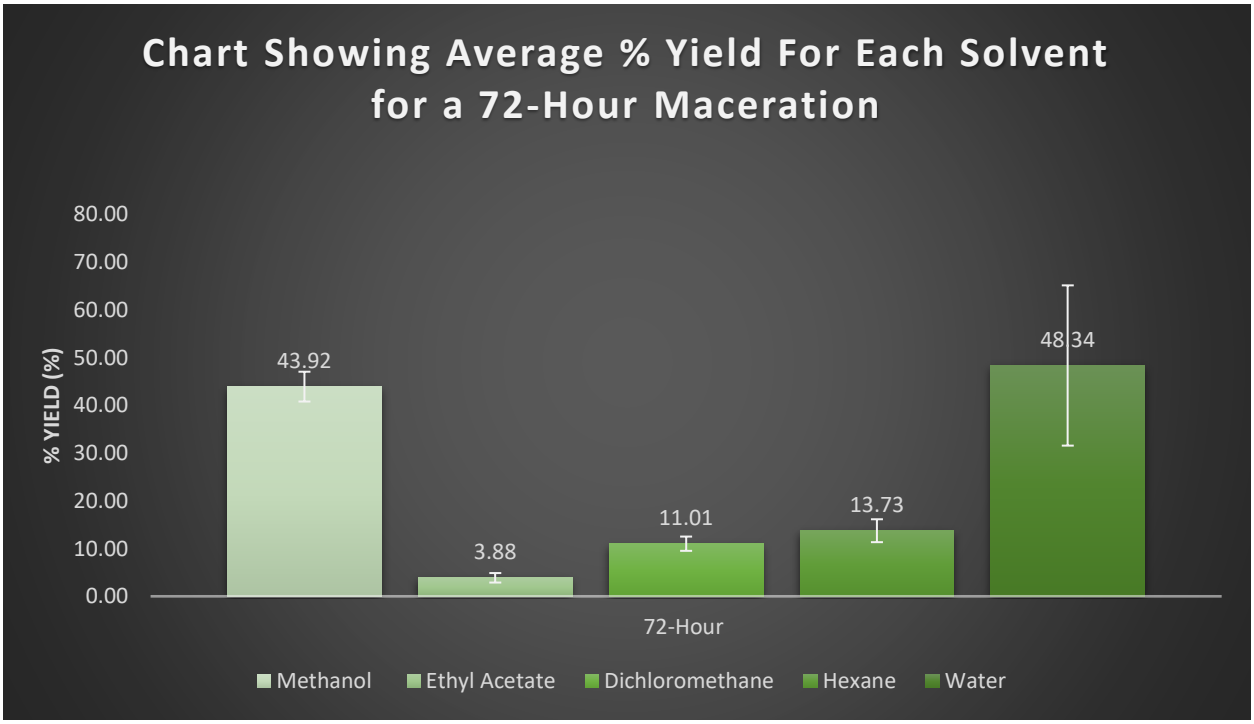

Figure 44: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 72hours. The data obtained from the maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

II. Mass Recovery of Ultrasound – Assisted Macerations

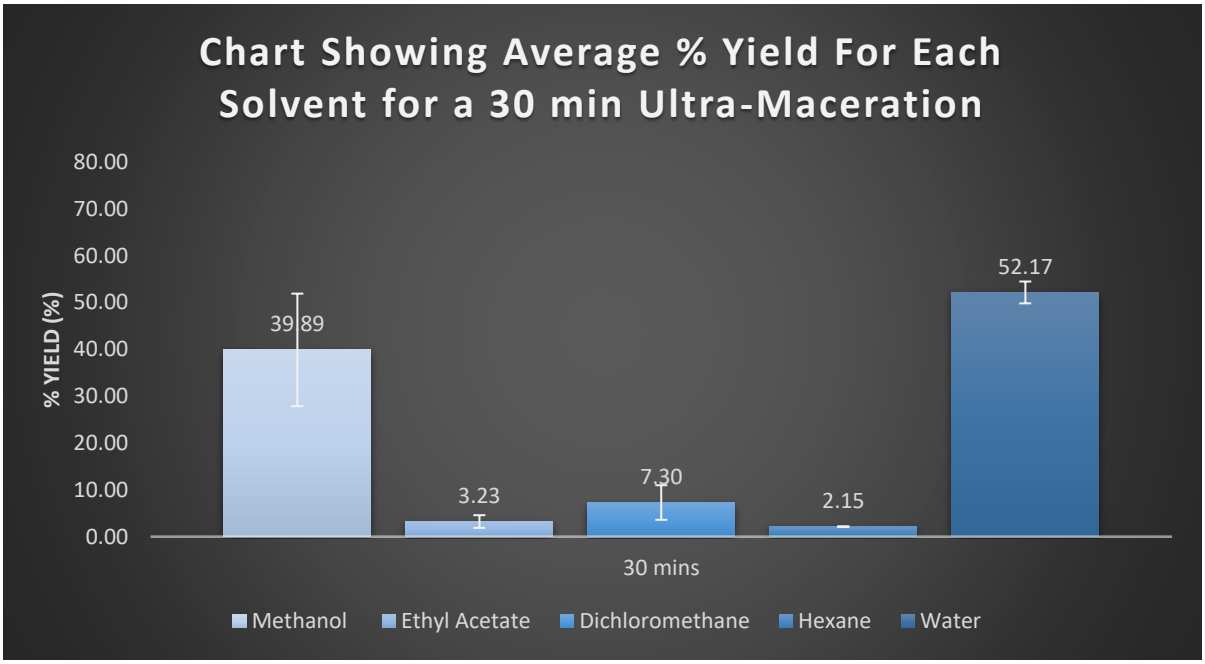

Figure 45: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 30 mins. The data obtained from the ultra-maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

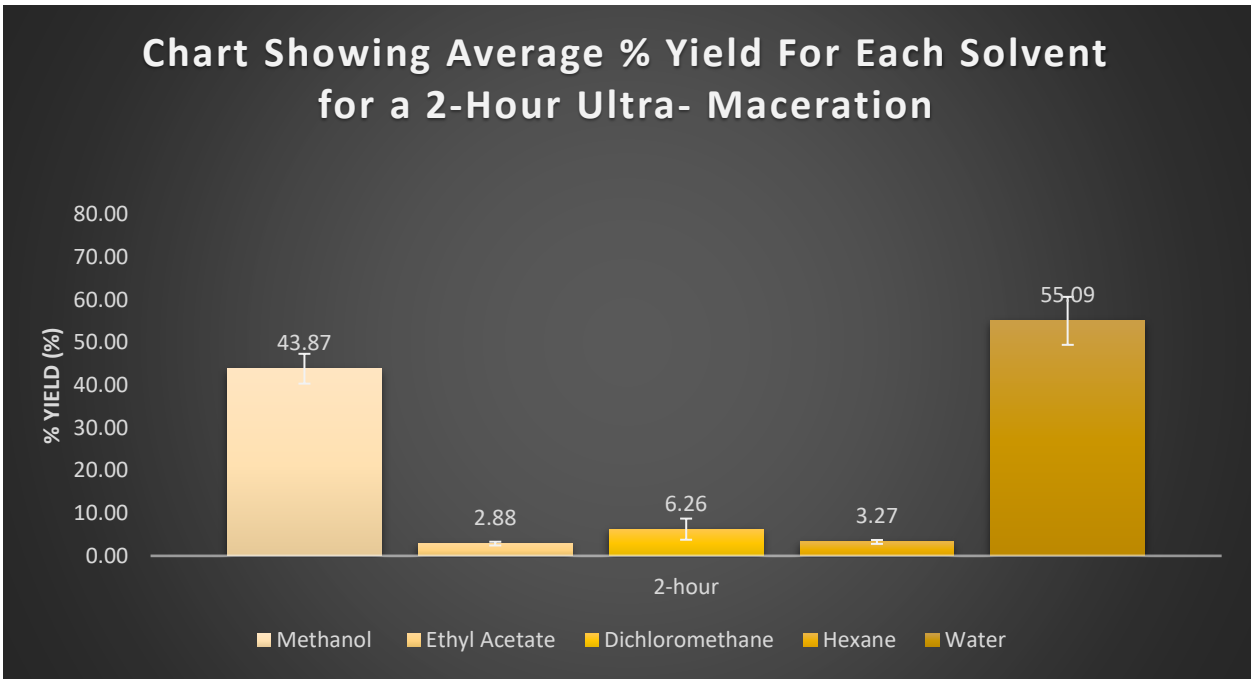

Figure 46: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 2 hours. The data obtained from the ultra-maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

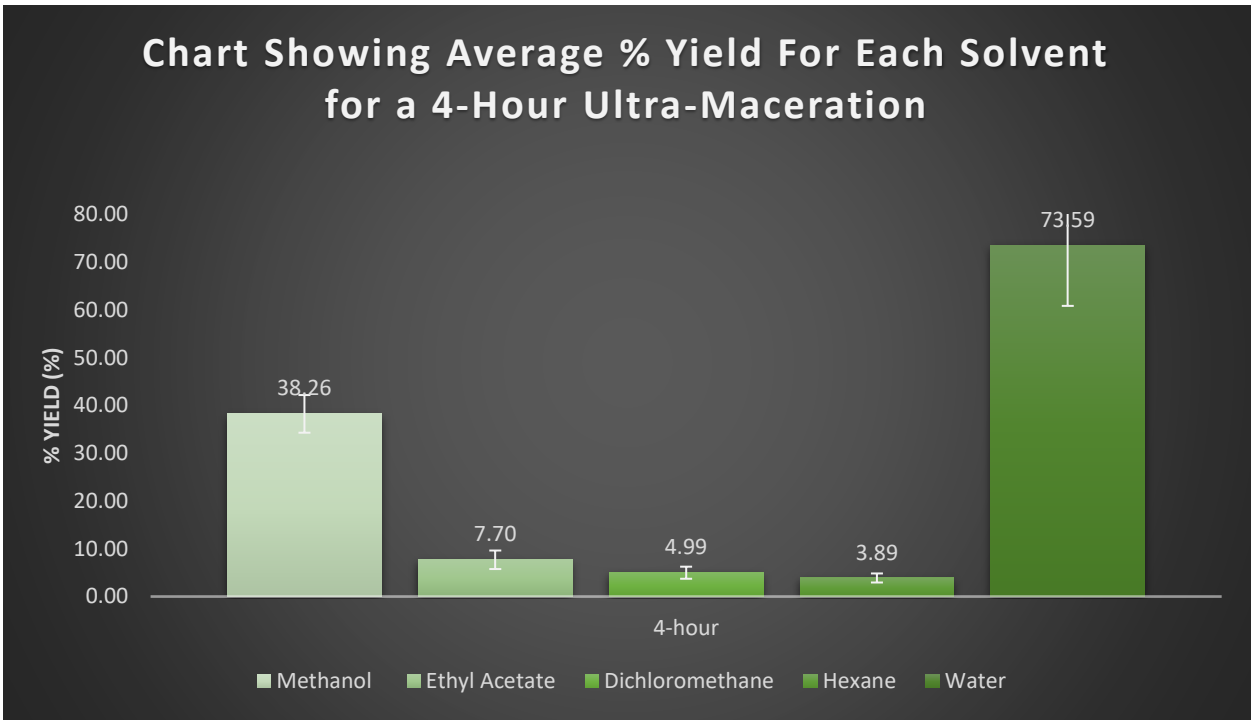

Figure 47: Average yield (%) produced for methanol, ethyl acetate, dichloromethane, hexane and water for 4 hours. The data obtained from the ultra-maceration of 1 g of Hericium erinaceus (lion's mane) mushroom with 200ml of solvent.

III. Mass Recovery of Soxhlet Extracts

Fresh lion's mane

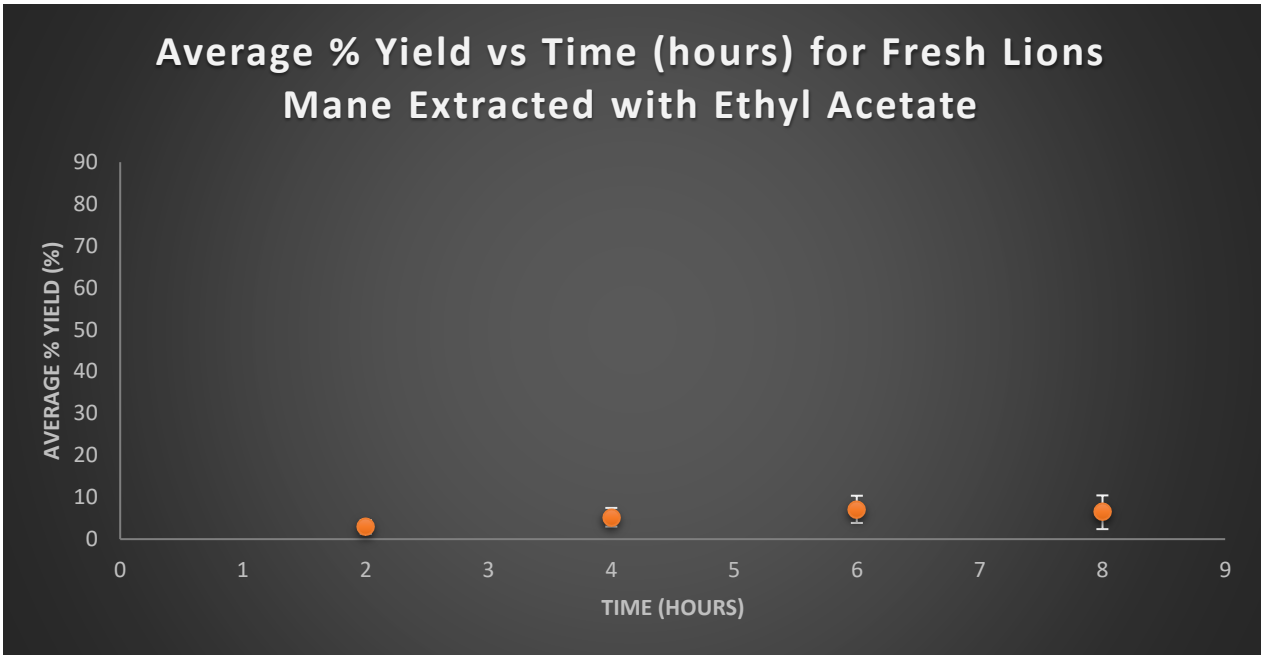

Figure 48: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh Hericium erinaceus (lion's mane) mushroom with ethyl acetate as the solvent for 8 hours. Produced a total percent yield of 5.8%.

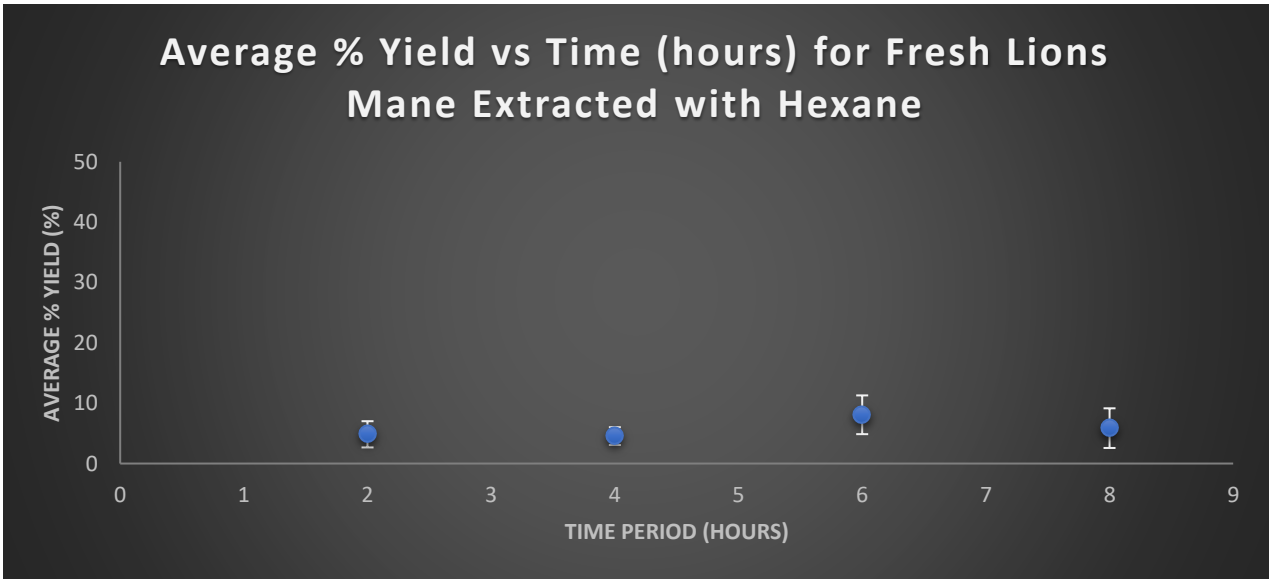

Figure 49: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh Hericium erinaceus (lion's mane) mushroom with Hexane as the solvent for 8 hours. Produced a total percent yield of 4.7%

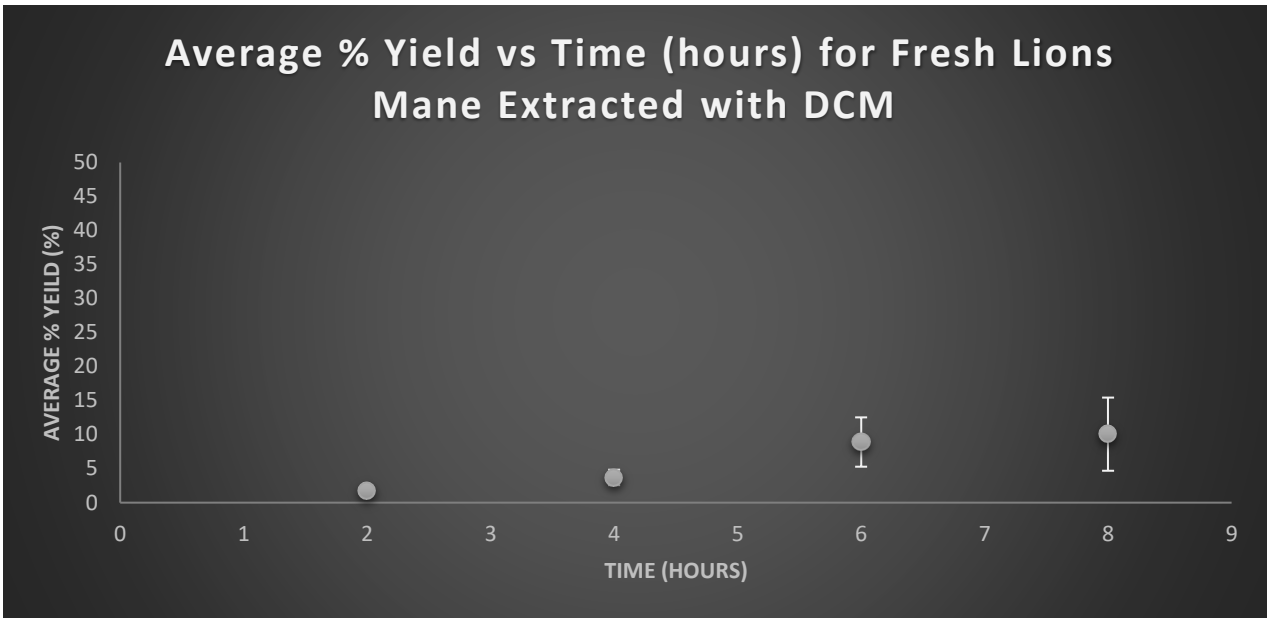

Figure 50: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh Hericium erinaceus (lion's mane) mushroom with DCM as the solvent for 8 hours. Produced a total percent yield of 3.06%

Dried lion's mane

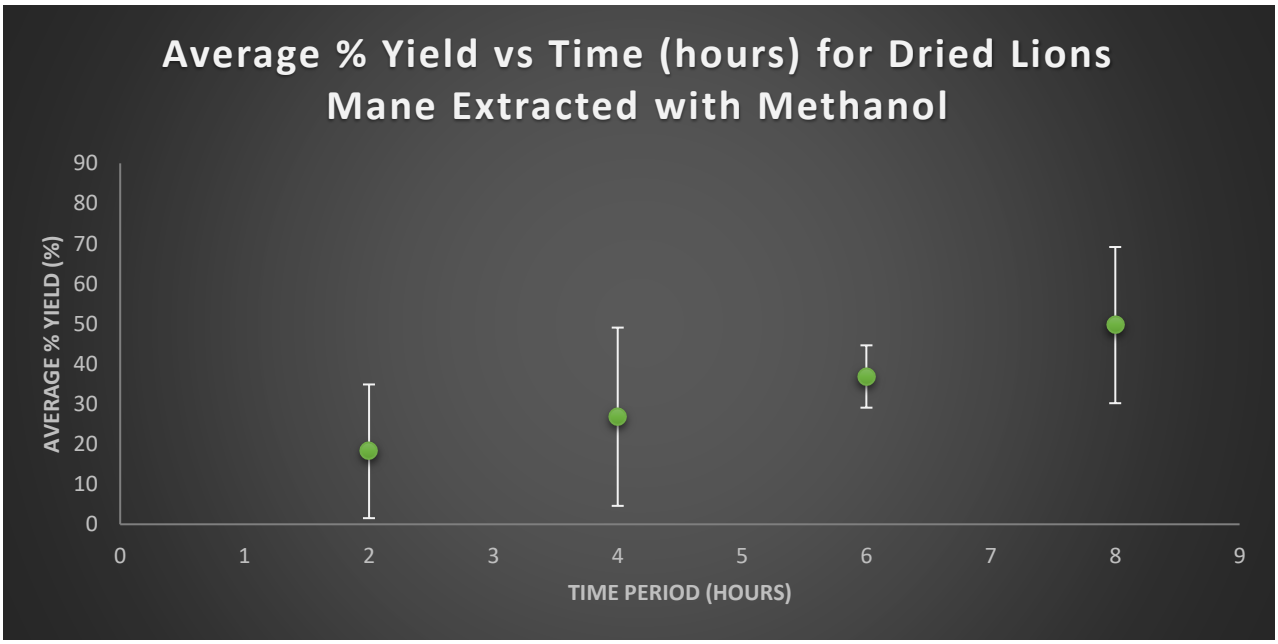

Figure 51: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried Hericium erinaceus (lion's mane) mushroom with methanol as the solvent for 8 hours. Produced a total percent yield of 31.3%.

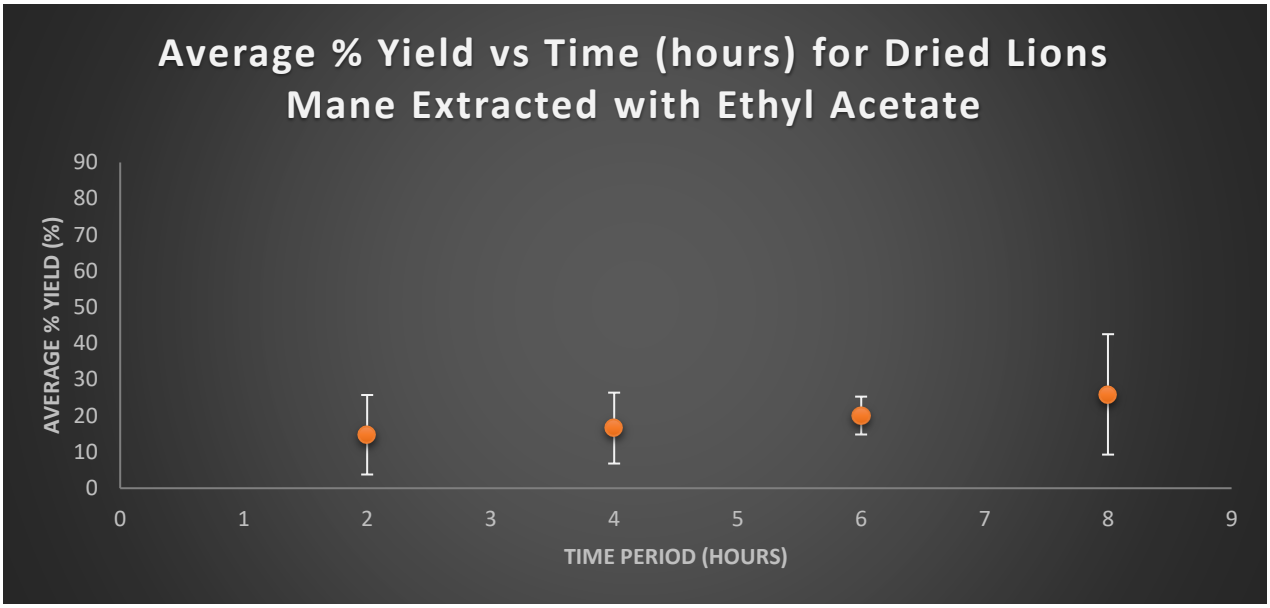

Figure 52: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried Hericium erinaceus (lion's mane) mushroom with ethyl acetate as the solvent for 8 hours. Produced a total percent yield of 8.8%.

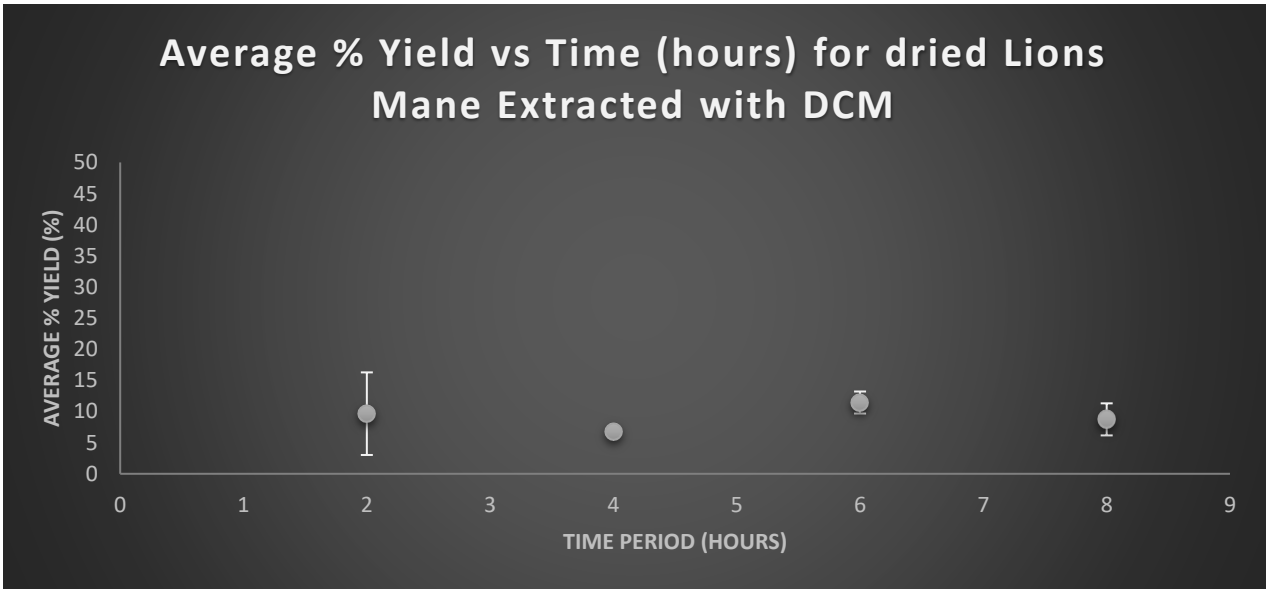

Figure 53: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried Hericium erinaceus (lion's mane) mushroom with DCM as the solvent for 8 hours. Produced a total percent yield of 11.7%.

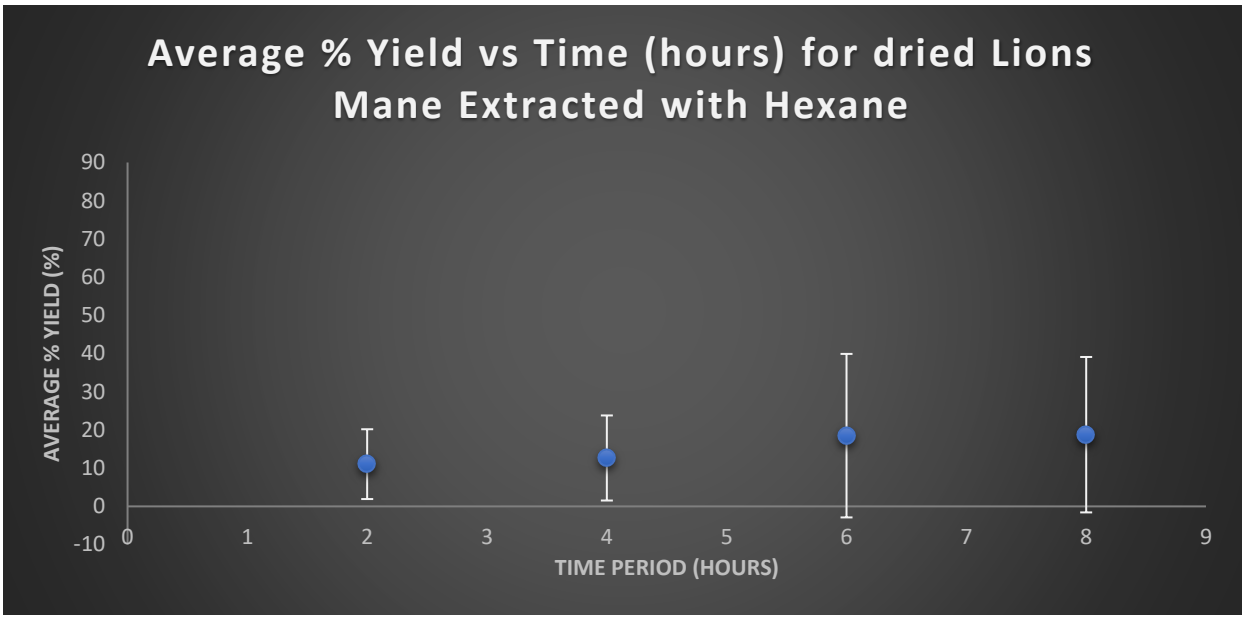

Figure 54: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried Hericium erinaceus (lion's mane) mushroom with hexane as the solvent for 8 hours. Produced a total percent yield of 9.2%.

Fresh reishi

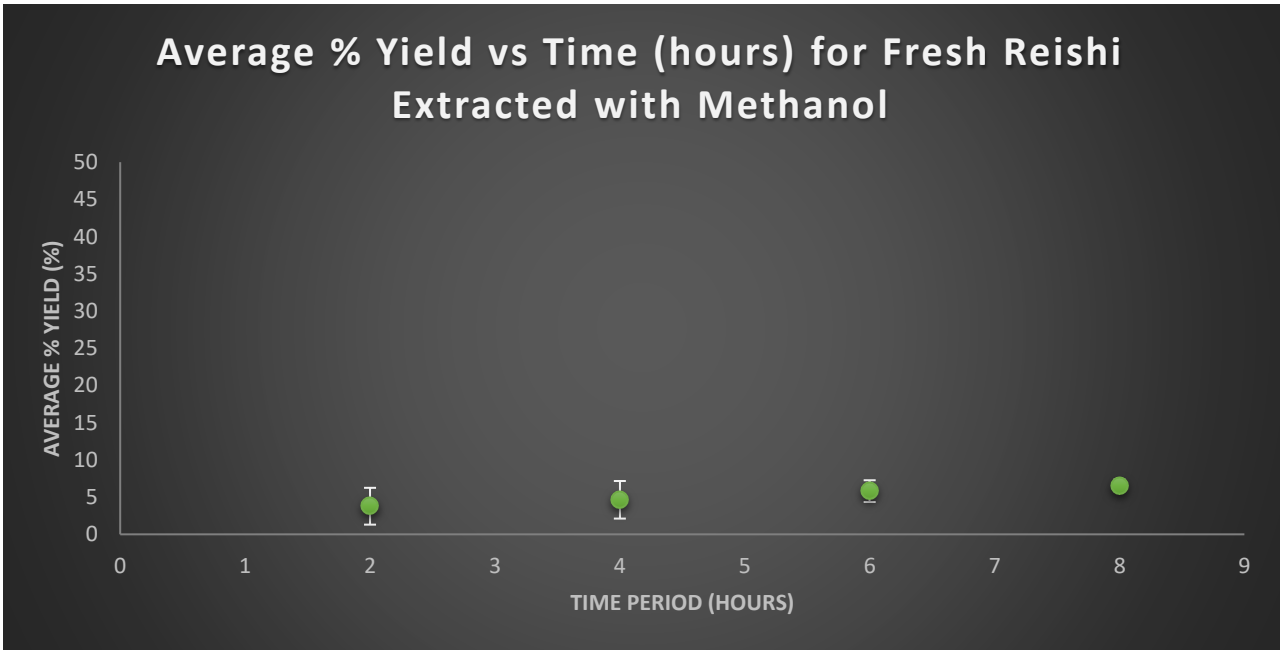

Figure 55: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh reishi (Ganoderma lucidum) mushroom with methanol as the solvent for 8 hours. Produced a total percent yield of 3.5%.

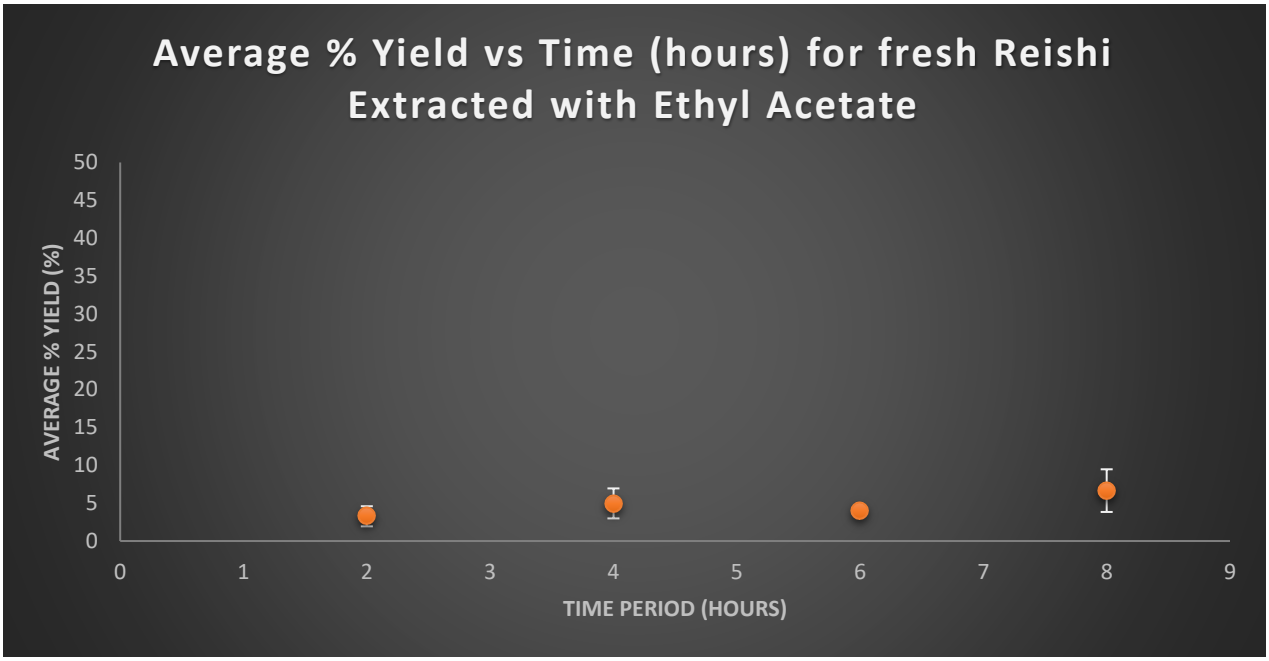

Figure 56: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh reishi (Ganoderma lucidum) mushroom with ethyl acetate the solvent for 8 hours. Produced a total percent yield of 3.7%.

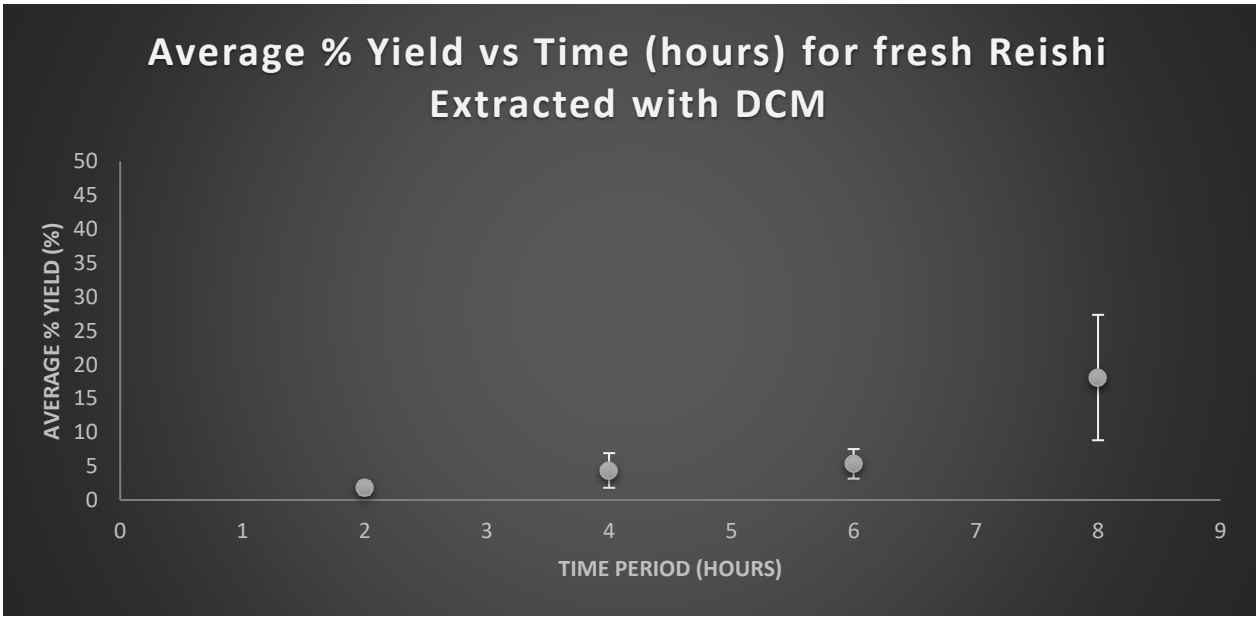

Figure 57: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh reishi (Ganoderma lucidum) mushroom with DCM the solvent for 8 hours. Produced a total percent yield of 2.8%.

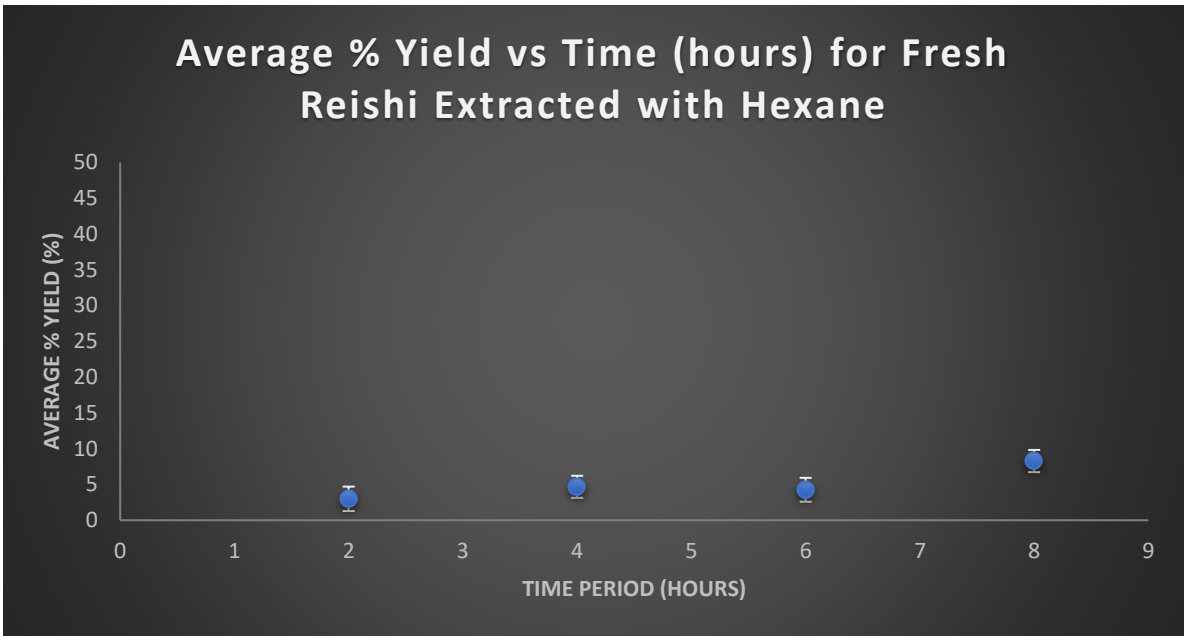

Figure 58: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of fresh reishi (Ganoderma lucidum) mushroom with hexane the solvent for 8 hours. Produced a total percent yield of 2%.

Dried reishi

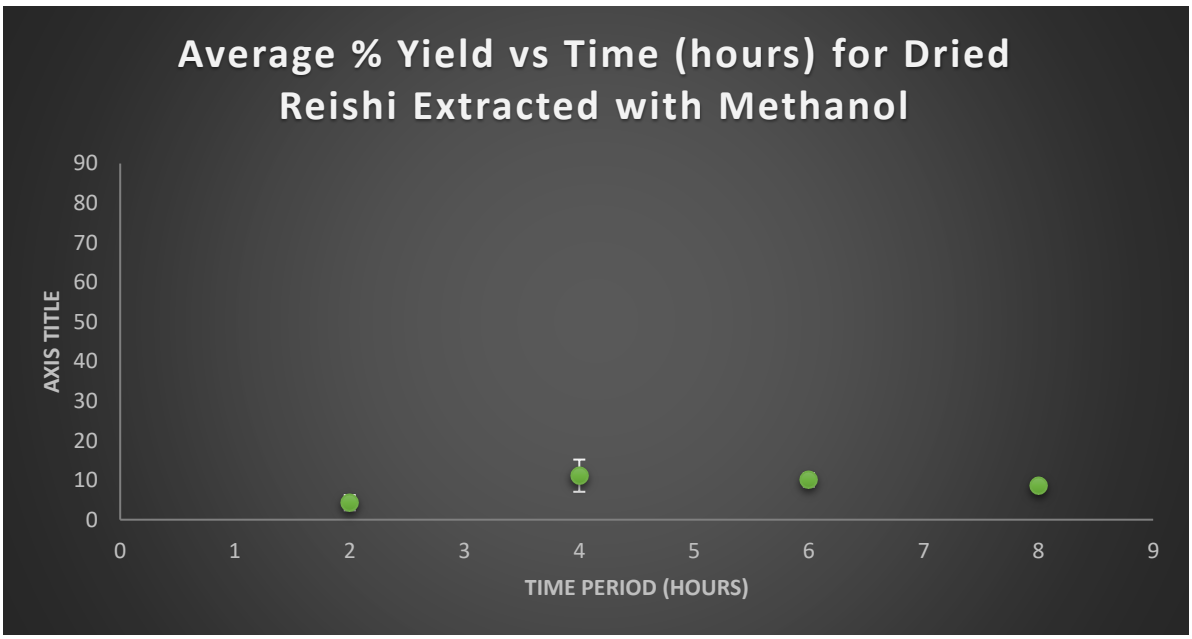

Figure 59: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried reishi (Ganoderma lucidum) mushroom with methanol the solvent for 8 hours. Produced a total percent yield of 7.8%.

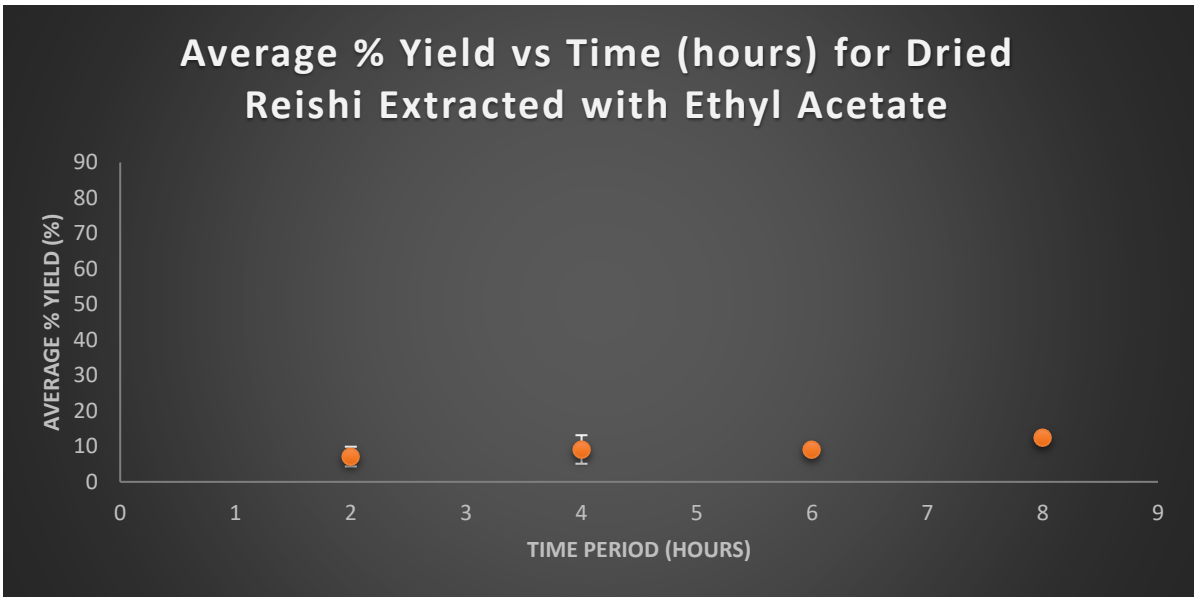

Figure 60: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried reishi (Ganoderma lucidum) mushroom with ethyl acetate the solvent for 8 hours. Produced a total percent yield of 4%.

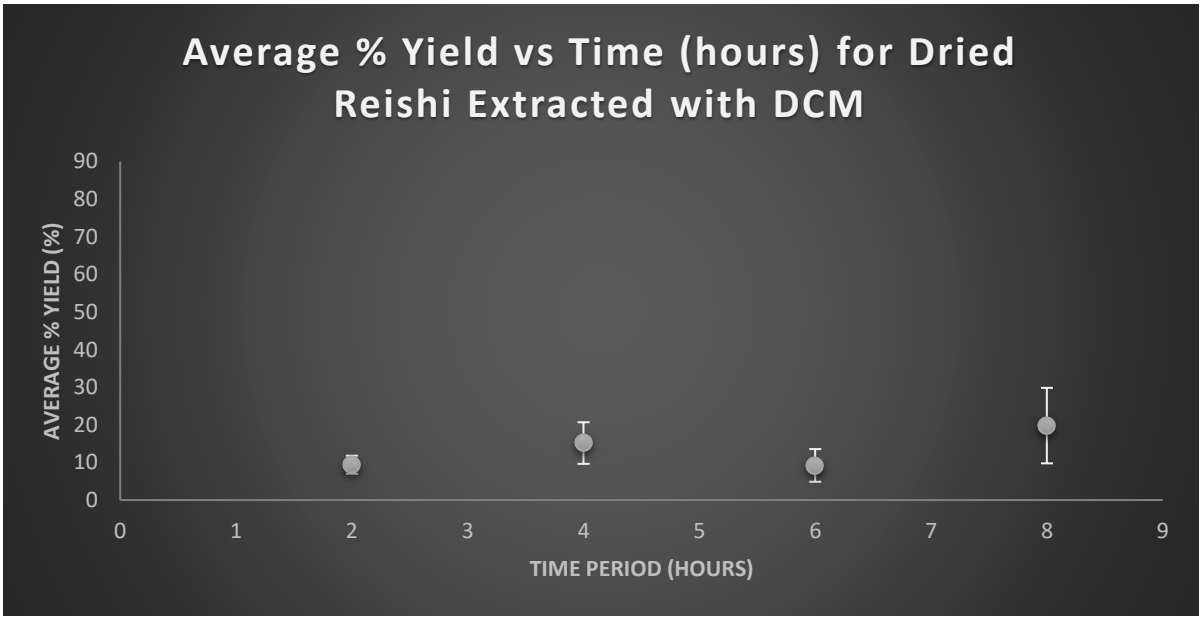

Figure 61: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried reishi (Ganoderma lucidum) mushroom with DCM the solvent for 8 hours. Produced a total percent yield of 5.5%.

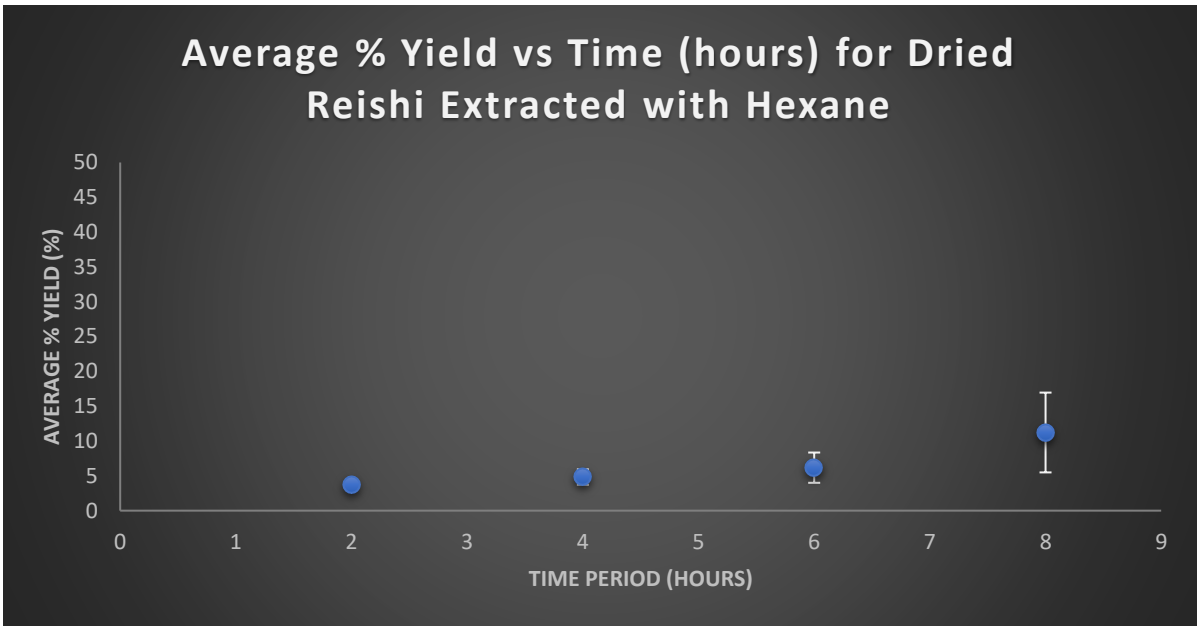

Figure 62: Average yield (%) plotted against Time Period (hrs). The data obtained from the extraction of 5.0 g of dried reishi (Ganoderma lucidum) mushroom with hexane the solvent for 8 hours. Produced a total percent yield of 2.8%.

IV. Protein Quantification Supporting Data

Fresh lion's mane

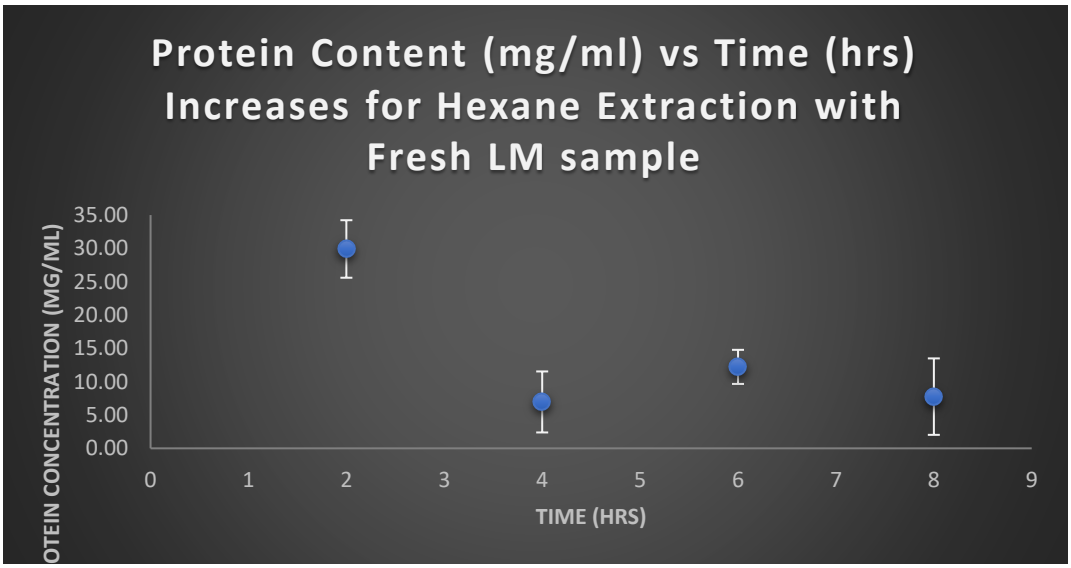

Figure 63: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with hexane. The data obtained from the extraction of 5.0 g of fresh lion's mane mushroom.

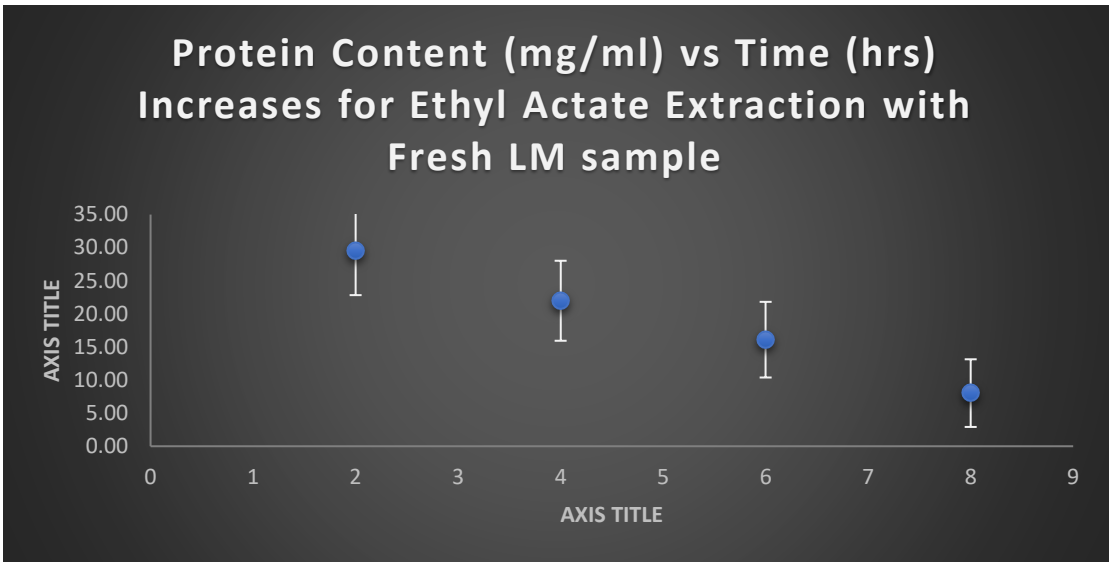

Figure 64: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with ethyl acetate. The data obtained from the extraction of 5.0 g of fresh lion's mane mushroom.

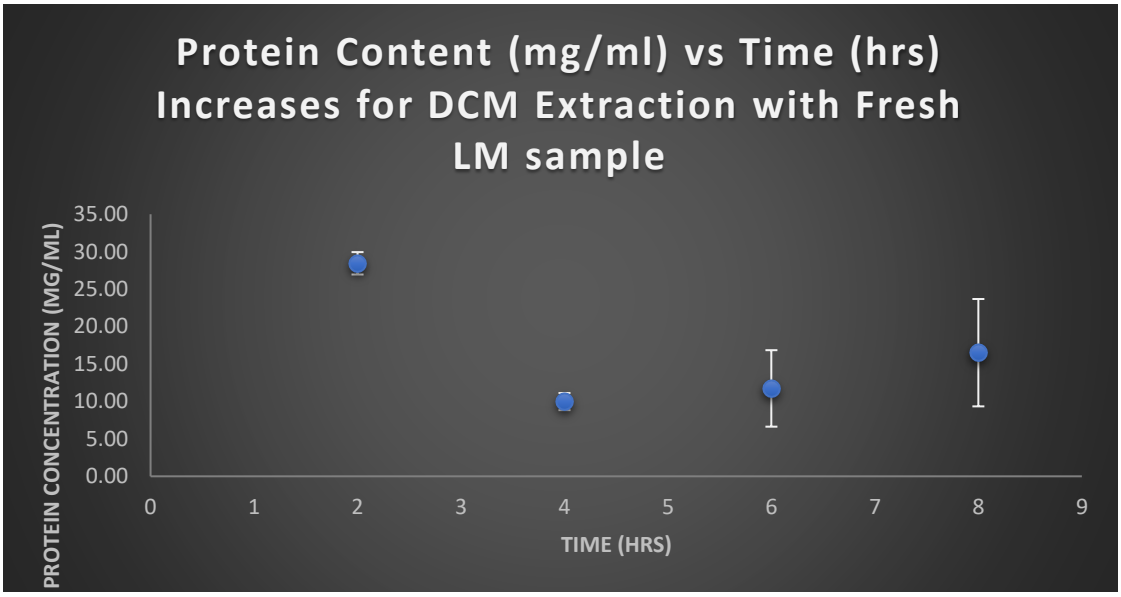

Figure 65: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with DCM. The data obtained from the extraction of 5.0 g of fresh lion's mane mushroom.

Dried lion's mane

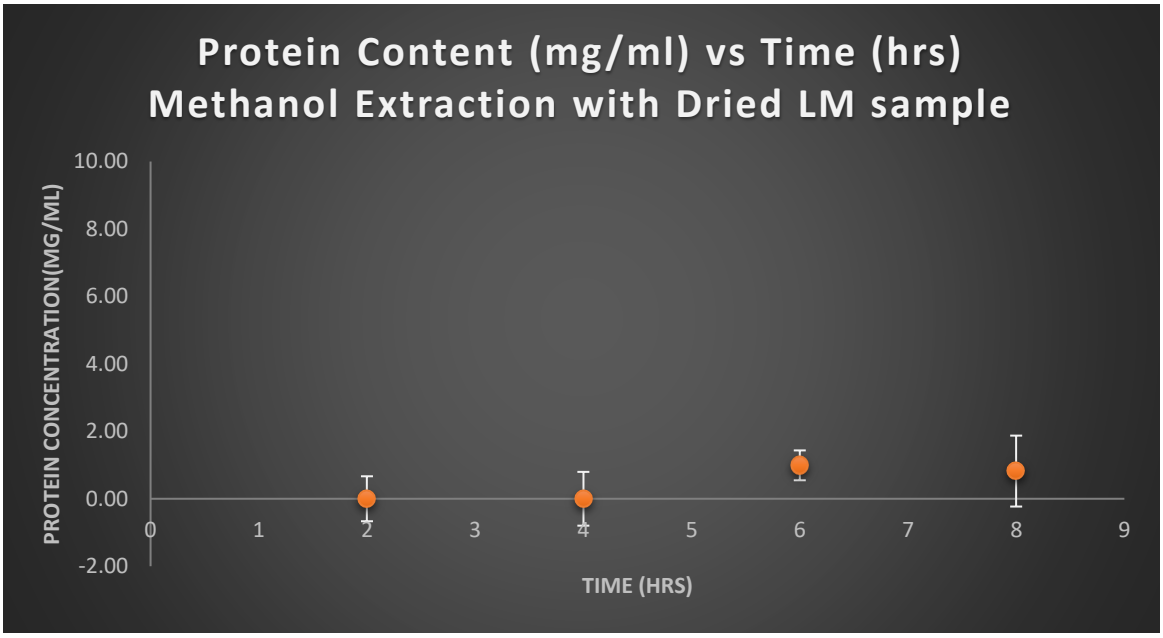

Figure 66: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with methanol. The data obtained from the extraction of 5.0 g of dried lion's mane mushroom.

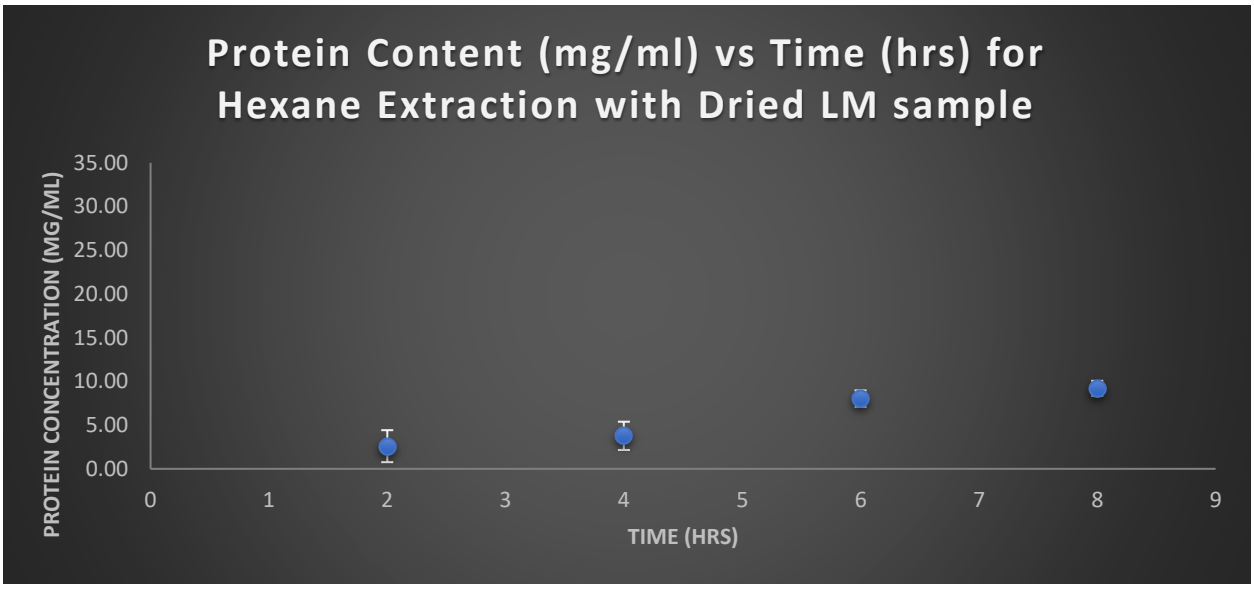

Figure 67: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with hexane. The data obtained from the extraction of 5.0 g of dried lion's mane mushroom.

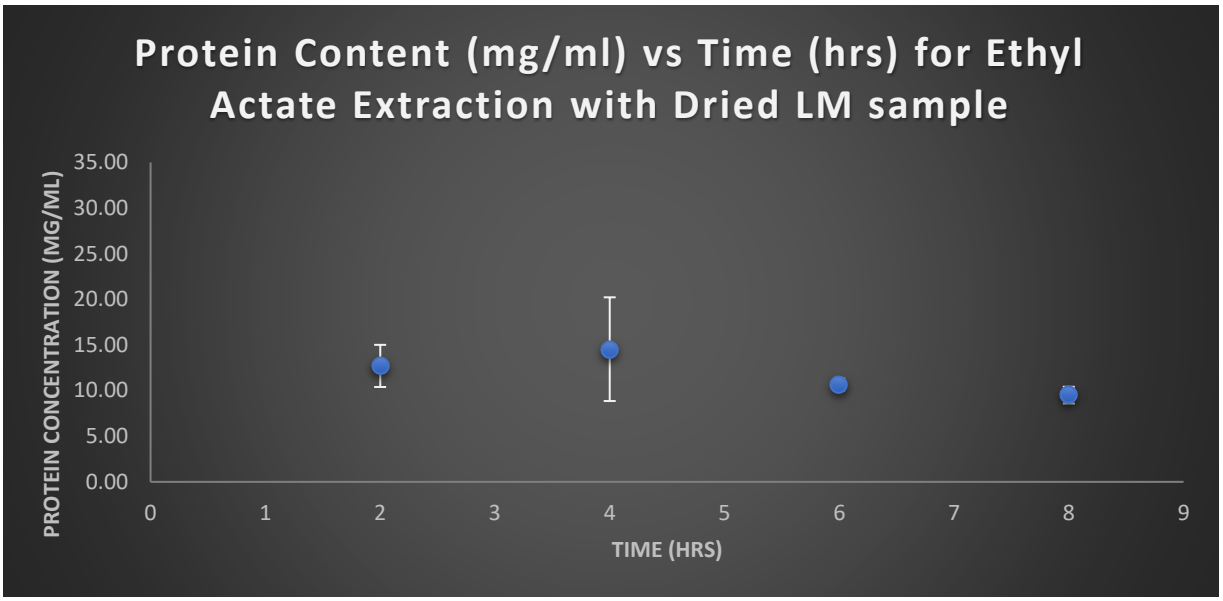

Figure 68: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with ethyl acetate. The data obtained from the extraction of 5.0 g of dried lion's mane mushroom.

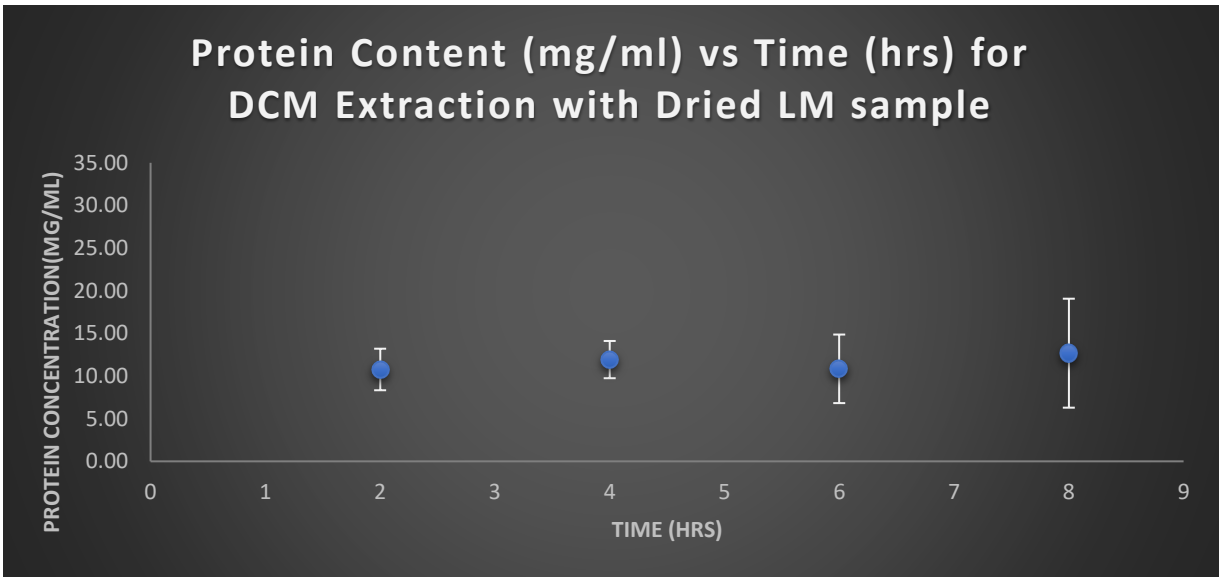

Figure 69: Protein Concentration (mg/ml) plotted against Time Period (hrs) for extraction with DCM. The data obtained from the extraction of 5.0 g of dried lion's mane mushroom.

Fresh reishi

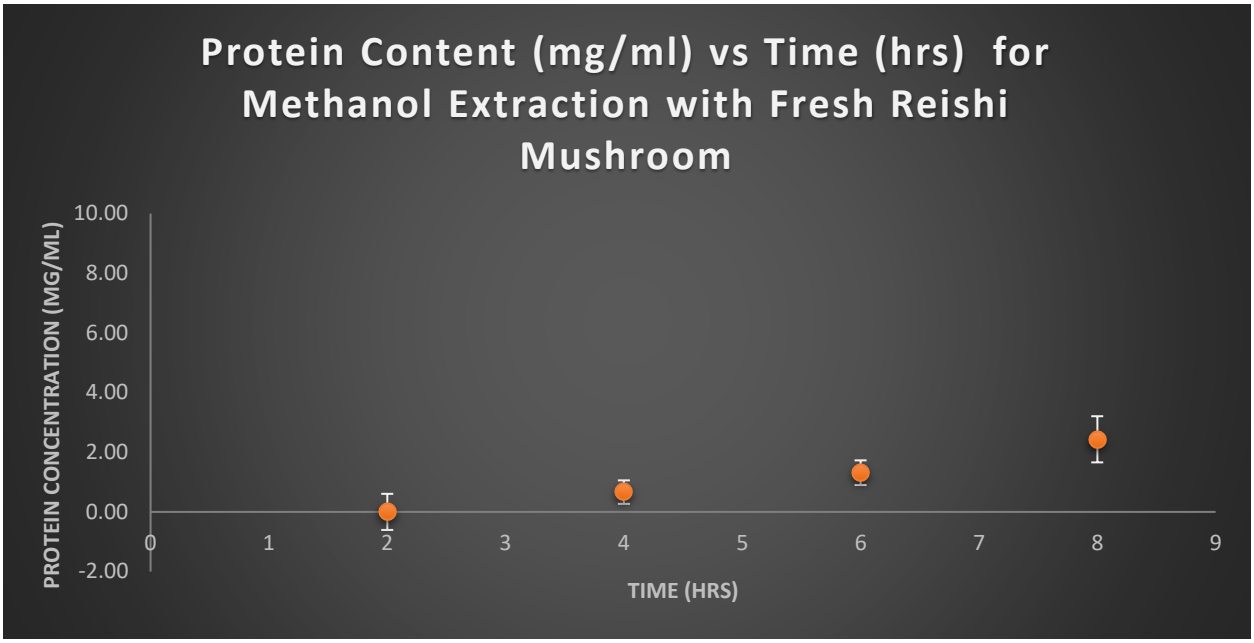

Figure 70: Average yield (%) plotted against Time Period (hrs) for extraction with methanol. The data is obtained from the extraction of 5.0 g of fresh reishi mushroom.

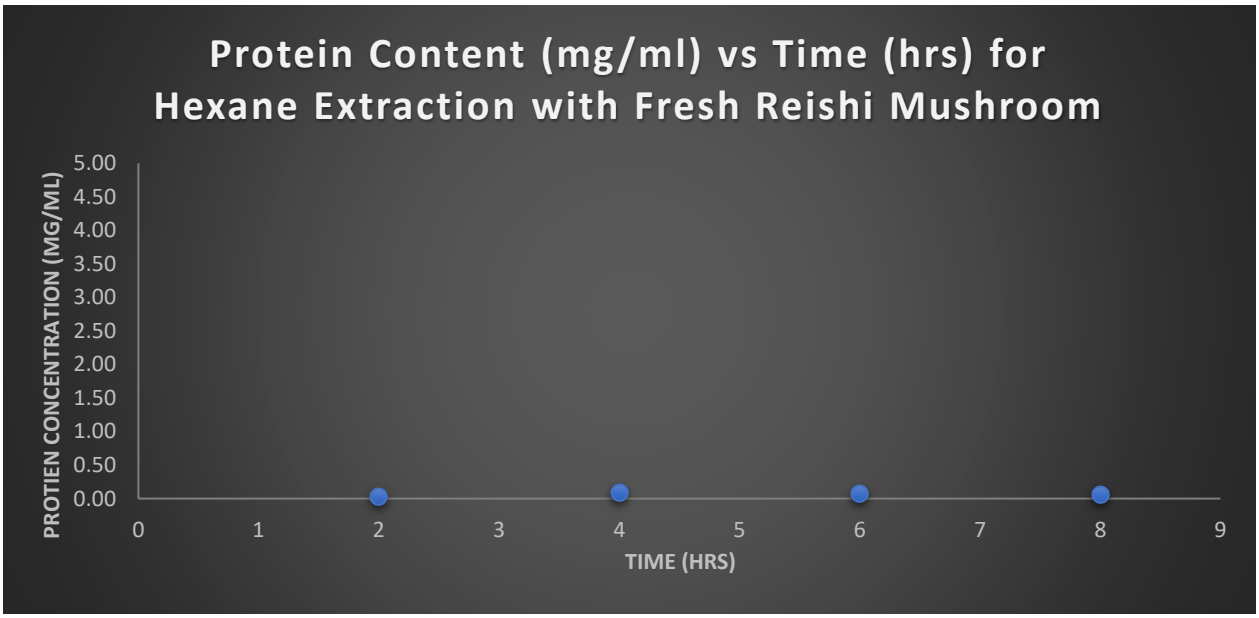

Figure 71: Average yield (%) plotted against Time Period (hrs) for extraction with hexane. The data is obtained from the extraction of 5.0 g of fresh reishi mushroom.

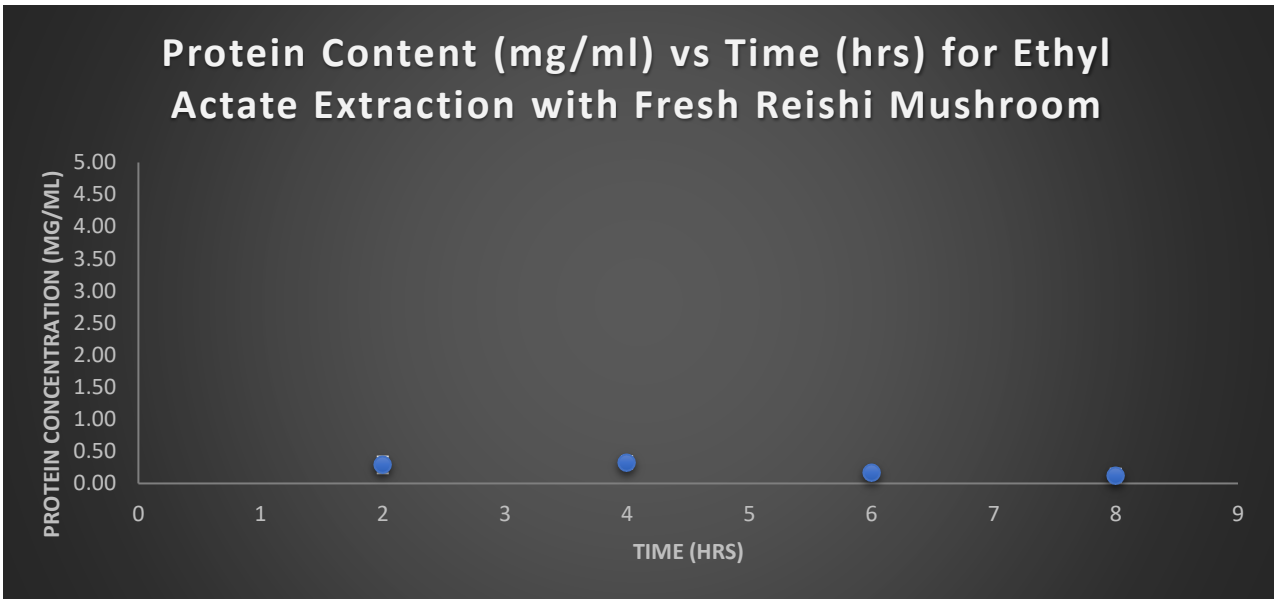

Figure 72: Average yield (%) plotted against Time Period (hrs) for extraction with ethyl acetate. The data is obtained from the extraction of 5.0 g of fresh reishi mushroom.

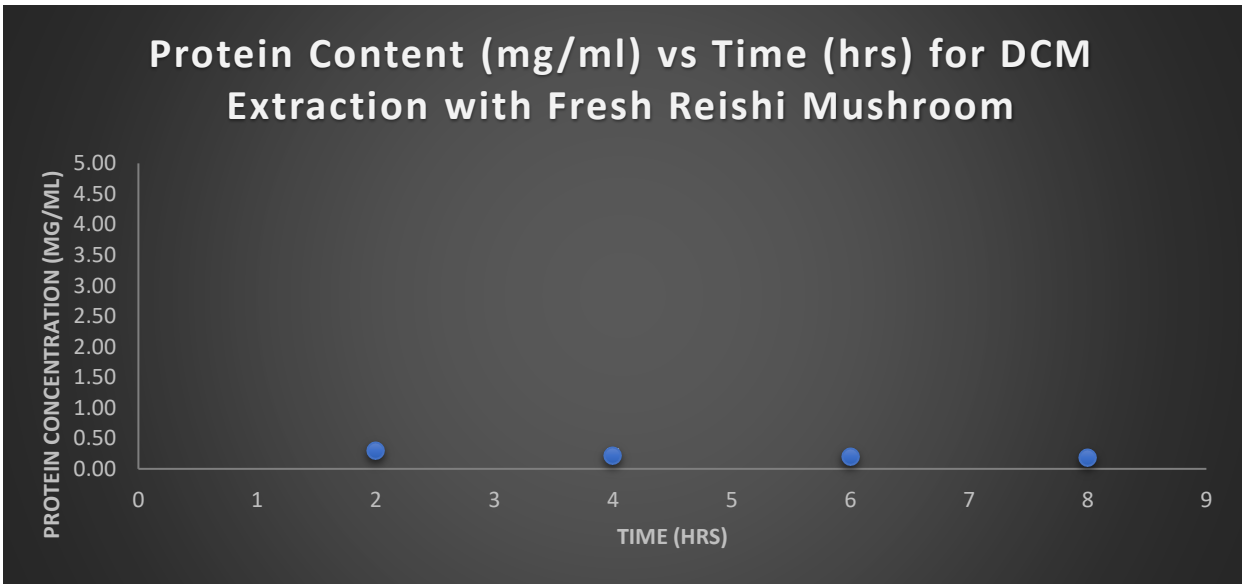

Figure 73: Average yield (%) plotted against Time Period (hrs) for extraction with DCM. The data is obtained from the extraction of 5.0 g of fresh reishi mushroom.

Dried reishi

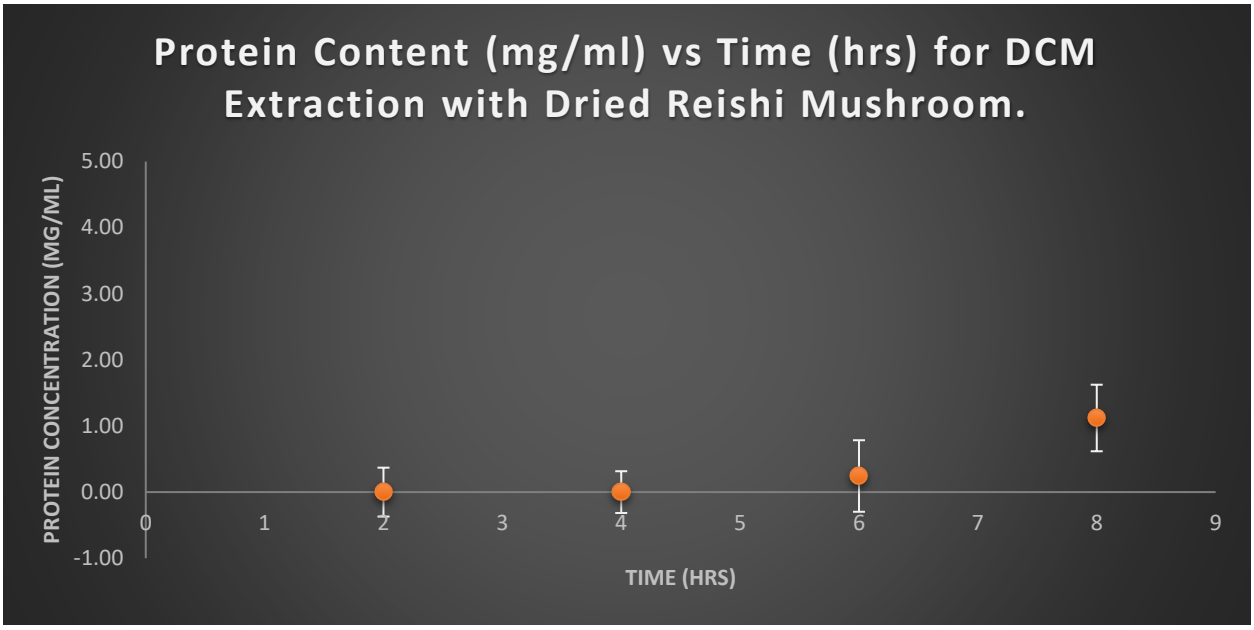

Figure 74: Average yield (%) plotted against Time Period (hrs) for extraction with methanol. The data is obtained from the extraction of 5.0 g of dried reishi mushroom.

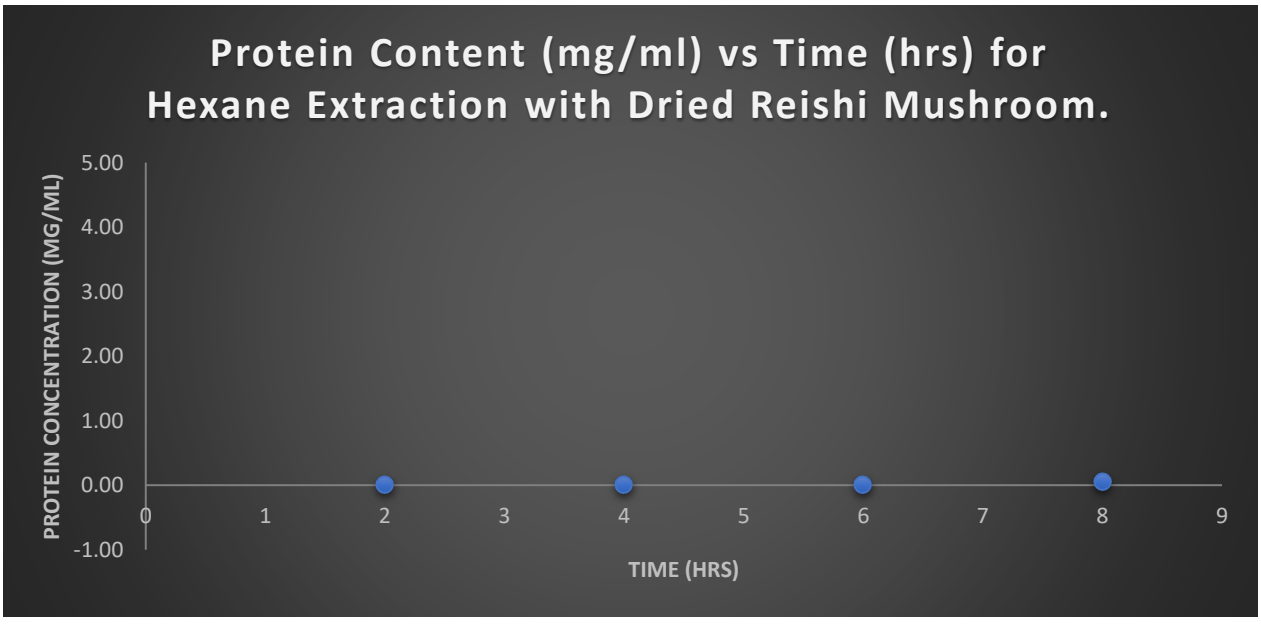

Figure 75: Average yield (%) plotted against Time Period (hrs) for extraction with hexane. The data is obtained from the extraction of 5.0 g of dried reishi mushroom.

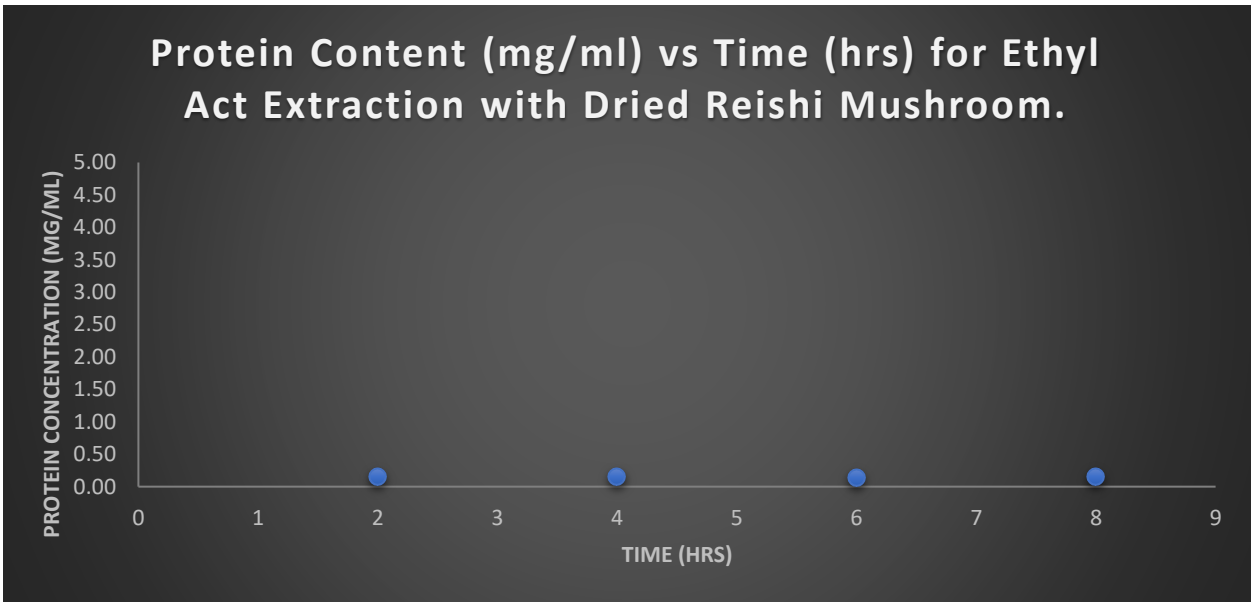

Figure 76: Average yield (%) plotted against Time Period (hrs) for extraction with ethyl acetate. The data is obtained from the extraction of 5.0 g of dried reishi mushroom.

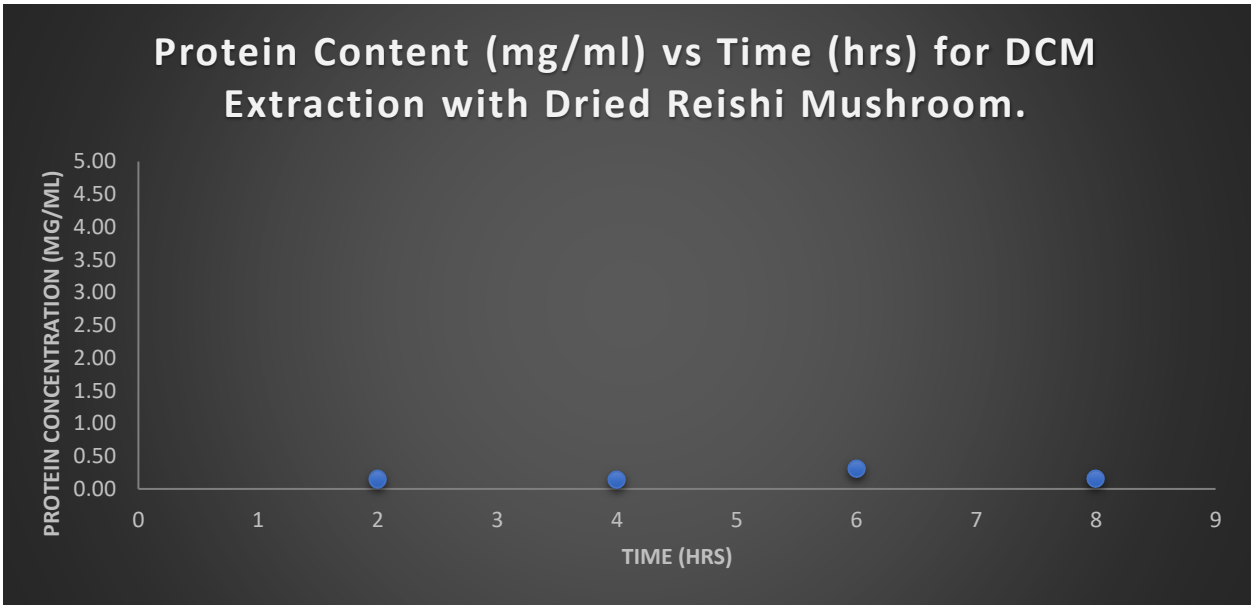

Figure 77: Average yield (%) plotted against Time Period (hrs) for extraction with DCM. The data is obtained from the extraction of 5.0 g of dried reishi mushroom.

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