Comparison of the phenolic components and their antioxidant activities in five common edible mushroom species in Manitoba

by

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Abstract

Background: Bioactive compounds found in mushrooms, particularly phenolic compounds, have demonstrated promising attributes such as anti-proliferative and antioxidant properties. However, until now, no comparative study between commercial and locally sourced fresh mushrooms has been undertaken.

Objectives: The objectives were i) to assess the differences in phenolic contents among different mushroom species supplied in Manitoba (*Agaricus bisporus, Cantharellus cibarius, Lentinus edodes, Inonotus obliquus*) and commercial mushrooms and how phenolic contents may contribute to antioxidant effects and potential antihypertensive efficacy *in vitro*; ii) to determine the impact of storage duration of *Inonotus obliquus* on phenolic content and their antioxidant properties.

Methods: The study used total phenolic content (TPC) and total flavonoid content (TFC) assays to determine the phenolic content in different mushrooms. Antioxidant activities were assessed using four antioxidant capacity assays. Potential anti-hypertensive effects were assessed *in vitro* by thiobarbituric acid reactive substances (TBARS) and angiotensin-converting enzyme (ACE) inhibition assays.

Results: *Inonotus obliquus* (Chaga) had the highest TPC of 6.98 mg GAE/g (dry wt.), which is six times higher than those obtained for other fresh mushrooms. The TPC in fresh Chaga mushrooms was four times higher than that of the corresponding commercial mushrooms. Chaga mushrooms also showed the highest antioxidant activity among different species of mushrooms, especially their total antioxidant activity level of 192.01 ± 14.67 mM TE/g DW, which was eight times higher than the total antioxidant activity level of other mushrooms. However, the ACE value of shiitake capsulated mushrooms was three times higher than corresponding fresh mushrooms, which had 47.03 ± 0.06%.

Conclusion:

This study showed varied phenolic content among the five mushrooms, with Chaga showing the highest content. However, phenolic content did not directly correlate with antioxidant properties and antihypertensive effects. More studies are required to understand how to connection between antioxidant and antihypertensive properties.

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Dedication

I dedicate this thesis to my beloved family, with special appreciation for my father, Mr. Chuangen Fan, and my mother, Mrs. Ming Xie. Their unwavering love, constant care, and boundless support have been my guiding light through every challenge and triumph. I am profoundly grateful for the sacrifices they have made to nurture and empower me along this journey.

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Chapter 1 Introduction

1.1 Introduction

Throughout history, mushrooms have been consumed since ancient times, and it is associated with many health benefits (Chang & Miles, 2004). In ancient Greece, people attributed mushrooms to providing strength for warriors in battle, while Chinese culture has also valued mushrooms for centuries as a health food (Valverde et al., 2015).

Mushrooms contain various nutrients, including protein, fibre, vitamins, minerals, and many bioactive molecules, while low in fat and calories. Due to their low-caloric value, low carbohydrates and sodium contents, and cholesterol-free, edible mushrooms have shown beneficial effects on heart health (Guillamón et al., 2010), metabolic syndrome and associated diseases, including diabetes (Harada et al., 2020). Edible mushrooms also have specific biochemical compounds such as polysaccharides; especially β-glucans, chitins, mannans (Singdevsachan et al., 2016), lectins, phenolic compounds, phytosterols, vitamins, and other bioactive compounds (Amirullah et al., 2018; Tan et al., 2022) that have shown promising results towards mitigating many diseases such as cancer, cardiovascular diseases (CVD), diabetes, and dementia (Chang & Wasser, 2012; Finimundy et al., 2013; Phan et al., 2017). These medicinal properties of mushrooms such as antioxidant, antitumor, antibacterial, and detoxification have been used for centuries in traditional medicine and have shown some promising results in conditions such as cancer (Grube et al., 2001; Twardowski et al., 2015; Wasser, 2017), obesity, type 2 diabetes mellitus (DM2) (Lindequist & Haertel, 2020; Lu et al., 2021), hypertension (HTN) (Agunloye & Oboh, 2022) and CVD (Petrisko et al., 2020). Furthermore, its diversified composition of polysaccharides, flavonoids, lectins, and vitamin D, aids in lowering the levels of

blood glucose and blood lipid, and improves insulin resistance (Das et al., 2022; Guo et al., 2019; Zhou et al., 2018).

The latest data (2017-2018) from the Canadian Chronic Disease Surveillance System (CCDSS) indicates that approximately 1 in 12 Canadian adults have heart disease (Public Health Agency of Canada, 2021). In addition, 10% of Canadians have been identified as having DM2 (Diabetes Canada, 2015). The mortality rate for people with diabetes is estimated by the Public Health Agency of Canada to be twice as high as the mortality rate for people without diabetes (2019). Annual statistics (2019-2020) from Manitoba Health indicate that up to 55.9 % of adults over 40 years old have one or more metabolic syndrome-related diseases including diabetes, HTN, heart failure, stroke, or chronic obstructive pulmonary disease (COPD) (Government of Manitoba, 2020). Significantly, there are over 30.8 % of adults (20 years and older) with HTN and 10.0 % of Manitobans over the age of one had diabetes during 2019/2020. It is valuable to identify the compounds present in mushrooms and their potential relationship with HTN, given the medicinal properties associated with mushrooms, particularly in managing conditions such as HTN and DM2.

In addition to the nutrients, mushrooms contain a wide variety of bioactive compounds, including phytosterols, glucan, acidic polysaccharides, phenolics, and lectins (Kumar et al., 2021). Several studies have shown that phenolic compounds have anti-proliferative, antioxidant, anti-cancer, antibacterial, and anti-hypertensive effects (Kähkönen et al., 1999; Nandi et al., 2007; Miklasińska-Majdanik et al., 2018; Boy et al., 2021). The main phenolic compounds extracted from mushrooms include flavonoids, tannins, and hydroxycinnamic acid (Abdelshafy et al., 2022). Among these, flavonoids have been shown to reduce the risk of myocardial infarction in young and middle-aged women (Cassidy et al., 2013). A prospective cohort study

by Jacques et al. (2013) found a negative relationship between flavonoid intake and diabetes incidence. Another polyphenol contained in mushrooms, tannic acid, has also been shown to exert cardiovascular protective effects, especially in HTN. In an animal study by Turgut Coşan et al. (2015), rats that consumed tannins showed a significant decrease in heart tissue malondialdehyde concentration, indicating an antihypertensive effect of tannic acid. In this study, our focus will be on investigating the potential effects of phenolic compounds found in mushrooms on a specific health condition, namely HTN.

Geographical and environmental factors may influence the growing conditions of wild mushrooms, and the information on the bioactive compounds of Manitoba-grown mushrooms is limited, especially their effects on HTN. Therefore, the literature review will provide a brief introduction to the mushrooms grown in Manitoba and provide an overview of the antioxidant properties of mushrooms and the effect of mushroom species on antioxidant properties.

Chapter 2 Literature Review

2.1 Cardiovascular Diseases Health Concerns in Manitoba

According to 2017–2018 data from the Canadian Chronic Disease Surveillance System (CCDSS), approximately 1 in 12 Canadian adults aged 20 and over, totalling 2.6 million individuals, live with diagnosed heart disease (Government of Canada, 2022). Additionally, in Manitoba, the Health Annual Statistic reported that 55.9% Manitobans had one or more of the following chronic conditions: HTN, ischemic heart disease, stroke, or chronic obstructive pulmonary disease (COPD) in the 2019/2020 period (Government of Manitoba, 2021). Furthermore, the prevalence of HTN was reported at 30.8% of the total population in 2019/2020. These data underscore a significant public health concern. Importantly, this prevalence varies across the province, with the Northern Health Region showing a notably higher proportion of hypertensive patients compared to the overall rate in Manitoba. As high blood pressure is a major risk factor for stroke and heart disease, addressing this regional disparity is crucial for targeted public health initiatives and interventions. By understanding the factors contributing to this variation, healthcare providers and policymakers can implement more effective strategies to mitigate the burden of HTN and its associated health risks in affected communities (Government of Manitoba, 2021). Mushrooms are commonly consumed by Manitobans and the relationship between mushroom consumption and CVD risk deserves to be explored. Mushrooms are a rich source of phenolic compounds, which are known for their potent antioxidant properties (Dimitrios, 2016). These phenolic compounds play a crucial role in scavenging free radicals, thereby reducing oxidative stress and inflammation in the body (Liu et al., 2023). Such antioxidant activities are essential for maintaining overall health and reducing the risk of CVD.

2.2 Phenolic Compounds and Cardiovascular Diseases

Phenolic compounds, widely distributed in various foods including vegetables, fruits, fungi, tea, wine, and vegetable oils, play a crucial role in human health due to their antioxidant properties (Dimitrios, 2016). These compounds exhibit significant antioxidant activity primarily due to the presence of benzene rings and hydroxyl (OH) groups. The effectiveness of their antioxidant action is intricately influenced by the structure and spatial arrangement of these chemical moieties, as the specific positioning and interaction of benzene rings and hydroxyl groups play a crucial role in stabilizing free radicals and preventing oxidative damage. Consequently, variations in the molecular architecture can lead to differences in the potency and efficacy of the antioxidant properties exhibited by these compounds (Lu et al., 2006). The antioxidant effects of phenolic compounds stem from their ability to scavenge free radicals and inhibit radical propagation reactions by donating hydrogen atoms or electrons from their hydroxyl groups to stabilize reactive species (Zeb, 2020). A clinical study by Hertog and colleagues (1993) has shown that phenolic compounds reduce the risk of death from coronary heart disease; the other study by Martín-Peláez and his colleagues (2017) has shown the enriched phenolic compounds diet can change the gene expression which was related to systolic blood pressure. The reason was related to the antioxidant properties of phenolic compounds. This also suggests that antioxidant activity is an important factor associated with cardiovascular disease.

2.3 Phenolic Compounds and Antioxidant Activity

Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are highly reactive molecules produced during normal cellular processes such as inflammation and respiration. These oxidants have the potential to damage various biological molecules including DNA and proteins.

The constant production of ROS during oxidative metabolism can lead to oxidative damage to biomolecules, contributing to the development of various diseases such as cancer, diabetes, cardiovascular diseases, and atherosclerosis (Urso & Clarkson, 2003; Halliwell, 2002). To counteract the harmful effects of RNS and ROS, the body employs antioxidant defense mechanisms, using antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), which play crucial roles in neutralizing these reactive molecules. Additionally, phenolic compounds act as antioxidants by scavenging ROS and inhibiting oxidative reactions. By reacting with free radicals, phenolic compounds help mitigate oxidative damage and contribute to overall health and well-being.

Balasundram et al. (2006) conducted a comprehensive analysis of phenolic compounds found in agro-industrial and plant products, emphasizing the significance of diets rich in these compounds for overall health. Their research indicated that diets containing high levels of phenolic compounds provide superior antioxidant activities compared to those lacking antioxidants due to the absorption and metabolism of these compounds. Studies by Fiorentino et al. (2008) and Hooper & Cassidy (2006) also have demonstrated that phenolic compounds possess preventive properties against various illnesses. Specifically, phenolic compounds such as flavonoids reduce low-density lipoprotein (LDL) oxidation, which protects against neuronal oxidation and inflammatory damage, so phenolic compounds demonstrate endothelium-independent vasodilator effects and decrease the risk of CVD (Perez-Vizcaino & Duarte, 2010).

Many studies have also demonstrated the effect of phenolic compounds contained in different foods on arterial HTN (Table 2-1). It has been shown that a daily intake of 425 mg of flavonoids from a variety of foods, including dark chocolate, tea, and dehydrated red apples, helped lower blood pressure in people with high blood pressure (de Jesús Romero-Prado et al., 2015).

Another study of hypertensive patients showed that consuming tomato phenolic extracts for four weeks at 213 mg per day can also improve blood pressure (Krasińska et al., 2018). Grassi and colleagues in 2015 found that black tea, which contains phenolic compounds, reduced blood pressure in adults with HTN when consumed at about 250 mg per day. Rull and colleagues (2015) found that daily consumption of 50 g of dark chocolate, with a low or high concentration of flavonoids, did not reduce the blood pressure of hypertensive individuals but may improve cardiovascular function.

In addition to the aforementioned foods, mushrooms also contain phenolic compounds, and the polyphenol content present in mushrooms holds promising potential for promoting cardiovascular health, particularly in the context of managing HTN (Rauf et al., 2023; Sun & Niu, 2020; Mohamed Yahaya et al., 2014). While ongoing research exploring the precise relationship between mushroom consumption and blood pressure is still evolving and not as extensive as studies on other dietary factors, there are indications suggesting that mushrooms may confer benefits for blood pressure regulation. For instance, in a study conducted by Kabir and Kimura (1989), spontaneously hypertensive rats (SHR) fed maitake mushrooms from the age of 10 weeks experienced a notable reduction in blood pressure after 8 weeks of continuous consumption. Additionally, shiitake mushrooms were found to significantly lower plasma levels of free cholesterol, triacylglycerides, and phospholipids, further suggesting a potential role in blood pressure management (Kabir & Kimura, 1989). In addition, Midoh et al., (2013) showed that Porcini (*Boletus aestivalis*) mushrooms reduced systolic and diastolic blood pressure in SHR during 18 weeks of oral consumption.

Table 2-1. Effect of phenolic compounds on arterial hypertension (HTN)

Study Type	Subject	Type of Intervention	Dose of Intervention	Duration	Results	References
Clinical Trial	37 men and 42 women with HTN (20-50 years old)	Dietary flavonoids in dark chocolate, red and green tea	425.8 ± 13.9 mg / day	6 months	SBP and DBP reduced -5/-4 mmHg	de Jesús Romero-Prado et al., 2015
Clinical Trial	44 men and 38 women with a high risk of HTN (28 – 74 years old)	Dietary phenolic compounds in tomato extract	213 mg/day	4 weeks	SBP, DBP and mean arterial pressure values reduced 8.8%	Krasińska et al., 2018
Clinical Trial	5 men and 14 women with HTN	Phenolic compounds in black tea	300 mg/day	8 days	SBP and DBP reduced -3.2/-2.6 mmHg, prevented blood pressure increase after a fat load	Grassi et al., 2015
Clinical Trial	60 men with HTN (24 – 72 years old)	Phenolic compounds in olive leaf extract	20 mg/day	6 weeks	SBP and DBP reduced −3.33/-3 mmHg. Plasma total, low-density lipoprotein and triglycerides reduced	Lockyer et al., 2017
Clinical Trial	53 women and 106 men with pre- or	Orange juice with a natural hesperidin content and hesperidin-	500 ml/day	12 weeks	SBP reduced −5.06 mmHg.	Valls et al., 2021

	stage 1 HTN (18 – 65 years old)	enriched orange juice				
Clinical Trial	32 adults with pre- or mild HTN	Dark chocolate with high concentrations of phenolics	50 g / day	6 weeks	Consumption of high flavonoid dark chocolate may improve cardiovascular function.	Rull et al, 2015
Clinical Trial	33 men and 27 women with HTN	Phenolic compounds in grape wine extract	800 mg polyphenols	4 weeks	SBP and DBP reduced -3/-1.9 mmHg.	Draijer et al., 2015
Animal Study	Male WKY rats or SHRs	HHQ and 5-CQA Diet	0.005% HHQ diet, 0.5% caffeoylquinic acid (CQA)	8 weeks	HHQ interferes with CQA-induced improvements in SHR blood pressure and endothelial function.	Suzuki et al., 2008
Animal Study	10 weeks old SHRs	Phenolic compounds in Maitake and Shiitake Diet	Fresh mushroom diet and 0.5% NaCl solution	8 weeks	reduce the plasma triglyceride 19.3 mg / 100 ml	Kabir and Kimura, 1989

SBP, systolic blood pressure; DBP, diastolic blood pressure; WKY, Wistar Kyoto; HTN, hypertension; HHQ, hydroxyhydroquinone; CQA, caffeoylquinic acid; SHR, spontaneously hypertensive rats; NaCl, sodium chloride.

2.4 Hypertension and Reactive Oxygen Species (ROS)

Hypertension stands as the primary contributor to CVD-related morbidity and mortality on a global scale (World Health Organisation [WHO], 2019). Oxidative stress emerges when there is an imbalance between the production of ROS and the body's antioxidant defense mechanisms. This imbalance leads to an excess of ROS, overwhelming the body's ability to neutralize them effectively. Oxidative stress is considered a pivotal pathway in the pathogenesis of HTN. When levels of ROS exceed those of antioxidants, it can trigger a cascade of events detrimental to vascular health (Zinkevich & Gutterman, 2011). For example, it can cause endothelial dysfunction. ROS can impair the function of endothelial cells, which line the inner walls of blood vessels. This dysfunction can lead to decreased vasodilation, increased vasoconstriction, and altered permeability, all of which contribute to vascular dysfunction. oxidative stress, inflammation, vascular remodelling, and endothelial cell apoptosis (Incalza et al., 2018). Oxidative stress can trigger inflammatory responses within the blood vessel walls, leading to the recruitment of immune cells and the release of inflammatory mediators. Chronic inflammation in the vascular system contributes to the development of atherosclerosis and other vascular diseases (Khansari et al., 2009). Furthermore, it can cause vascular remodelling. ROS-mediated oxidative stress can induce structural changes in blood vessels, leading to vascular remodelling. This can include thickening of the vessel walls, proliferation of smooth muscle cells, and formation of atherosclerotic plaques, all of which contribute to vascular dysfunction and increase the risk of cardiovascular events (Xu & Touyz, 2006). A plant-based and fungi-based diet with a high intake of nutrient-dense foods such as fruits, vegetables, and mushrooms can reduce the risk of diseases associated with oxidated stress (Johnson, 2004; Hever & Cronise, 2017; Cha et al., 2024). For example, oyster mushrooms

contain high levels of phenolic compounds, flavonoids, and alkaloids, making them a rich source of antioxidants and potential candidates for the development of new therapies to treat various oxidative stress-related diseases (Effiong et al., 2024).

Moreover, many studies have shown that angiotensin-converting enzyme (ACE) inhibitors are beneficial for ROS (Kim et al., 2013; Chen et al., 2008). Specifically, the renin-angiotensin system (RAS) interacts with ROS and contributes to the development of CVD, including HTN, chronic kidney disease, and heart failure (Koumallos et al., 2023). Overall, ACE inhibitors are a class of medications commonly used to treat HTN, as well as other cardiovascular conditions like heart failure, chronic kidney disease, and post-myocardial infarction (Azizi, 1999; Gallois & Piot, 1999; Matchar, 2007). In addition to antioxidant properties, some edible mushrooms, due to their rich content of ACE inhibitory peptides, significantly reduce blood pressure (Lau et al., 2013)..

In conclusion, mushrooms are rich in compounds with antioxidant properties (Mwangi et al., 2022). ROS can induce endothelial dysfunction, promote inflammation, contribute to vascular remodeling, and negatively interacts with RAS, all of which are implicated in the development and progression of HTN. These processes underscore the importance of maintaining a balance between ROS, antioxidants and RAS to safeguard vascular health and mitigate the risk of HTN and its associated complications. ACE inhibitors can treat HTN, and other CVD; however, a food-based ACE may have less adverse effects compared to synthetic ACE medications commonly used to treat HTN. Thus, understanding the antioxidant properties of mushrooms and their compounds can help us recognize their potential cardiovascular health benefits.

2.5 Mushroom and Antioxidant compounds

Mushrooms are renowned for their antioxidant properties, attributed to a diverse array of bioactive compounds present in their extracts (Kozarski et al., 2015; Podkowa et al., 2021; Naim, 2024). These compounds can vary between mushroom species and may include phenolics, polysaccharides, flavonoids, and carotenoids. These antioxidant compounds are not only confined to the fruit bodies of mushrooms but are also found in other parts such as the mycelium and culture (Kozarski et al., 2015; Venturella et al. 2021; Liu et al. 2024). The presence of these antioxidants contributes to the overall health-promoting properties associated with mushroom consumption, including their potential to combat oxidative stress, inflammation, and various chronic diseases (Table 2-2). Therefore, the consumption of mushrooms may reduce oxidative stress and protect cells from damage caused by ROS, leading to potential antihypertensive effects.

Table 2-2. Some of the antioxidant compounds in different types of mushrooms

Mushroom Scientific Name	Common names	Antioxidant compounds	Reference
Agaricus bisporus	White button mushroom, portobello	α- and β-glucans, catechin (Polysaccharides), gallic acid, caffeic acid (Polyphenols)	Barros et al., 2008; Robaszkiewicz et al., 2010
Cantharellus cibarius	Chanterelles	Flavonoids (Polyphenols)	Puttaraju et al., 2006; Barros et al., 2008; Robaszkiewicz et al., 2010
Calocybe indica	Milky white mushroom	Gallic acid, caffeic acid (Polyphenols)	Alam et al., 2019
Lentinus edodes	Shiitake	Gallic acid (Polyphenols)	Kim et al., 2008; Mau et al., 2002
Inonotus obliquus	Chaga	Flavonoids (Polyphenols)	Kim et al., 2008
Hericium erinaceus	Lion's Mane mushroom	Ergosterol, hericenone C, and hericene A, polyphenols	Tachabenjarong et al., 2022
Grifola frondosa	Maitake	Polysaccharides	Bai et al., 2019

Overall, mushrooms represent a promising natural source of antioxidants with potential health benefits, and ongoing research continues to explore their antioxidant properties and applications in various industries.

2.6 Some Types of Edible Mushrooms Available in Manitoba

Many Asian countries and regions have been using edible mushrooms as a part of their culture for health and medical purposes (Sanmee et al., 2003; Valverde et al., 2015). In recent years, Western countries have also been studying the benefits of mushrooms for chronic diseases such as heart disease and HTN (Krittanawong et al., 2021) because of their rich bioactive components and health benefits related to their antioxidant properties. Manitoba has 195,000

square feet to produce mushrooms (CTV News, 2022), and one acre of land can produce 1 million pounds of mushrooms annually (Mushroom facts, 2023). So based on these statistics, Manitoba can produce 5 million pounds of mushrooms per year for economic benefits. The following section provides an overview of the characteristics of four species of mushrooms available in Manitoba.

2.6.1 *Agaricus bisporus* (*A. bisporus*): White button and Portobello mushrooms

Manitoba is a province that produces large quantities of *Agaricus bisporus* (Loveday Mushroom Farms, 2022). Both white button and Portobello mushrooms belong to the mushroom species *A. bisporus*, although there is a large difference between these two mushrooms in color and size (Blumfield et al., 2020). The cap of the Portobello mushroom is longer than the white button mushroom as it is a mature-stage mushroom compared to the white button mushroom (Petruzzello, 2022). *A. bisporus* is the most cultivated mushroom in the world (McGee, 2018). *A. bisporus* is rich in beta-glucans, lectins, ergosterol, flavonoids, lactic acid, gallic acid, vitamins C and D, and other phenolic bioactives facilitating its potent antioxidant (Ghahremani-Majd & Dashti, 2015), antimicrobial (Ndungutse et al., 2015), anticancer (Chen et al., 2006), antidiabetic (Mao et al., 2013), antihypertensive (Sun & Niu, 2020), and anti-hemolytic (Kakoti et al., 2021) properties. According to the clinical study by Sun and Niu (2020), the consumption of 100 g of *A. bisporus* per day has been shown to decrease the risk of pregnancy-induced HTN. The mushroom diet group decreased the incidence of gestational HTN dramatically (normal diet: 48% vs. mushroom group: 24%).

2.6.2 *Cantharellus cibarius* (*C. cibarius*): Chanterelles mushroom

Chanterelles are wild edible mushrooms commonly found in forest environments in Manitoba. They are notable for their nutritional composition, being rich in protein and carbohydrates (Walde et al., 2006). Studies have shown that Chanterelles, particularly *C. cibarius*, contain significant antioxidant components derived from phenolic compounds, including flavonoids (Kozarski et al., 2015). Moreover, research suggests that Chanterelles may exhibit therapeutic effects against certain types of cancer, such as breast cancer and leukemia (Nowakowski et al., 2021; Patel & Goyal, 2012). Additionally, animal studies have indicated that ointments containing extracts from Chanterelles can enhance wound healing rates (Nasiry et al., 2017). These findings highlight the potential health benefits and biomedical applications of Chanterelle mushrooms. Nasiry and his colleagues (2017) showed that male Wistar rats were randomly divided into four groups to treat two different wound shapes: round and linear. They were treated with an ointment containing *C. cibarius* extract, Madecassol medication, and vehicle-treated or non-treated. The result showed that the rats treated with the extract containing *C. cibarius* had faster repair capacity than the other three groups. The study by Ebrahimzadeh et al. (2015) underscores the antioxidant properties of *C. cibarius*, highlighting its potential therapeutic applications in addressing diseases associated with reactive oxygen species, such as CVD, wound healing, and aging. Specifically, the methanolic extract of *C. cibarius*, particularly the ethyl acetate fraction, demonstrated significant scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, suggesting its efficacy in combating oxidative stress-related conditions. Furthermore, Lemieszek et al. (2018) revealed the neuroprotective effects of polysaccharides found in *C. cibarius*. These polysaccharides were shown to protect against neuronal death and excitotoxicity induced by glutamate, indicating a potential role in preserving neuronal health and

function. Collectively, these studies highlight the diverse antioxidant and bioactive properties of *C. cibarius*, suggesting its potential as a natural remedy for various oxidative stress-related ailments and neurodegenerative conditions.

2.6.3 Lentinus edodes (*L. edodes*): Shiitake mushroom

The mushroom species *L. edodes*, commonly known as shiitake, is cultivated for both edible and medicinal purposes. It was shown through human studies by Dai et al. (2015) that shiitake mushrooms improved the proliferation of anti-tumor immune cells, specifically, γ- δ-T- and NK-T cells and improved T-cell effector functions (60% and 2-fold increase, respectively). The result also showed a decrease in the inflammatory marker serum C-reactive protein (CRP) after four weeks of *L. edodes* consumption, suggesting that shiitake mushrooms also have potential anti-inflammatory activity. Spim et al. (2021) and Olawuyi and Lee (2019) noted that the inclusion of shiitake mushrooms in the processing of cereals improved the nutritional properties of cereal products, such as antioxidant and anti-diabetic activities, due to the high content of phenolic components within *L. edodes*. In another study conducted by Kabir and colleagues (1987), spontaneously hypertensive rats were fed a diet containing 5% shiitake mushroom powder for 9 weeks, with the results demonstrating a reduction in blood pressure levels among the rats.

2.6.4 *Inonotus obliquus (I. obliquus):* Chaga mushroom

The mushroom species *I. obliquus*, also known as Chaga, is a parasitic fungus that grows on birch trees (Ryvarden & Gilbertson, 1993). Chaga can be found in the forest environments in Manitoba. Chaga contains biological compounds such as polysaccharides, triterpenes, and polyphenols (Ma et al., 2013). The extracts of *I. obliquus* are used in China, Korea, Japan, and

other Asian countries and regions to treat health problems such as dyslipidemia and heart function (Shashkina et al., 2006). For example, Chaga has been observed to lower triglyceride levels and elevate the high-density lipoprotein-cholesterol levels (HDL)/low-density lipoprotein-cholesterol levels (LDL) ratio in mice (Chou et al., 2016). Chaga also showed inhibitory activity against lipopolysaccharide (LPS)-induced inflammatory cytokines such as RAW 264.7 macrophages (Alhallaf & Perkins, 2022). When the level of nitric oxide (NO) reached 44.53 ± 0.23 μM, the accelerated solvent extraction extract contained in Chaga reduced ($p < 0.05$) the level of NO production, suggesting potent anti-inflammatory properties (Alhallaf & Perkins, 2022). In addition, *I. obliquus* was found to have a hypoglycemic effect in animal experiments (Sun et al., 2008). At the end of the experimental period of 3 weeks of both 500 mg/kg and 1000 mg/kg consumption, the blood glucose levels of diabetic mice decreased by 31.30%. Sun and colleagues (2008) also found that consumption of *I. obliquus* significantly reduced LDL levels and increased HDL levels ($p < 0.01$). When higher doses of *I. obliquus* were consumed, the lipid-lowering effect was shown to be more pronounced. For example, when 500 mg of *I. obliquus* /kg was added to 1000 mg/kg of food, LDL decreased from 1.39 ± 0.19 to 1.22 ± 0.17 mmol/l.

In Sharp and colleagues' comparison study (2021), they showed that *I. obliquus* has a higher antioxidant activity than five other edible mushroom species, which are *Grifola frondosa* (Maitake), *Ganoderma lucidum* (Reishi species), *Lentinula edodes* (Shiitake), *Trametes versicolor* (Turkey tail) and *Hericium erinaceus* (Lion's mane). Sharpe *et al*., (2021) showed that the total phenolic content (TPC) of Chaga was 12 times more than Turkey tail, which was 48.3 ± 3.9 μmol gallic acid equivalents (GAE)/mg compared to 4.8 ± 0.9 μmol GAE/mg. The outcome of alcoholic and aqueous extract types showed that Chaga would be a potential mushroom species to decrease oxidative stress and prevent diseases such as atherosclerosis, CVD, and diabetes ($p < 0.05$).

In summary, Table 2-3 provides the health benefits of five edible mushrooms that were analyzed in this study.

Table 2-3. The effect of five types of mushrooms in Manitoba and their health benefit

Mushroom type (Common name)	**Study Type**	**Subjects**	**Form of mushroom**	**Duration**	**Compounds**	**Known health benefit**	**Reference**
***Agaricus bisporus* (White button mushrooms)**	Human Study	Healthy men (n=10) with a body mass index between 20 and 35 kg/m^2, LDL cholesterol < 150 mg /dL, TG < 200, and BP < 140/80, were studied	Freeze-dried mushroom powder (containing 14 ug ergocalciferol/serving)	½ cup = 87.9 g/day for 6 weeks	Ergothioneine	Antioxidant capacity and Anti-inflammatory	Weigand-Heller et al., (2012)
		Participants with pre-diabetics with 2 or more features of metabolic syndrome	Cooked, sliced mushrooms	100 g fresh white button mushroom daily for 16-weeks	Ergothioneine	Anti-inflammatory and antioxidant	Calvo et al., (2016)
	Animal Study	8 weeks old mice (n=30)	Frozen lyophilized mushroom powder (Stored at −20 °C)	300, 600, and 1200 mg/kg body weight ethanolic extract per day respectively for 30 days	Gallic acid, protocatechuic acid, catechin, caffeic acid, ferulic acid and myricetin	Antioxidant	Liu et al., (2013)
	In Vitro	Fresh cultivated Agaricus bisporus		N/A			

***Agaricus bisporus* (Portobello)**	Animal Study	6 weeks old C57BL/6J male mice (n=30)	A basic supplemented diet with 5% Freeze-dried white button mushroom / Portobello mushroom powder	Fed a regular diet or a diet with 5% white button mushroom powder or a diet with 5% portobello mushroom powder for 15 weeks	N/A	Antioxidant, anti-inflammatory, and regulation of microbiota	García-Sanmartín et al., (2022)
***Cantharellus cibarius* (Chanterelles)**	In Vitro	Fresh; Blanched; Fermented mushrooms	Freeze-dried mushroom powder	N/A	Phenolic acid contents	Antioxidant	Jabłońska-Ryś et al., (2016)
		0.2 mL different concentrations of the extracts (2-10 mg/mL)	Dried fruit bodies of Cantharellus cibarius	N/A	Phenolic compounds: homogentisic acid and gallic acid; Carotenoidic compound: lycopene		Vamanu & Nita, (2014)
	In Vivo	Two yeast strains: *Saccharomyces cerevisiae* and *Pichia pastoris*		Cultivated in YPG medium, obtained cells by centrifuging, treated the cells with different		Anti-inflammatory	

				concentration extracts (2, 4, 6, 8, and 10 mg/mL)			
***Lentinus edodes* (Shiitake)**	In Vitro	The fruiting bodies of Lentinus edodes	N/A	N/A	Polysaccharides	Antioxidant	Chen et al. (2012)
		The fruiting bodies of Lentinus edodes	Heat treatment of raw Shiitake	N/A	free polyphenolic, flavonoids	Antioxidant activity	Choi et al., (2006)
		Micrococcus luteus and Bacillus cereus	Shiitake extracted from high-pressure operations and low-pressure methods	N/A	Phenolic compounds	Antioxidant activity and antimicrobial activity	Kitzberger et al., (2007)
	Human Study	42 healthy, elderly Caucasian subjects	2.5 mg (1-6,1-3)- β-glucan (lentinan) from shiitake	16 weeks	Soluble β-glucan	Increase immune response	Gaullier et al., (2011)
	Animal Study	10 healthy mice	10 mg/mL Bioprocessed polysaccharide isolated from edible L.edodes	60 hours	Polysaccharides	Anti-inflammatory	Kim et al., (2013)
		Adult male Spragus-Dawley rats	β-glucan extracts from L.edodes	48 hours	β-glucan	Immunomodulatory effect	Masterson et al., (2020)
	In Vivo	Liver	Acidic-hydrolysis L.edodes	N/A	Polysaccharides	Antioxidant and hepatoprotective effects	Song et al., (2021)

	In Vitro	Cancer cell lines and the macrophage cell line	Dried mushroom powder	N/A	Triterpenoids	Anti-Proliferative Activity	Kim et al., (2020)
	Animal Study	20 ± 2 g Male the Institute of Cancer Research mice	The dry matter of culture broth	21 days	N/A	Antihyperglycemic and anti-lipid peroxidative	Sun et al., (2008)
	In Vitro study	The dried mushroom powder	The dried mushroom powder	N/A	Water-soluble and alkali-soluble polysaccharide constituents	Antioxidant	Mu et al., (2012)
		Human lung carcinoma (A549); Colon adenocarcinoma (HT-29), and rat glioma (C6) cell cultures	The dried fruiting bodies	N/A	IO_4^- fraction from Chaga	Anticancer	Lemieszek et al., (2011)
		Herpes simplex virus and in Vero cells	The dried fruiting bodies	N/A	The aqueous extract from *I.obliquus*	Anti-virus: Herpes simplex virus (HSV)	Pan et al., (2013)

***Inonotus obliquus*(Chaga)**		Chaga, Agaricus blazei Mycelia, Ganoderma lucidum and Phellinus linteus	The fruiting body and Scleroyoim part.	N/A	Phenolic ingredients	Antioxidant activity	Nakajima et al., (2007)
		Pseudomonas aeruginosa; tumour cells (MCF-7, NCl-H460, HeLa and HepG2) and non-tumor liver cells	The dried fruiting bodies	N/A	Oxalic acid and phenolic compounds	Antioxidant activity; Antimicrobial activity and cytotoxicity	Glamočlija et al., (2015)

N/A, Not Available; LDL, low-density lipoprotein; TG, triglycerides; BP, blood pressure

2.7 Common Methods of Extraction of Phenolic Compounds from Mushrooms

The process of extracting phenolic compounds from mushrooms typically involves using solvents like methanol or ethanol to extract the bioactive compounds from the mushroom biomass. This is due to the higher polarity and enhanced solubility of phenolic compounds in these solvents, which makes them effective for this purpose. (Zhao et al., 2006). These extracted compounds can then be further purified and concentrated for various applications in food, pharmaceuticals, and nutraceuticals.

Various methods have been used for extracting the phenolic compounds contained in different species of mushrooms. Table 2-4 provides a summary of several extraction methods that have been previously used for phenolic compounds from different mushroom species. Phenolic compounds are more easily dissolved in polar organic solvents because they contain hydroxyl groups in their chemical structure (Wang and Weller, 2006). As a result, solvents such as methanol, ethanol, and others have been utilized to extract phenols from plant materials (Dai and Mumper, 2010; Xu and Chang, 2007; Do et al., 2014). The type of solvent and its concentration (i.e., the water content) influence the quantity and rate of phenol extraction (Dai and Mumper, 2010; Xu and Chang, 2007). For example, Gursoy and his colleagues (2009) used 100% methanol concentration to extract phenolic compounds in seven mushroom species, and found *M. conica* had the highest phenolic content (25.38 ± 0.70 μg GAE/mg) among seven mushroom species. However, when Alfaleh & Sindi (2024) used 70% methanol concentration to extract phenolic compounds in plants, they found that date palm seeds had 20.78 ± 4.8 μg GAE/mg. Previous studies have demonstrated that phenolic compounds can be extracted from mushrooms or plants whether they are extracted with methanol at a concentration of 70% or 100%. However, the

extraction of phenolic compounds from the same mushroom with different concentrations of methanol has not been studied. The optimal methanol concentration for phenolic extraction remains unclear. To resolve this issue, two common solvents (70% and 100% methanol) that have appeared in previous articles, were used in this work.

Table 2-4. The extraction methods and solvents for isolating phenolic compounds using five species of dietary mushroom

Mushroom types	Solvent	Extraction methods	Outcomes	Reference
Agaricus bisporus	80% ethanol	2 g of *Agaricus bisporus* extracted by stirring with 100 ml of 80% ethanol.	TPC was 6.18 mg per gram of dry weight	Liu et al., 2013
Cantharellus cibarius	96% ethanol	50 g of *Cantharellus cibarius* was extracted twice by stirring with 500 mL of 96% ethanol.	TPC was 11.27 mg per gram of dry weight	Zavastin et al., 2016
Lentinus edodes	80% ethanol	50 g of *Lentinus edodes* extracted by stirring with 100 ml of 80% ethanol.	TPC was 29 mg per gram of dry weight	Choi et al., 2006
Inonotus obliquus	70% aqueous methanol	5000 g of *Inonotus obliquus* extracted twice with 70% aqueous methanol for 12 hours at 60 °C.	N/A	Hwang et al., 2016
Morchella	100% methanol	5 g mushroom samples extracted by 100 ml 100% methanol.	TPC were ranged from 12.36 to 25.38 μg per mg of dry weight	Gursoy et al., 2009

TPC, Total phenolic content

2.8 Common Methods of Analysing Antioxidant Properties from Mushrooms

Phenolic compounds are known for their antioxidant properties, so the total phenolic content (TPC) assay measures the concentration of phenolic compounds in a sample (Tsao et al., 2005). Flavonoids are another group of compounds with antioxidant activity, and the total flavonoid content (TFC) assay quantifies the concentration of flavonoids in a sample (Shraim et al., 2021). This can be done using colorimetric methods based on the reaction of flavonoids with specific reagents.

Chromatographic methods separate and analyze components of a mixture based on differences in their partitioning between a stationary phase and a mobile phase (Smith, 2013). High-performance liquid chromatography (HPLC) is a widely used chromatographic method for determining individual phenolic compounds within plants (Christopoulou and Perkins, 1989; Määttä et al., 2003; Manzano Durán et al., 2020). HPLC allows for the separation of specific compounds in a mixture by modulating factors such as high flow rates and mobile phase pressures, and the identification of these compounds when coupled with detection methods such as diode array detection (DAD) (Munteanu & Apetrei, 2021). Overall, HPLC analysis allows for separation and confirmation of the identity of the phenolic compounds and provides quantitative results.

In addition, analyzing the antioxidant properties of mushrooms typically involves various laboratory techniques aimed at measuring their ability to neutralize free radicals or inhibit oxidative processes. To investigate the antioxidant capacity of mushrooms, the common methods are spectroscopic and chromatographic (Moharram et al., 2014). Spectroscopic techniques analyze the interaction of electromagnetic radiation with matter (Feltes et al., 2023), including a test for one-electron transfer, such as the ferric reducing antioxidant power (FRAP) test, which measures the ability of antioxidants to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) (Amarowicz & Pegg,

2019). Additionally, spectroscopic methods include tests for the transfer of a mixture of one hydrogen atom and one electron, such as the 2,2'-azidobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test, which is the assay that measures the ability of antioxidants to scavenge free radicals. The decrease in absorbance at a specific wavelength is used to quantify antioxidant activity. The DPPH assay is a commonly used method for assessing the ability of antioxidants to scavenge free radicals (Ozgen et al., 2006).

Moreover, the lipid peroxidation inhibition assay evaluates the ability of antioxidants to prevent the oxidative degradation of lipids, a process known as lipid peroxidation (Ayala et al., 2014). Lipid peroxidation can be measured by assessing the formation of TBARS or by monitoring changes in the levels of lipid hydroperoxides. These are the methods commonly used to analyze the antioxidant properties of mushrooms.

2.9 The Relationship Between Antioxidant Activity and Mushroom's Storage Period

It is well known that the storage period of a plant affects its antioxidant activity; the antioxidant activity of some plants decreases with increasing storage period, such as bitter melon Momordica charantia L. (MC). and Momordica charantia var. abbreviata Ser. (MCVAS), the latter being one of the wild species of bitter melon (Lin et al., 2020). Moreover, 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging ability, and ferric reducing power (FRAP) decreased more rapidly in MC than in MCVAS during 24 days of storage. It may be because MCVAS has a richer composition (Lin et al., 2020). In addition, Julia and her colleagues found that preserving the phytochemicals in fruits and vegetables is very complex. They showed the effect of storage time on the stability of these compounds.

Mushroom's antioxidant effects may also be related to its storage period. The relationship between antioxidant activity in mushrooms and the mushroom's storage period time can vary depending on several factors including the species of mushroom, environmental conditions, and cultivation methods. Generally, mushrooms undergo changes in their biochemical composition as they preserving time, which can influence their antioxidant properties (Marçal et al., 2021).

In conclusion, it is important to note that the relationship between antioxidant activity and mushroom storage time is complex and can vary depending on the specific antioxidant compounds studied and the conditions under which the mushrooms are grown (Chun et al., 2021; Mwangi et al., 2022). Further studies are needed to fully understand how mushroom storage time affects antioxidant activity in different species and cultivation methods.

Chapter 3 Study Rationale, Objectives, and Hypotheses

3.1 Rationale

Mushrooms have long been recognized for their nutritional value and potential health benefits (Valverde et al., 2015). Among the numerous bioactive compounds found in mushrooms, phenolic compounds have garnered significant attention due to their diverse health-promoting properties (Chang & Wasser, 2018). Studies have demonstrated the antioxidant, anti-inflammatory, antimicrobial, and anticancer activities of phenolic-rich mushroom extracts, underscoring their potential as functional foods and nutraceuticals (Stastny et al., 2022).

The hydroxyl groups in phenolic compounds act as electron donors, enhancing their strong antioxidant properties (Bendary et al., 2013, Abdelshafy et al., 2022, Do et al., 2014). The antioxidant activities include playing a crucial role in scavenging free radicals, thereby protecting cells from oxidative damage and reducing the risk of chronic diseases, including cardiovascular diseases and cancer (Abdelshafy et al., 2022). Phenolic compounds include phenolic acids (e.g. hydroxybenzoic and hydroxycinnamic acids), polyphenols (hydrolyzable and condensed tannins), and flavonoids (e.g., flavones, isoflavones and anthocyanins) (Sim & Sil, 2008, Do et al., 2014). Studies have shown that TPC; which consists mainly of phenolic acids and flavonoids, contributes the most to a plant's antioxidant activity (Mulihah et al., 2021). The TPC level is shown to be directly proportional to their antioxidant activity (Do et al., 2014, Liu et al., 2009). Similar to TPC, the TFC also showed a positive correlation with antioxidant activity (Rosyantari et al., 2021). There are a variety of phenolic compounds in mushrooms, such as phenolic acids, flavonoids, and tannins (Abdelshafy et al., 2022). Understanding the variation in phenolic content and antioxidant effects among different mushroom species is essential for harnessing their therapeutic potential and promoting human health (Brown & Miller, 2021).

Despite the increasing number of studies on the bio-phenolic content and antioxidant effects of mushrooms (Sharpe et al., 2021; Kim et al., 2008; Chu et al., 2023), comparative studies on different mushroom species grown in North America and comparative studies of mushroom storage times remain scarce. This study aims to fill this gap by investigating the phenolic compounds and antioxidant effects of five types of edible mushrooms growing in North America and to compare antioxidant effects between mushroom ages by investigating the mushroom type with the highest phenolic compounds. Namely *Agaricus bisporus* (white button mushroom, Portobello), *Cantharellus cibarius* (chanterelle), *Lentinus edodes* (shiitake), and *Inonotus obliquus* (Chaga) were used for this study. These mushrooms represent a diverse range of culinary and medicinal mushrooms with varying degrees of popularity and commercial availability. Moreover, in previous studies, Chaga showed superior antioxidant performance in comparison to other mushroom species (Sharpe et al., 2021; Ma et al., 2013).

3.2 Hypothesis

Inonotus obliquus (Chaga) will have the highest content of polyphenolic compounds and the strongest antioxidant effect among the five mushroom types. Furthermore, fresh Chaga will have a higher content of polyphenolic compounds and a stronger antioxidant effect than aged ones.

3.3 Objectives

The overall objective is to assess if there are variations in phenolic content among different mushroom types (white *Agaricus bisporus*, brown *Agaricus bisporus, Cantharellus cibarius, Lentinus edodes, Inonotus obliquus*), and how the phenolic contents contribute to antioxidant effects and potential anti-hypertensive benefits *in vitro*. In addition, the highest phenolic content of one of five different mushrooms will be identified and studied to determine if

the antioxidant properties of this mushroom may have an effect on storage time. Thus, specific objectives are:

1. To compare the levels of polyphenolic compounds amongst five fresh mushroom types (*Agaricus bisporus* [white and brown], *Cantharellus cibarius, Lentinus edodes, Inonotus obliquus*), and to compare the levels of phenolic compounds in three of the fresh mushrooms (white *Agaricus bisporus, Lentinus edodes, Inonotus obliquus*) to corresponding commercial capsules.
2. To compare the antioxidant activities in the five fresh mushroom types, and to compare the antioxidant activities in three of fresh mushrooms to corresponding commercial capsules.
3. To assess the potential *in vitro* antihypertensive benefits of three different types of fresh mushrooms to corresponding commercial capsules.
4. To identify the mushroom with the highest content of phenolic compounds among the five mushroom types and to compare its antioxidant capacity at different methanol concentrations (70% and 100%) in different storage periods (fresh, 5 years old, and 10 years old).

Chapter 4 Material and Methods

4.1 Samples

Five edible mushroom types commonly consumed in Manitoba were selected for the current project, including two wild (*Cantharellus cibarius* and *Inonotus obliquus*) and three cultivated (white and brown *Agaricus bisporus*, and *Lentinus edodes*). To eliminate source specificity, *Agaricus bisporus* (white button mushrooms, and portobello), *Cantharellus cibarius* (Chanterelles), and *Lentinus edodes* (shiitake) were purchased from three different local markets in southern, central, and northern Winnipeg; at Country Root's Market & Garden, Jardins St-Léon Gardens, and Vic's Market (Winnipeg, Manitoba, Canada), respectively, between August to September, 2022. Fresh *Inonotus obliquus* (Chaga) mushrooms were acquired from the northern Manitoba First Nations community of Opaskwayak Cree Nation (OCN) (The Pas, Manitoba, Canada) Aug, 2022. Also, some processed Chaga mushrooms were acquired from the Manitoba Food Development Centre (FDC) (Portage la Prairie, Manitoba, Canada) in March, 2022. For each mushroom type, the different sources of mushroom samples were pooled together to make representative samples. For positive controls, commercial mushroom capsules were donated from Nutritional Fundamentals for Health (NFH, Quebec, Canada). Four commercial capsules, including mushroom complex Scientific Advisory Panel (SAP) {contains equal proportions of Reishi, Maitake, Shiitake, Coriolus versicolor}, Shiitake SAP, *Agaricus bisporus* SAP, and Chaga SAP were used in this study.

4.2 Methods

4.2.1 Freeze-Drying Samples

The length and width of the fresh mushroom samples were measured randomly upon initial acquisition leaving out any bias. Subsequently, three mushrooms from each species were chosen arbitrarily for measurement. Mushrooms were stored in a freezer at -80°C to maintain freshness before freeze-drying. The Chaga samples were much harder than other types of fresh mushrooms, therefore, it was ground into a powder form with a manual grinder and micro-mill grinder (SP Bel-Art, Warminster, PA, USA). The commercial capsules were ground into powder form with a manual grinder and other fresh mushrooms (white button mushrooms, portobello, chanterelles, and shiitake) were cut into small pieces, before freeze-drying. Figure 4-1 summarizes the freeze-drying process by showing that the mushroom samples and capsules were processed in a FreeZone 4.5-liter freeze dryer (Labconco Corporation, Kansas City, MO, USA) at 0.14 mBar and -55°C for four days. The freeze-dried mushrooms and capsules were used for the extraction of phenolic compounds. The phenolic extracts were tested for various antioxidant activities.

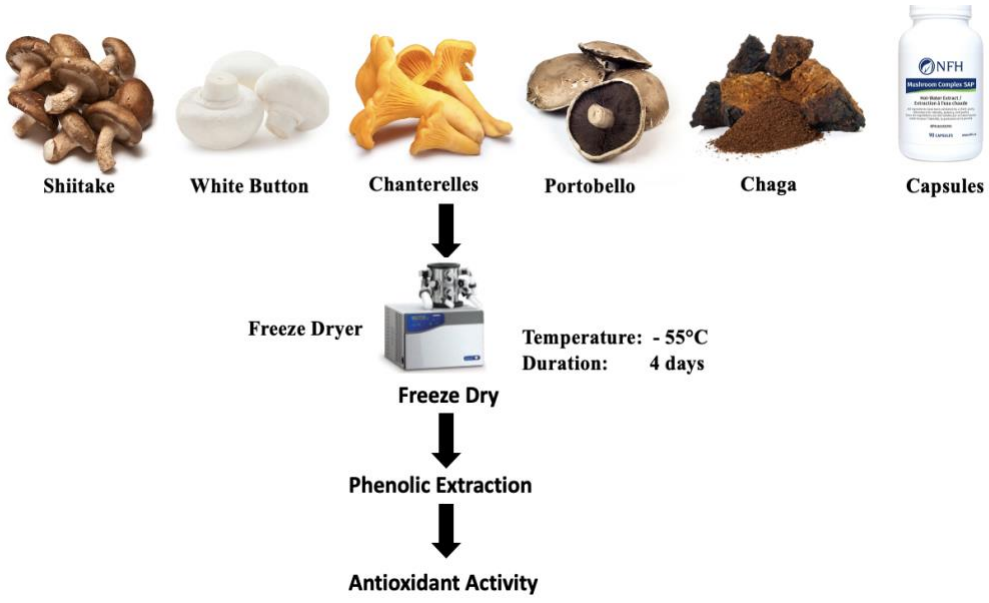


Figure 4-1. Summarized experimental steps for samples.

4.2.2 Phenolic Extraction of Samples

Phenolic compounds, due to their hydroxyl groups, are more soluble in polar organic solvents like methanol and ethanol (Wang & Weller, 2006). The type and concentration of the solvent significantly influence the extraction efficiency of phenolic compounds from plant materials (Dai & Mumper, 2010, Xu & Chang, 2007, Truong et al., 2019, Salih et al., 2021, Do et al., 2014). Methanol, particularly in high concentrations, is effective for extracting lower molecular weight phenols (Dai & Mumper, 2010). The addition of water to methanol can also impact the extraction yield (Zhao et al., 2006). In this study, two methanol concentrations (70% and 100%) were used to extract phenolic compounds from mushroom samples. For each mushroom sample, 0.1 g of freeze-dried mushroom powder or capsule powder was transferred into a 2 mL test tube, and 1 mL of 70% methanol or 1 mL of 100% methanol added to the tube. For each type of mushroom sample, there were six samples of 0.1 g per species. The samples were divided into two methanol concentration groups and made three replicates for each group. In one group, 1 mL of 70% methanol was added; in the other group, 1 mL of 100% methanol was added to each tube. All tubes were then mixed thoroughly using a vortex. (Figure 4-2).

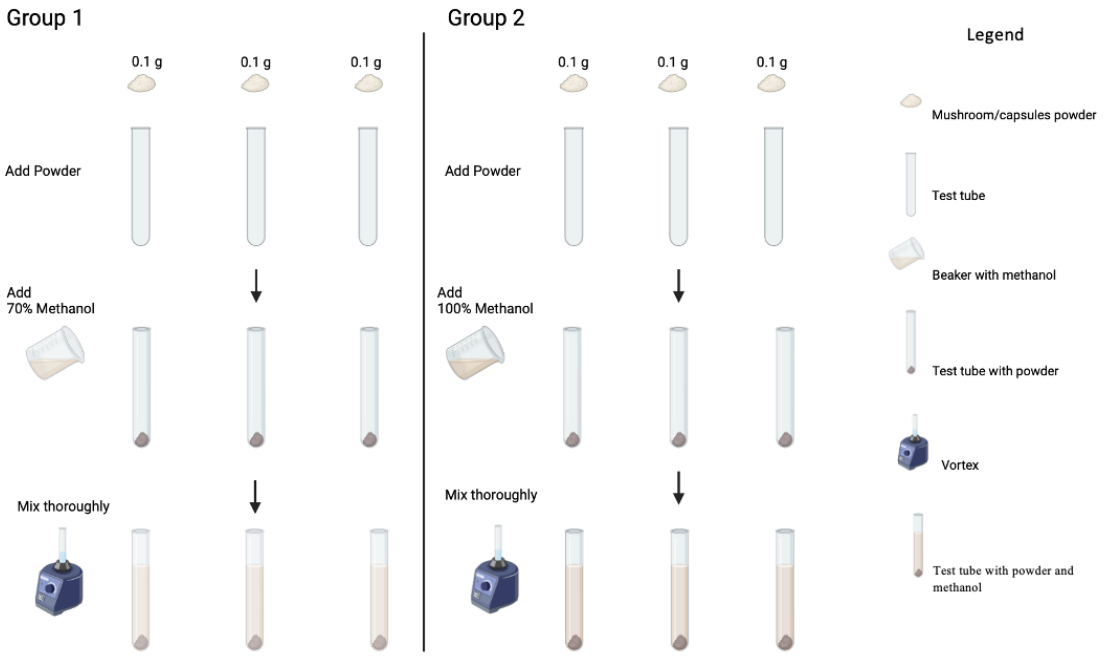


Figure 4-2. The phenolic extraction of mushroom samples using two methanol concentrations.

The homogenate was incubated at 60°C, for 24hr, using a Microplate Shaker (VWR International, Mississauga, CA) with intermittent shaking of 500 rpm. After 24 hrs the samples were centrifuged at 5000 rpm for 20 min at 4°C (Sorvall™ Legend™ Micro 17R Microcentrifuge, Thermo Fisher Scientific, Ottawa, CA). The supernatant, comprised of methanol and polyphenols, was collected, and the precipitate was subjected to two additional extractions using the same procedure. Overall, three extractions were conducted for each sample in an attempt to maximize the extractions of all of the methanol-soluble polyphenols within a sample. The final extract was about 1 mL, which was preserved at -80°C for subsequent analyses. The commercial mushrooms extracted at 70% methanol showed minimal phenolic content during the extraction process. Therefore, this experiment completed the extraction of phenolics from commercial mushroom capsules using only 100% methanol concentration.

4.3 Phenolic Content in Mushrooms

4.3.1 Total phenolic content (TPC)

The most commonly used assay for the determination of TPC in mushrooms is the Folin-Ciocalteu method (Chun et al., 2003; Lee et al., 2004; Lawag et al., 2023, Fadairo et al., 2021). In this study, 40 µL of the mushroom sample extracts were added to 40 µL of Folin-Ciocalteu reagent (Fisher Scientific, Ottawa, ON, Canada) and 120 µL of deionized water into a Corning 9017 96-well microplate (Fisher Scientific, Ottawa, ON, CA). After incubating the mixture at 25°C for 5 mins, 40 µL of sodium carbonate (Na_2CO_3) solution was added, Na_2CO_3 can bring the pH level to basic conditions, because the phenolic compounds only react with the Folin-Ciocalteu reagent in basic conditions (Pérez et al., 2023). And the mixture was incubated at room temperature in the dark for 60 mins. Then, a microplate reader (Bio-Tek Powerwave XS, New England, VT, USA)

was used to measure the absorbance at 640 nm. The TPC values were calculated from the calibration curve of gallic acid, which uses a TPC standard solution of 1000 mg/mL (Fisher Scientific, Ottawa, CA). TPC values are expressed as GAE in milligrams per gram (mg/g) of dry weight (DW) of the sample.

4.3.2 Total flavonoid content (TFC)

The total flavonoid content (TFC) assay used in this study has been slightly modified from the method of Zhishen et al. (1999). In this study, we changed the standard from rutin to quercetin, because quercetin has higher antioxidant activities than rutin (Nguyen et al., 2013). Measured by the colorimetric method, 25 µL of the mushroom extract was added into a Corning 9017 96-well microplate (Fisher Scientific, Ottawa, ON, Canada) with 100 µL of deionized water. Then, 7.5 µL of 5% (w/v) sodium nitrite ($NaNO_2$) was added. After incubating at room temperature (25°C) for 6 min, 7.5 µL of 10% (w/v) aluminum chloride ($AlCl_3$) was added followed by incubation of the mixture for 5 min, then the addition of 50 µL of 1 M sodium hydroxide (NaOH) to the mixture. Subsequently, a microplate reader (Bio-Tek Powerwave XS, New England, VT, USA) was used to measure the absorbance at 510 nm. TFC used quercetin as a standard for the calibration curve (Fisher Scientific, Ottawa, CA). TFC values are expressed as quercetin equivalents (QE) in milligrams per gram (mg/g) of DW of the sample.

4.4 Antioxidant Activity Assays in Mushrooms

4.4.1 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity

The radical scavenging power of the extracted solution was evaluated using the DPPH method described by Nandasiri et al. (2019). In summary, 290 µL of 0.05 mM DPPH radical solution (Fisher Scientific, Ottawa, ON, Canada) was added to 10 µL of the mushroom extract

solution, and the reaction carried out at room temperature (25°C) in the dark for 5 min. A microplate reader (Bio-Tek Powerwave XS, New England, VT, USA) was used to measure the absorbance at 516 nm.

Methanol was used as the blank in the microplate. The free radical scavenging activity was calculated as follows:

Scavenging effect (%) = [(the absorbance of the solvent control - the absorbance of the sample) × 100] / the absorbance of the solvent control.

4.4.2 Ferric ion-reducing antioxidant power (FRAP)

The ferric ion-reducing/antioxidant power of the mushroom extracts was measured using the modified method of Dharmishtha et al. (2009). The reagents for FRAP were prepared by mixing 25 mL of phosphate buffer (300 mM, pH = 3.6), 2.5 mL of iron (III) chloride hexahydrate ($FeCl_3$) (0.1%), and 2.5 mL of 2,4,6-tripyridyl-S-triazine (TPTZ) solution (10 mL, 40 mM hydrogen chloride (HCl)). In this experiment, 90 µL of FRAP solution was mixed with 3 µL of mushroom extract and 90 µL of deionized water, and the mixture was incubated for 8 min in a dark place at room temperature. Finally, the absorbance of the mixture was measured at 593 nm using a microplate reader (Bio-Tek Powerwave XS, New England, VT, USA). The reducing power of the mushroom extracts was measured using Trolox solution (1.0 mM) for the standard curve, and activity was expressed as moles of Trolox equivalent per gram of dried sample (M TE/g DW).

4.4.3 Ferrous-ion-chelating activity assay antioxidant capacity

The chelating activity of the metals within the mushroom extracts was determined according to the method used by Dharmishtha et al.(2004). For this study, 10 µL of mushroom extracts were mixed with 50 µL of 2.0 mM iron (II) chloride hexahydrate ($FeCl_2$) and 20 µL of 5.0 mM

ferrozine solution and added to a Corning 9017 96-well microplate (Fisher Scientific, Ottawa, ON, Canada). In a microplate, the total volume was adjusted to 4 mL with deionized water and then incubated for 10 min at room temperature (25°C). Methanol was used as the blank in the microplate. Methanol was used as the blank in the microplate. The absorbance was measured at 562 nm using a microplate reader (Bio-Tek Powerwave XS, New England, VT, USA). The results were compared with disodium ethylenediaminetetraacetate dihydrate solution (Na_2EDTA, 1.0mM, Fisher Scientific, Ottawa, ON, Canada) as a standard.

4.4.4 Total antioxidant capacity (TAC) assay

Total antioxidant capacity (TAC) is used to assess the response of antioxidants in mushroom samples to free radicals produced in specific diseases. In this experiment, the TAC assay kit (Project No. Cay709001-96; Cayman Chemicals, Ann Arbor, Michigan, USA) was used to assess the total antioxidant capacity in mushroom samples by detecting the conversion capacity of Cu^{2+} and Cu^{+}. The assay kit included antioxidant assay buffer (10X), antioxidant assay chromogen, antioxidant assay metmyoglobin, and antioxidant assay Trolox. When performing the assay, 10 µL of samples was added to 10 µL of metmyoglobin and 150 µL of chromogen. Methanol was used as the blank in the microplate. The assay was standardized on Trolox (1.0 mM), and the absorbance was measured at 570 nm using a microplate reader (Bio-Tek Powerwave XS, Michigan, USA). TAC values are expressed as Trolox equivalents (TE) in millimoles of Trolox per gram (mM TE/g) of dry weight (DW) of the sample.

4.5 Profiling of Phenolic Compound by High-performance Liquid Chromatography (HPLC) Analysis

The major phenolic compounds present in mushrooms were identified using high-performance liquid chromatography with Vanquish™ diode-array detection (HPLC-DAD) (Thermo Fisher Scientific, Ottawa, Canada). Some modifications were made according to the HPLC analysis of Abellan et al. (2021). In this experiment, a Kinetex® Biphenyl C18 100 RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, USA) was used for the chromatographic separation of phenolic compounds in mushroom extract. The flow rate was 0.8 mL/min and the injection volume was 20 μL. The gradient flow consisted of mobile phase A (1% formic acid in water) and mobile phase B (acetonitrile). The gradient was employed to achieve a composition of 25% B after 25 min and 60% B after 40 min. The ionization conditions were set at 350°C for capillary temperature and 4 kV for voltage. The nebulizer pressure was maintained at 60 psi, and the nitrogen flow rate was set to 11 L/min. Full scan mass spectra covered the range from m/z 100 to 1000. The phenolic compounds within the mushroom samples and quantified by photodiode array (PDA) chromatograms (330 nm and 270 nm). The standards sinapinic acid (Thermo Fisher Scientific, Ottawa, Canada) and quercetin-3-rutinoside (Thermo Fisher Scientific, Ottawa, Canada) were used.

4.6 Lipid Peroxidation Assay and In Vitro Blood Pressure Assessment

4.6.1 Thiobarbituric acid reactive substances (TBARS)

TBARS was used in this experiment to detect lipid peroxidation products in mushroom samples. This experiment was modified from the assay of Kumar et al. (2018), where the 0.2 mM malondialdehyde (MDA) standard was prepared as a reference along with diluted standards. MDA

reacted with thiobarbituric acid (TBA) to form a pink chromogen. Finally, a microplate reader (Bio-Tek Powerwave XS, New England, VT, USA) was used to measure the absorbance at 535 nm. TBARS values are expressed as MDA in nanomoles per gram (nmol/g) of dry weight (DW) of the sample (0.1 g per each).

4.6.2 In Vitro Angiotensin-converting enzyme (ACE) Inhibitor Assay

To evaluate the potential blood pressure lowering effect of the mushroom extracts, an angiotensin-converting enzyme (ACE) inhibitor assay was performed using angiotensin-converting enzyme from rabbit lung (Sigma-Aldrich, Burlington, Massachusetts, USA). The ACE inhibitory activities of fresh and commercial mushroom samples were evaluated using the methods of Cinq-Mars et al. (2007), with some modifications by Nandasiri et al. (2019), the spectrophotometric absorbance used 360 and 500 nm instead of 228 nm. Within 2 mL tubes, 21 µL of mushroom sample extract was added to 150 µL substrate Hippuryl-L-Histidyl-L-Leucine (HHL). Then, 30 µL ACE was added to each tube and the mixture was incubated at 37°C in a Symphony oven (VWR International, Radnor, USA) for an hour. Three control groups were prepared for comparison to the mushroom samples. Control 1 tube was 21 µL captopril mixed with 150 µL HHL and 30 µL ACE enzyme. Control 2 tube was 21 µL captopril mixed with 150 µL HHL and 30 µL buffer. Control 3 tube was 21 µL buffer mixed with 150 µL HHL and 30 µL enzyme. The blank unit was 201 µL buffer which was equal to the total volume of each sample unit. After 1 hour of incubation of the samples and control groups, 150 µL of 0.35 mM NaOH solution was added into each tube to stop the enzymatic reaction. This was followed by addition of 100 µL *O*-phaldiadehyde into each tube to make the fluorescent adduct. The solution was kept at room temperature for 15 min, and then 50 µL of 3 M HCl solution was added into the tube to stop the reaction. Finally, 100 µL aliquot from each sample was added to a 96-well plate and

fluorescence measured by BioTek multi-mode reader (Thermo Fisher Scientific, Ottawa, Canada) at excitation (360 nm) and emission (500 nm). ACE values are expressed as percentage (%) of the sample.

4.7 Statistical Analysis

To compare the phenolic compound content, antioxidant capacity and anti-hypertensive activity among common edible fresh mushrooms and commercial mushrooms, data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, using Python programming language version 3.11.9 (Python Software Foundation, New York, US). The two main factors were concentrations of MeOH and mushroom types. Three groups were analyzed:

1. Two-way ANOVA was used to analyze TPC, TFC and antioxidant activity of fresh mushrooms extracted at different concentrations of MeOH (70% and 100%).
2. Two-way ANOVA was used to analyze TPC, TFC, antioxidant activity and in vitro assay for blood pressure parameters of three different fresh mushrooms and their corresponding commercial mushrooms extracted at the same concentration of MeOH (100%).
3. Two-way ANOVA was used to analyze the antioxidant activity of different mushroom storage duration period (Fresh, 5 years old and 10 years old) of Chaga with the highest phenolic compound content at different concentrations of of MeOH (70% and 100%).

In this study, a $p<0.05$ was set significant difference level between the samples. The data were presented as mean ± standard error of mean (SEM).

Chapter 5 Results Section

5.1 Total Phenolic and Flavonoid Contents in Mushrooms

The antioxidant properties of mushroom extracts are expected to be related to their concentrations of total phenolic and flavonoid compounds; thus, this study examined the concentrations of TPC and TFC within the mushroom extracts. Tables 5-1 and 5-2 show the results of the total polyphenols and total flavonoids concentrations, respectively, in five different types of mushroom samples.

The effects of mushroom species and extraction methods on TPC were tested. The methanol concentration had a significant effect on TPC levels ($p=0.002$), showing 100% methanol increased three times higher TPC in comparison to the 70% methanol. The mushroom species also had a significant effect on TPC levels ($p=0.0001$), showing Chaga containing the highest and whit button mushroom having the lowest. An interaction effects existed between the methanol concentration and the mushroom type ($F(4,10) = 6.61$, $p = 0.007$). In summary, as shown in Table 5-1, the highest TPC values amongst the fresh mushroom types were shown for Chaga in 100% concentration of methanol extraction, measuring at 6.98 ± 0.75 mg GAE/g DW, which is significantly higher than Chaga in 70% concentration of methanol extraction, measuring at 2.16 ± 0.03 mg GAE/g DW. The lowest TPC values were for the white button mushroom, where samples extracted in 100% methanol concentration, had 0.31 ± 0.01 mg GAE/g DW, which is significantly higher than those extracted using 70% methanol concentration; measured at 0.13 ± 0.01 mg GAE/g DW.

Table 5-1 also shows the effect of mushroom forms (fresh mushroom and commercial capsulated mushroom extracts) and mushroom species; on TPC levels when extracted with 100% methanol. A two-way ANOVA determined that fresh mushrooms were statistically significantly

different from capsulated mushroom extracts ($F(2,12) = 244.03$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) had a significant effect on TPC levels ($p = 0.01$), and the type of mushroom also had a significant effect on TPC levels ($p < 0.0001$). Post hoc comparisons showed that TPC levels significantly differ ($p<0.05$) between the fresh and capsulated mushroom types (Table 5-1). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that fresh Chaga extracted with 100% methanol continued to exhibit the highest TPC value, measured at 6.98 ± 0.75 mg GAE/g DW, which is significantly higher than commercial capsulated Chaga, measured at 1.51 ± 0.06 mg GAE/g DW. However, the lowest TPC levels were observed in white button mushrooms extracted with 100% methanol with a value of 0.31 ± 0.01 mg GAE/g DW, which is significantly lower than commercial capsulated white button samples, measured at 1.01 ± 0.01 mg GAE/g DW.

Table 5-1. Total phenolic contents (mg GAE/g DW) of mushroom samples

Forms	**Fresh**	**Fresh**	**Capsulated**		
Extraction **Mushroom Species**	**70% MeOH**	**100% MeOH**	**100% MeOH**	**Effects of (P-value[ş])**	**Effects of (P-value[ɣ])**
White button	0.13 ± 0.01^{c}	0.31 ± 0.01^{cg}	1.01 ± 0.01^{g}	Mushroom species: $p<0.0001$ MeOH: $P = 0.002$ Mushroom* MeOH: $P = 0.007$	Species $p<0.0001$ Forms $P = 0.01$ Species * Form $p<0.0001$
Shiitake	0.25 ± 0.01^{c}	0.36 ± 0.03^{cg}	3.61 ± 0.24^{f}		
Chanterelle	0.20 ± 0.01^{c}	0.53 ± 0.01^{cg}	n/a		
Portobello	0.20 ± 0.01^{c}	2.03 ± 1.59^{bf}	n/a		
Chaga	2.16 ± 0.03^{b}	6.98 ± 0.75^{ae}	1.54 ± 0.06^{g}		

Results expressed as mean ± SEM (n=3). [ş]Two-way ANOVA was tested to test the effects of mushroom types and methanol (MeOH) concentration for extraction.. [ɣ]Two-way ANOVA was used to test the effects of mushroom species and mushroom forms(fresh and capsulated samples). Both two way ANOVA was followed by the Tukey test for multiple comparisons, at the level of $p<0.05$. [a-c] Means in the 70% and 100% MeOH columns with different letters are significantly

different, [e-g] Means in the fresh (100% MeOH) and capsulated columns with different letters are significantly different n/a: not applicable, DW: dry weight, GAE: gallic acid equivalents

The effects of mushroom species and extraction methods on TFC were tested. The results revealed that TFC values exhibited similar concentration patterns to TPC values (Table 5-2). Specifically, two-way ANOVA analysis of the effect of methanol concentration and fresh mushroom type on TFC values revealed that there was a significant interaction between the methanol concentration and the mushroom type ($F(4,10)=25801.50$, $p <0.0001$). Simple main effects analyses showed that the methanol concentration has a significant effect on TFC levels ($p <0.0001$), and the type of mushroom also had a significant effect on TFC levels ($p < 0.0001$). Post hoc comparisons using the Tukey HSD test showed that TFC levels significantly differ ($p<0.05$) between the mushroom types (Table 5-2). In summary, as shown in Table 5-2, for comparison of fresh mushroom samples, Chaga displayed the highest TFC levels after extraction with 100% methanol with a value of 8.23 ± 0.36 mg QE/g DW, which is significantly higher than the 70% methanol extract (3.21 ± 0.05 mg QE/g DW). The lowest TFC levels were observed in white button mushrooms, with a value of 0.25 ± 0.01 mg QE/g DW after 100% methanol extraction, which is significantly higher than the 70% methanol extract at 0.13 ± 0.01 mg QE/g DW.

As shown in Table 5-2, a two-way ANOVA determined that fresh mushrooms were statistically significantly different from capsulated mushroom extracts ($F (2,6) = 6374.80$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) had a significant effect on TFC levels ($p < 0.0001$), and the type of mushroom also had a significant effect on TFC levels ($p < 0.0001$). Post hoc comparisons using the Tukey HSD test showed that TFC levels significantly differ ($p<0.05$) between the fresh and capsulated mushroom types (Table 5-2). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that fresh Chaga continued to exhibit the highest TFC value after

100% methanol extraction (8.23 ± 0.36 mg QE/g DW), which is significantly higher than Chaga commercial samples (1.58 ± 0.21 mg QE/g DW). The lowest TFC levels were observed in white button mushrooms, measuring 0.25 ± 0.01 mg QE/g DW after 100% methanol extraction, significantly lower than white button commercial samples (1.07 ± 0.04 mg QE/g DW).

Table 5-2. Total flavonoid contents (mg QE/g DW) of mushroom samples

Forms	**Fresh**	**Fresh**	**Capsulated**		
Extraction **Mushroom Species**	**70% MeOH**	**100% MeOH**	**100% MeOH**	**Effects of (P-value[§])**	**Effects of (P-value[γ])**
White button	0.13 ± 0.01^{b}	0.25 ± 0.01^{bg}	1.07 ± 0.04^{g}	Mushroom species: p<0.0001 MeOH: p<0.0001 Mushroom* MeOH: p<0.0001	Species p<0.0001 Forms p<0.0001 Species * Form p<0.0001
Shiitake	0.20 ± 0.02^{b}	0.28 ± 0.05^{bg}	1.66 ± 0.12^{f}		
Chanterelle	0.23 ± 0.03^{b}	0.39 ± 0.03^{bg}	n/a		
Portobello	0.22 ± 0.01^{b}	0.37 ± 0.04^{bg}	n/a		
Chaga	3.21 ± 0.05^{a}	8.23 ± 0.36^{ae}	1.58 ± 0.21^{f}		

Results are expressed as mean ± SEM (n=3); [§]Two-way ANOVA was used to compare samples extracted with 70% methanol vs. 100% methanol. [γ]Two-way ANOVA was used to compare fresh and capsulated samples. [a-b]Means in the 70% and 100% MeOH column with different letters are significantly different ($p < 0.05$; Tukey test); [e-g]Means in the 70% and 100% MeOH column with different letters are significantly different ($p < 0.05$; Tukey test); n/a, not applicable; DW, dry weight; QE, quercetin equivalents

5.2 Phenolic Profile in Mushrooms

In the current study, we aimed to characterize phenolic compounds in mushroom species by using HPLC methodology. The phenolic compounds chlorogenic acid, isoquercetin, and kampferol were detected in fresh Chaga extracts, but the levels were too low to be identified within other mushroom samples. Specifically, Chaga's phenols extracted with 100% methanol contained 1.02 μg/g DW of chlorogenic acid, 0.42 μg/g DW of isoquercetin, and 0.23 μg/g DW of kampferol. Additionally, Chaga extracted with 70% methanol contained 0.92 μg/g DW of chlorogenic acid, 0.22 μg/g DW of isoquercetin, and 0.15 μg/g DW of kampferol.

5.3 Antioxidant Activities in Phenolic Fractions of Mushrooms

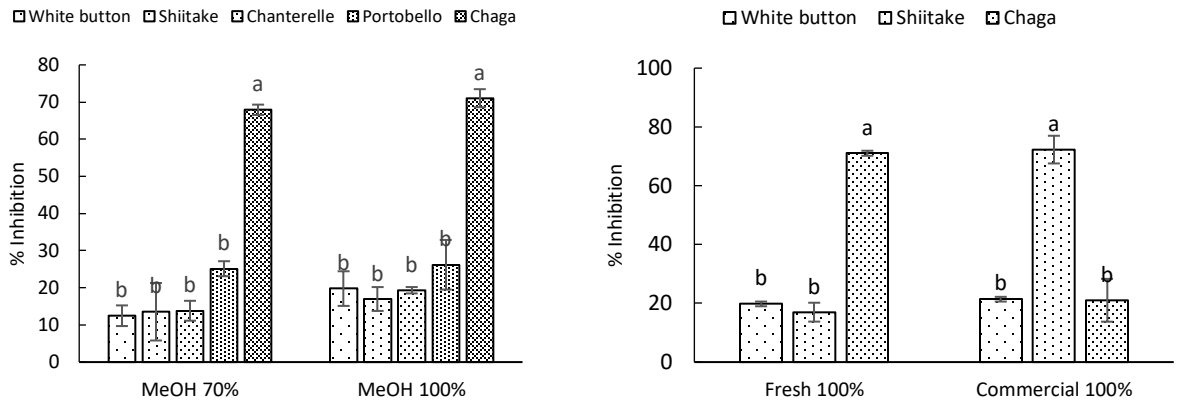
Since phenolic compounds are a collective of compounds with different structures and active sites, each compound may have different mechanisms of action for antioxidant activity. Thus, to assess the antioxidant capacity of the mushroom samples, this study employed various methods, that assess different antioxidant mechanisms, including DPPH, FRAP, Ferrous-ion-chelating activity, and TAC assays. The results of these assessments are depicted in Figure 5-1 to 5-4.

5.3.1 DPPH radical-scavenging activity of mushrooms

A two-way ANOVA was performed to analyze the effect of the methanol concentration and fresh mushroom species on the DPPH radical-scavenging activity of mushrooms, and the results revealed that there was a significant interaction between the methanol concentration and the mushroom type ($F(4,10)=365.18$, $p < 0.0001$). Simple main effects analyses showed that the methanol concentration has significant effect on DPPH levels ($p < 0.0001$), and the type of mushroom also had a significant effect on DPPH levels ($p < 0.0001$). Post hoc comparisons

using the Tukey HSD test showed that DPPH radical-scavenging activity significantly differed ($p < 0.05$) between the mushroom types (Figure 5-1 (a)). In summary, as shown in Figure 5-1 (a), the highest DPPH activity values amongst the fresh mushroom types were shown for Chaga after extraction with 100% methanol, measuring at 71.03 ± 2.43%, which is higher than Chaga extracted with 70% methanol, measuring at 69.65 ± 1.39%. The lowest DPPH activity values were for the shiitake mushroom, where samples extracted with 100% methanol, measured at 16.98 ± 3.21%, and were significantly higher than those extracted using 70% methanol, which measured at 12.12 ± 7.73%.

Figure 5-1 (b) shows the mushroom form and mushroom species on DPPH. to commercial capsulated mushroom extracts. A two-way ANOVA determined that fresh mushrooms were statistically significantly different from capsulated mushroom extracts ($F(2,6) = 571.15$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) had a significant effect on DPPH levels ($p < 0.05$), and the type of mushroom also had a significant effect on DPPH levels ($p < 0.0001$). Post hoc analysis with Tukey HSD revealed that DPPH activity levels significant differences ($p < 0.05$) within fresh mushrooms and capsulated mushrooms respectively (Figure 5-1 (b)). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that shitake commercial samples exhibited the highest DPPH radical scavenging activity, measured at 72.27 ± 7.29%, which is significantly higher than shitake mushrooms extracted with 100% methanol, and having a value of 16.98 ± 3.21%. The lowest DPPH scavenging levels were observed in white button mushrooms, measuring 19.79 ± 4.66% for the 100% methanol extract, which is significantly lower than white button commercial samples with 21.39 ± 4.71%.



(a) (b)

Figure 5-1. The antioxidant activity as measured by DPPH radical scavenging activity in the phenolic–rich fractions from (a) five different fresh mushrooms with phenolic extracted with 70% methanol vs. 100% methanol, and (b) fresh mushrooms and corresponding commercial capsules with phenolic extracted with 100% methanol.

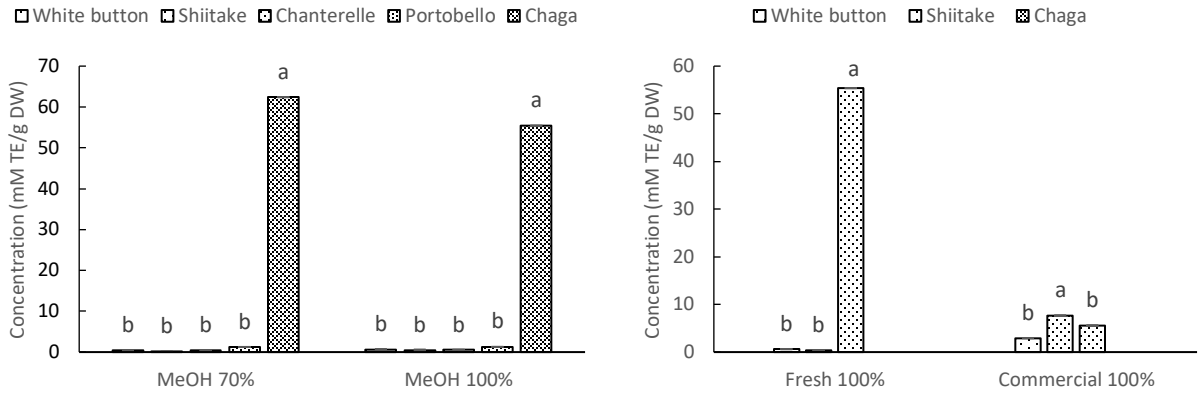
The bars represent means ± SEM (n = 3). (a) The different letters for different mushrooms in 70% and 100% methanol concentrations indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). (b) The different letters in fresh and commercial capsules indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). MeOH 70%, 70% methanol extract; MeOH 100%, 100% methanol extract; Fresh 100%, fresh mushroom extracted with 100% methanol; commercial 100%, commercial capsulated mushroom extracted with 100% methanol; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

5.3.2 Ferric-reducing antioxidant power of mushrooms

The FRAP results, as depicted in Figure 5-2a, showed that the antioxidant capacity of the mushroom extracts ranged from 0.04 to 62.41 M TE /g DW. Two-way ANOVA analysis of the effect of methanol concentration and fresh mushroom type on FRAP values revealed that there was a significant interaction between the methanol concentration and the mushroom type ($F(4,10) = 7.00$, $p = 0.006$). Simple main effects analyses showed that the methanol concentration ($p=0.045$) and type of mushroom ($p < 0.0001$) have significant effects on FRAP levels. Post hoc comparisons using the Tukey HSD test showed that FRAP value significantly differed ($p < 0.05$)

between the mushroom types (Figure 5-2a). As shown in Figure 5-2a, fresh Chaga extract exhibited the highest FRAP value (62.41 M TE/g DW) after 70% methanol extraction and 55.39 M TE/g DW for the 100% methanol extract among all the mushroom extracts.

Figure 5-2 also showed the comparison of fresh mushroom samples to commercial capsulated mushroom extracts. A two-way ANOVA determined that fresh mushrooms were statistically significantly different from capsulated mushroom extracts ($F(2,6) = 577.16$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) had a significant effect on FRAP levels ($p < 0.0001$), and the type of mushroom also had a significant effect on FRAP levels ($p < 0.0001$). Post hoc analyses using Tukey HSD showed that there was a significant difference in FRAP levels between fresh and capsule mushrooms, respectively ($p < 0.05$) (Figure 5-2b). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that fresh Chaga mushrooms exhibited the highest FRAP value, measured at 55.39 M TE/g DW, which is significantly higher than Chaga capsulated mushrooms, measuring 5.55 M TE/g DW. The lowest FRAP values were observed in fresh shiitake mushrooms, measuring 0.43 M TE/g DW for the 100% methanol extract, which is significantly lower than shiitake commercial samples, measured at 7.65 M TE/g DW.



(a) (b)

Figure 5-2. The antioxidant activity as measured by the Ferric-reducing antioxidant power (FRAP) antioxidant assay in the phenolic–rich fractions from (a) five different fresh mushrooms with phenolic extracted with 70% methanol vs. 100% methanol, and (b) fresh mushrooms and commercial capsules with phenolic extracted with 100% methanol.

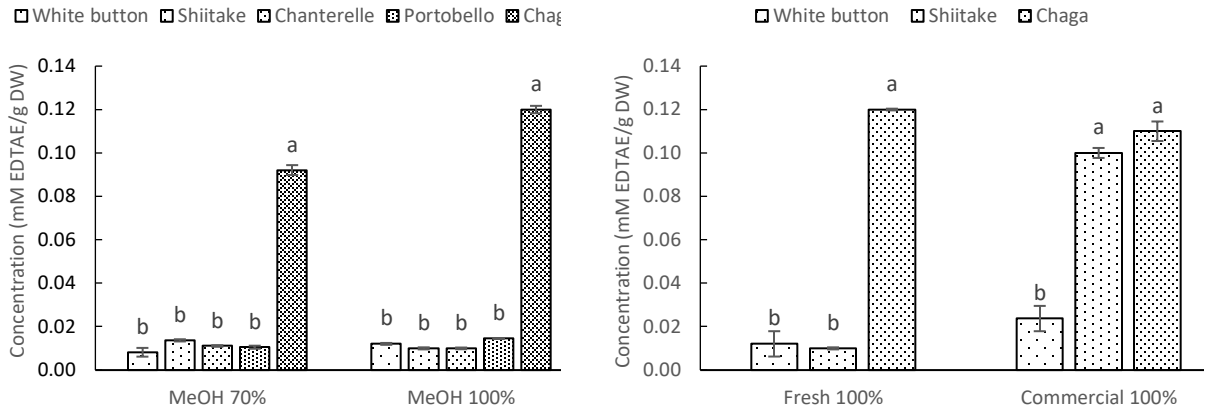
The bars represent means ± standard error of the mean (n = 3). (a) The different letters for different mushrooms in each methanol concentration indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). (b) The different letters in fresh and commercial capsules indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). mM, millimoles; g, gram; TE, Trolox equivalent; DW, dry weight; MeOH 70%, 70% methanol extract; MeOH 100%, 100% methanol extract; Fresh 100%, fresh mushroom extracted with 100% methanol; commercial 100%, commercial capsulated mushroom extracted within 100% methanol ; FRAP, ferric reducing antioxidant power.

5.3.3 Ferrous-ion-chelating activity of mushrooms

Two-way ANOVA analysis of the effect of methanol concentration and fresh mushroom type on Ferrous-ion-chelating activity values revealed that there was a significant interaction between the methanol concentration and the mushroom type ($F(4,10) = 110.94$, $p < 0.0001$). Simple main effects analyses showed that the methanol concentration and the type of mushroom had significant effects on Ferrous-ion-chelating activity levels ($p < 0.0001$). Post hoc compare sons using the Tukey HSD test showed that Ferrous-ion-chelating activity value significantly differed ($p < 0.05$) between the mushroom types (Figure 5-3a). As shown, Chaga extracted with

100% methanol displayed the highest Ferrous-ion-chelating activity, measuring 0.12 mM EDTAE/g DW, which is significantly higher than the 70% methanol extract with 0.09 mM EDTAE/g DW. The white button, shiitake, chanterelle, and Portobello mushrooms had similar Ferrous-ion-chelating activity levels, measuring 0.01 mM EDTAE/g DW after 100% and 70% methanol extractions.

Figure 5-3b compared fresh mushroom samples to commercial capsulated mushroom extracts. A two-way ANOVA determined that fresh mushrooms were statistically significantly different from capsulated mushroom extracts ($F(2,6) = 756.88$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) and the type had significant effects on Ferrous-ion-chelating activity levels ($p < 0.0001$). Post hoc analysis with Tukey HSD revealed that Ferrous-ion-chelating activity levels significant differences ($p < 0.05$) within fresh mushrooms and capsulated mushrooms respectively (Figure 5-3b). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that fresh Chaga mushrooms exhibited the highest Ferrous-ion-chelating activity value, measured at 0.12 mM EDTAE/g DW, which is higher than Chaga capsulated mushrooms, measuring 0.11 mM EDTAE/g DW. The lowest Ferrous-ion-chelating activity values were observed in fresh white button mushrooms, measuring 0.01 mM EDTAE/g DW after 100% methanol extraction, which is lower than white button commercial samples, measured at 0.02 mM EDTAE/g DW.



(a) (b)

Figure 5-3. The antioxidant activity as measured by the chelating ability of the metals in the phenolic–rich fractions from (a) five different fresh mushrooms with phenolics extracted with 70% methanol vs. 100% methanol, and (b) fresh mushrooms and commercial capsules with phenolics extracted with 100% methanol.

The bars represent means ± standard error of the mean (n = 3). (a) The different letters for different mushrooms in each methanol concentration indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). (b) The different letters in fresh and commercial capsules indicate significant differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). mg, milligram; g, gram; EDTA, ethylenediaminetetraacetic acid; DW, dry weight; MeOH 70%, 70% methanol extract; MeOH 100%, 100% methanol extract; Fresh 100%, fresh mushroom 100% methanol extract; commercial 100%, commercial capsulated mushroom 100% methanol extract.

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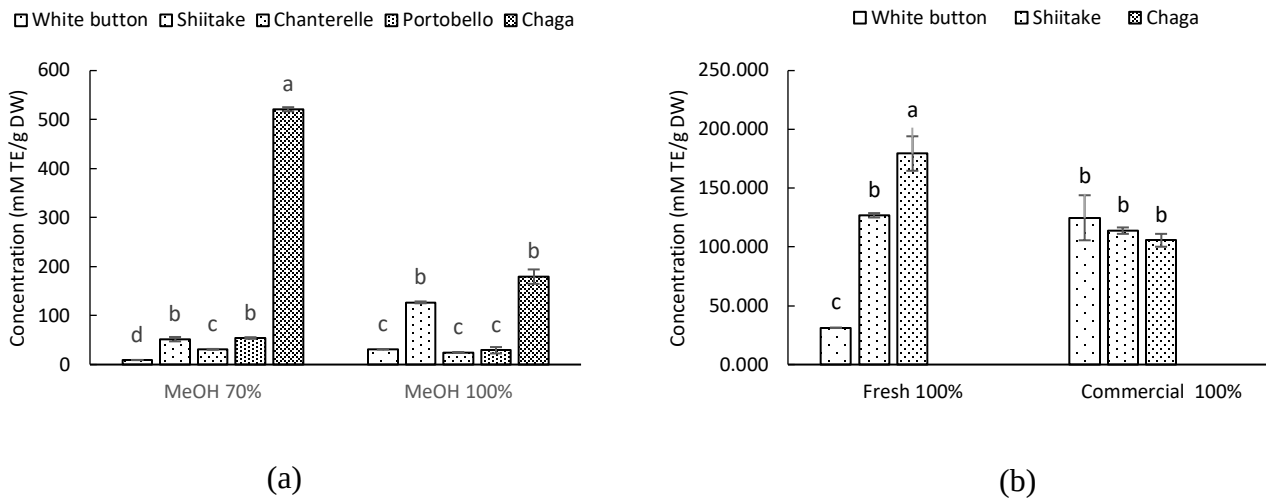
5.3.4 Total Antioxidant Capacity of Mushrooms

Two-way ANOVA analysis of the effect of methanol concentration and fresh mushroom type on total antioxidant capacity values revealed that there was a significant interaction between the methanol concentration and the mushroom type ($F(4,10)=419.05$, $p < 0.0001$). Simple main effects analyses showed that the methanol concentration and the type of mushroom had a significant effect on total antioxidant capacity levels ($p < 0.0001$). Post hoc comparisons using the Tukey HSD test showed that total antioxidant capacity value significantly differed ($p < 0.05$)

between the mushroom types (Figure 5-4a). As shown, Chaga displayed the highest total antioxidant capacity levels when extracted with 70% methanol with a value of 521.05 ± 4.19 mM TE/g DW, which is significantly higher than Chaga extracted with 100% methanol and measuring at 192.01 ± 14.67 mM TE/g DW. The white button has the lowest total antioxidant capacity levels, measuring 9.24 ± 0.21 mM TE/g DW for the 70% methanol extract, which is significantly lower than white button mushrooms extracted with 100% methanol and measuring 31.26 ± 0.21 mM TE/g DW.

Figure 5-4b compared fresh mushroom samples to commercial capsulated mushroom extracts. A two-way ANOVA determined that fresh mushrooms were significantly different from capsulated mushroom extracts ($F(2,6) = 34.57$, $p < 0.05$). Simple main effects analyses showed that the methanol concentration ($p < 0.05$) and the type of mushroom ($p < 0.0001$) have significant effects on total antioxidant capacity levels. Post hoc analysis with Tukey HSD revealed that total antioxidant capacity levels of fresh mushrooms and capsulated mushrooms were significantly different ($p < 0.05$) as shown in Figure 5-4b. In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that Chaga mushrooms from 100% methanol, measured at 192.01 ± 14.67 mM TE/g DW, which is significantly higher than Chaga capsulated samples, measured at 105.66 ± 5.47 mM TE/g DW. The lowest total antioxidant capacity values were observed in fresh white button mushrooms, measuring 31.26 ± 0.21 mM TE/g DW after 100% methanol extraction, which is significantly lower than the white button capsulated samples, measuring 113.87 ± 19.15 mM TE/g DW.



(a) (b)

Figure 5-4. The antioxidant activity as measured by the 2,2'-azidobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay in the phenolic–rich fractions from (a) five different fresh mushrooms with phenolic extracted with 70% methanol vs. 100% methanol, and (b) fresh mushrooms and commercial capsules with phenolic extracted with 100% methanol.

The bars represent means ± SEM (n = 3). (a) The different letters for different mushrooms in each methanol concentration indicate significant differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). (b) The different letters in fresh and commercial capsules indicate significant differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). mM, millimoles; TE, Trolox; g, gram; DW, dry weight; MeOH 70%, 70% methanol extract; MeOH 100%, 100% methanol extract; Fresh 100%, fresh mushroom extracted with 100% methanol; commercial 100%, commercial capsulated mushroom extracted with 100% methanol.

5.4 Lipid Peroxidation and Angiotensin-Converting Enzyme Activity of Mushrooms

In Figure 5-5, a two-way ANOVA determined that fresh mushrooms were significantly different from capsulated mushroom extracts ($F(2,6) = 255.39$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) had a significant effect on lipid peroxidation activity levels ($p = 0.003$), and the type of mushroom also had a significant effect on lipid peroxidation activity levels ($p < 0.0001$). Post hoc analysis with Tukey HSD revealed that lipid peroxidation activity levels had significant differences ($p < 0.05$) within fresh mushrooms and capsulated mushrooms (Figure 5-5). In summary, a comparison of fresh mushrooms and commercial mushroom extracts revealed that fresh Chaga has the lowest lipid peroxidation response with the highest value at 2462.63 ± 152.44 nmol/g DW, which is significantly higher than Chaga capsulated mushrooms, measuring 379.10 ± 49.75 nmol/g DW, that means the Chaga capsulated mushrooms had the highest lipid peroxidation.

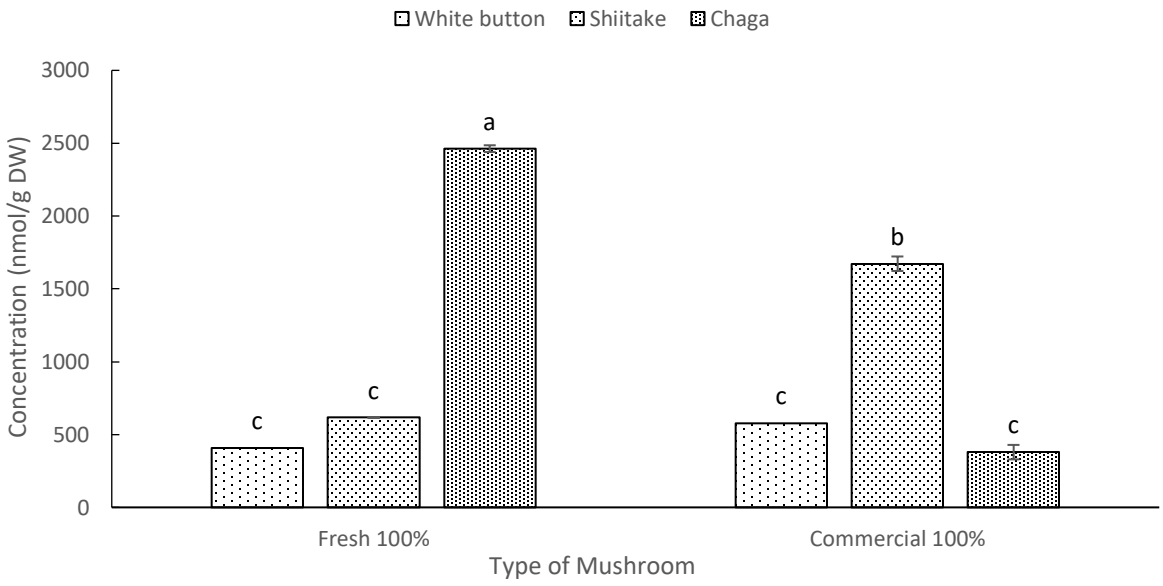


Figure 5-5. The lipid peroxidation activity as measured by thiobarbituric acid reactive substances (TBARS) assay in the phenolic–rich fractions from fresh mushrooms and commercial capsules with phenolic extracted with 100% methanol.

Mean ± SEM (n=3). The different letters in fresh and commercial capsules indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). Fresh 100%, fresh mushroom extracted with 100% methanol; commercial 100%, commercial capsulated mushroom extracted with 100% methanol; DW, dry weight.

In Figure 5-6, a two-way ANOVA determined that fresh mushrooms were significantly different from capsulated mushroom extracts ($F(2,12) = 134.67$, $p < 0.0001$). Simple main effects analyses showed that the mushroom forms (fresh compared to capsulated) and the type of mushroom had significant effects on angiotensin-converting enzyme inhibitory levels ($p < 0.0001$). Post hoc analysis with Tukey HSD revealed that angiotensin-converting enzyme activity levels had significant differences ($p < 0.05$) within fresh mushrooms and capsulated mushrooms (Figure 5-6). The highest angiotensin-converting enzyme inhibitory activity value was observed in shiitake capsulated mushrooms, measuring 47.03 ± 0.06%, which was significantly higher than fresh shiitake mushrooms, measuring 14.56 ± 3.57%. The lowest angiotensin-converting enzyme inhibitory activity values were observed in Chaga capsulated mushrooms, measuring 6.26 ± 1.25%, which is significantly lower than fresh Chaga mushrooms extracted with 100% methanol and measuring 39.16 ± 1.25%.

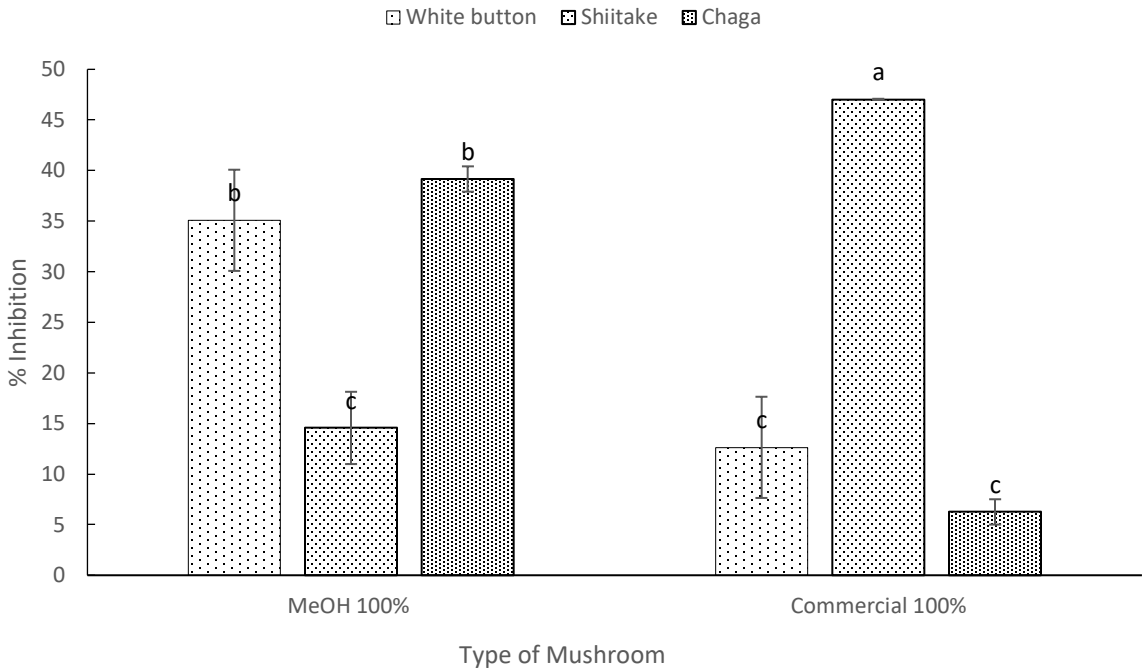


Figure 5-6. The *in vitro* inhibition of angiotensin-converting enzyme (ACE) by phenolic–rich fractions from fresh mushrooms and commercial capsules with phenolics extracted with 100% methanol.

Mean ± SEM (n=3). The different letters in fresh and commercial capsules indicate statistical differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). Fresh 100%, fresh mushroom extracted with 100% methanol; commercial 100%, commercial capsulated mushroom extracted with 100% methanol.

5.5 Chaga Storage Time and Its Total Phenolic and Flavonoid Contents

In Figure 5-7, a two-way ANOVA was performed to analyze the effect of the methanol concentration and storage duration of the Chaga mushrooms on TPC levels, and the results revealed that there was a significant interaction between methanol concentration and mushroom age ($F(2,6) = 14.29$, $p = 0.005$). Simple main effects analyses showed that the methanol concentration and age of Chaga mushrooms had significant effects on TPC levels ($p < 0.0001$). Post hoc comparisons using the Tukey HSD test showed that TPC levels significantly differ ($p<0.05$) between the mushroom ages (Figure 5-7). In summary, as shown in Figure 5-7, The highest TPC value was observed in fresh Chaga mushrooms, measuring 9.94 ± 0.61 mg GAE/g DW for the 100% methanol extract, which is significantly higher than fresh Chaga mushrooms extracted with 70% methanol, and measuring 3.82 ± 0.17 mg GAE/g DW. The lowest TPC values were found in 10-year-old Chaga mushrooms, with 2.09 ± 0.09 mg GAE/g DW for the 70% methanol extract, which is significantly lower than the 10-year-old Chaga mushrooms extracted with 100% methanol, and measuring 5.74 ± 0.83 mg GAE/g DW.

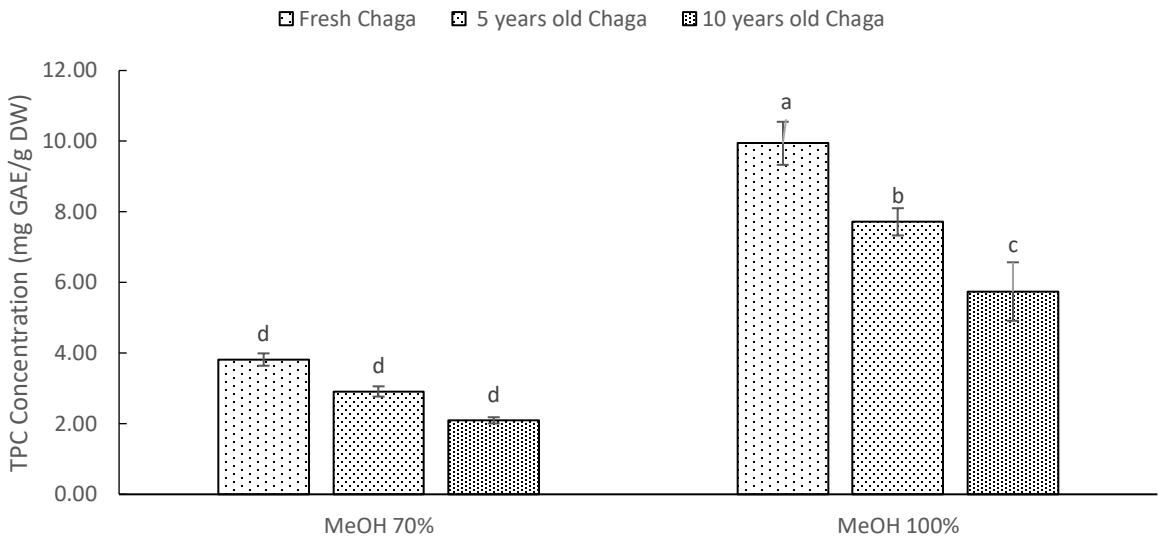


Figure 5-7. Total phenolic content (TPC) of Chaga at different mushroom storage time.

The bars represent mean ± SEM (n=3). Different letters between the different age groups of Chaga indicate significant differences according to two-way ANOVA and Tukey's post hoc test ($p < 0.05$) GAE: gallic acid equivalent; DW, dry weight; Extraction 70%, Chaga extracted with 70% methanol; Extraction 100%, Chaga extracted with 100% methanol.

The results indicated a similar concentration pattern for the TFC as was seen for TPC values, as illustrated in Figure 5-8. A two-way ANOVA was performed to analyze the effect of the methanol concentration and the age of the Chaga mushrooms on TFC levels, and the results revealed that there was a significant interaction between the methanol concentration and the mushroom age ($F(2,6) = 65.71$, $p < 0.0001$). Simple main effects analyses showed that the methanol concentration ($p < 0.05$) and the age of Chaga mushrooms ($p = 0.001$) had significant effects on TFC levels. Post hoc comparisons using the Tukey HSD test showed that TFC levels significantly differ ($p<0.05$) between the mushroom ages (Figure 5-8). In summary, as shown in Figure 5-8, the highest TFC value (6.39 ± 0.26 mg QE/g DW) was observed in fresh Chaga

mushrooms extracted with 100% methanol, which is significantly higher than fresh Chaga mushrooms extracted with 70% methanol, and measuring 5.71 ± 0.18 mg QE/g DW. The lowest TFC value was observed in 10-year-old Chaga mushrooms, measuring 2.02 ± 0.17 mg QE/g DW for the 100% methanol extract, which is significantly lower than 10 years old Chaga mushrooms extracted with 70% methanol, and measuring 3.70 ± 0.32 mg QE/g DW.

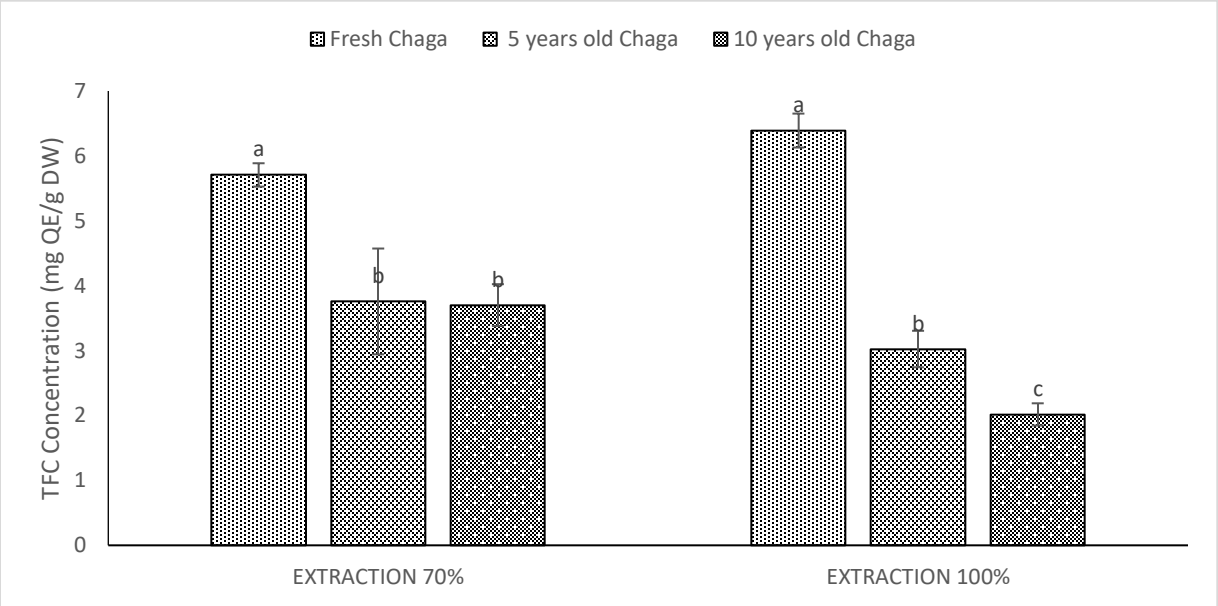


Figure 5-8. Total flavonoid content (TFC) of Chaga at different mushroom storage time.

Mean ± SEM (n=3); The different letters between the ages of Chaga indicate significant differences based on a two-way analysis of variance followed by the Tukey post hoc test ($p < 0.05$). QE, quercetin equivalent; DW, dry weight; Extraction 70%, Chaga extracted with 70% methanol; Extraction 100%, Chaga extracted with 100% methanol.

5.6 Chaga Storage Time and Its Antioxidant Activity

The antioxidant activity of Chaga mushroom extracts was assessed using four different spectrophotometric assays, and the summarized results can be found in Table 5-3. A two-way ANOVA was performed to analyze the effect of the methanol concentration and the storage time of the Chaga mushrooms on DPPH radical scavenging levels, and the results revealed that there was a significant interaction between the methanol concentration and the mushroom storage time ($F(2,6) = 104.08$, $p < 0.0001$). Simple main effects analyses showed that the methanol concentration and the storage time of Chaga mushrooms had significant effects on DPPH radical scavenging levels ($p < 0.0001$). Post hoc comparisons using the Tukey HSD test showed that DPPH levels significantly differ ($p<0.05$) between the Chaga mushroom storage time (Table 5-3). In summary, as shown in Table 5-3, the highest DPPH radical scavenging value was observed in fresh Chaga mushrooms, measuring 72.9 ± 0.30% for the 100% methanol extract, which is significantly higher than fresh Chaga mushrooms extracted with 70% methanol, and measuring 71.4 ± 0.64%. The lowest DPPH radical scavenging value was observed in 10-year-old Chaga mushrooms, measuring 68.2 ± 0.21% for the 70% methanol extract, which is lower than 10-year-old Chaga mushrooms extracted with 100% methanol, and measuring 68.6 ± 0.17%. Furthermore, in the DPPH, ABTS, and FRAP assays, the fresh Chaga emerged as the most active, indicating its strong antioxidant potential across multiple tests. There are significant differences among three different storage time of mushrooms in the same methanol extraction ($p < 0.05$). These results highlight the varying antioxidant activities of different Chaga mushroom extracts, with fresh Chaga extract standing out as the most active in multiple assays.

Table 5-3. Chaga mushrooms storage time and the association to its antioxidant activities

Extract	Storage duration	DPPH Assay Inhibition %	FRAP Assay (M TE/g DW)	Metal chelating assay (mM EDTAE/g DW)	ABTS Assay (mM TE/g)
70% MeOH	Fresh	71.36 ± 0.64^{a}	0.25 ± 0.00^{a}	0.06 ± 0.00^{b}	336.61 ± 8.72^{a}
	5 years old	69.97 ± 0.11^{b}	0.17 ± 0.00^{b}	0.02 ± 0.00^{c}	252.42 ± 10.72^{b}
	10 years old	68.23 ± 0.21^{b}	0.12 ± 0.03^{b}	0.08 ± 0.00^{a}	182.70 ± 10.82^{c}
100% MeOH	Fresh	72.86 ± 0.30^{a}	0.25 ± 0.01^{a}	0.07 ± 0.00^{b}	387.38 ± 6.61^{a}
	5 years old	71.91 ± 0.57^{a}	0.23 ± 0.02^{a}	0.02 ± 0.00^{c}	320.67 ± 9.11^{a}
	10 years old	68.58 ± 0.17^{b}	0.18 ± 0.01^{b}	0.09 ± 0.00^{a}	263.49 ± 8.83^{b}

Mean ± SEM (n=3); Different letters between the different storage duration groups of Chaga at the 70% and 100% MeOH indicate significant differences according to two-way ANOVA and Tukey's post hoc test ($P < 0.05$).M, moles; mM, millimoles; DW, dry weight; TE, Trolox equivalents; EDTA, ethylenediaminetetraacetic acid; Fresh, Fresh Chaga; 5 years old, Chaga preserved for 5 years; 10 years, Chaga preserved for 10 years. ABTS, 2,2'-azidobis-(3-ethylbenzothiazoline-6-sulfonic acid).

Chapter 6 Discussion

6.1 Total Phenolic and Flavonoid Contents in Mushrooms

The findings of this study underscore the significant variation in TPC and TFC among different species of mushrooms tested. We used 70% and 100% methanol concentrations to extract TPC and TFC from five different mushrooms, which are Chaga, white button, shiitake, chanterelle, and Portobello. Various studies have shown that methanol has a higher extraction rate and the highest content of bioactive compounds, including phenolics, compared to ethanol (Gursoy et al, 2009; Vambe et al, 2020; Liu et al, 2013; Zavastin et al, 2016; Choi et al, 2006). In our study, we found that there were significant differences in TPC and TFC yields between 70% and 100% methanol extractions, indicating that the methanol concentrations alter the extraction effect: methanol of higher concentration allowed more phenolic in mushrooms to be dissolved in the solvent, thus yielding a more thorough extraction. The result agrees with the findings of Hashim and his colleagues (2016), who concluded that a higher methanol concentration, 50 %, showed superior performance in the extraction of TPC from Orthosiphon Stamineus when they compared 0%, 25%, and 50% methanol concentrations. Thus, it is recommended to use higher concentrations of methanol, 100%, in the extraction of TPC for comparison of the antioxidant capacity of different plant or fungi species.

Amongst the five different types of fresh mushrooms used in this study, Chaga has the highest total phenolic and flavonoid contents. The TPC values measured in fresh Chaga extracts, reaching up to 6.98 ± 0.75 mg GAE/g DW in 100% methanol concentration, are comparable to the findings of Sharpe et al. (2021), who also reported the highest TPC values for Chaga among different types of mushrooms (48.3 ± 3.90 μmol GAE/mg, which is approximately equal to 8.21 ± 0.66 mg GAE/g DW). The results of our study showed that there were differences in TPC

values for different types of mushrooms, which is in line with the study of Sharp et al. (2021), who showed that compared to Chaga, the other mushrooms extract in 100% methanol had lower TPC values, for example, the TPC of shiitake had 5.3 ± 1.20 μmol GAE/mg, which is approximately 0.90 ± 0.20 mg GAE/g DW. In our study, the shiitake value was 0.36 ± 0.03 mg GAE/g DW in 100% methanol, which is lower than the shiitake mushroom in Sharp's study (2021). The TPC values for white button (0.31 ± 0.01 mg GAE/g DW) and Portobello (2.03 ± 1.59 mg GAE/g DW) are lower than those in the study of Soceanu and coworkers (2024), who showed that the TPC values for white button and Portobello were 3.58 mg GAE/g DW and 3.12 mg GAE/g DW, respectively. In our study, the TPC value for Chanterelle (0.53 ± 0.01 mg GAE/g DW) is in agreement with the findings of Fogarasi and colleagues (2021), who found a TPC of 0.79 ± 0.01 mg GAE/g DW for Chanterelle. Some of the reasons for the differences between the data from previous studies and ours may be due to the different types of extracts, they used ethanol (Soceanu et al., 2024), hydro-alcoholic (Fogarasi et al., 2021, Sharpe et al., 2021), or aqueous (Fogarasi et al., 2021) extracts, respectively.

Similar to TPC, the current study's findings illuminate the significant variation in TFC among different mushroom species, with the most pronounced value observed in fresh Chaga, reaching 8.23 ± 0.36 mg TE/g DW in 100% methanol extractions. The pattern observed in our study aligned with the study by Thoo and his colleagues (2013) that reported Chaga had the highest data in TFC value (17.89 ± 1.70 mg QE/g) in the fall season, which is the same season that the samples used in the current study were harvested. In our study, in contrast to Chaga, the other types of mushrooms exhibited TFC values that were about 30 folds lower: white button mushroom's TFC value was 0.25 ± 0.01 mg QE/g DW, shiitake was 0.28 ± 0.05 mg QE/g DW, chanterelle was 0.39 ± 0.03 mg QE/g DW, Portobello was 0.37 ± 0.04 mg QE/g DW. The study

by Fogarasi and his colleagues (2021) showed that the TFC of *Boletus edulis* (porcini mushrooms) was 0.20 ± 0.00 mg QE/g DW, which is similar to the TFC of *Lentinus edodes* (shiitake) in the current study. Moreover, in the current study, Chanterelle's TFC value was 0.39 ± 0.03 mg QE/g DW, which is higher than 0.03 ± 0.00 mg QE/g DW reported in Fogarasi's study (2021). The results of Fogarasi's study (2021) and Thoo's study (2013) differed from the current results because of differences in extraction solvents; Fogarasi's study (2021) used acidic water, ethanol/water/acetic acid (15:76.5:8.5, v/v/v), hexane, and diethyl ether to extract mushroom powders. Thoo's study (2013) used ethanol to extract Chaga mushrooms, indicating the importance of the choice of extraction solvent.

Our study compared fresh and capsulated mushrooms to determine if the processing involved in creating capsules affects the levels of TPC and TFC. Findings from this study indicate that there are significant differences in the TPC and TFC values between fresh and capsulated mushrooms (p-value < 0.05). This suggests that the processes of drying and encapsulating mushrooms significantly change these bioactive compounds (Gąsecka et al., 2020).

6.2 Phenolic Profile in Mushrooms

The current study underscores the rich phenolic profile of Chaga mushrooms in terms of chlorogenic acid, isoquercetin, and kaempferol content. Chlorogenic acid has been previously detected in mushrooms such as *T. letestui* with concentrations ranging from 4.55 μg/g (Woldegiorgis et al. 2014), 26.00 μg/g (Kim et al. 2008), and 63.73 μg/g (Palacios et al. 2011). In the current study, the content of fresh Chaga mushroom was 1.02 μg/g DW for the 100% methanol extract, and 0.92 μg/g DW for the 70% methanol extract. Chlorogenic acid, known for its anti-inflammatory, antiviral, and hypoglycemic effects (Clifford, 2000), was found in

substantial amounts in Chaga extracts, highlighting its therapeutic potential. Similarly, kaempferol has been previously found in plants such as leaves with contents ranging from 325 mg/kg (Dabeek et al. 2019), to 832.0 mg/kg (Miean & Mohamed, 2001). In contrast, the kaempferol content we found in Chaga was lower than in the leaves, where the value in fresh Chaga was 0.23 μg/g DW, which is about 0.23 mg/kg for the 100% methanol extract, and 0.15 μg/g DW, equivalent to about 0.15 mg/kg for the 70% methanol extract. Isoquercetin and kaempferol are known for their cardiovascular protective and anticarcinogenic properties (Cushnie & Lamb, 2005), suggesting that Chaga could be beneficial in managing chronic diseases and health maintenance. The difference between previous studies and current study might be related to the method of HPLC analysis; for example, the current study used the Biphenyl C18 100 RP (2.6 mm, 150 × 4.6 mm) separation column; however, Woldegiorgis et al. (2014) used a RESTECK reverse phase C18 column (5 μm, 250 mm × 4.6 mm). Moreover, the growth condition affects the results as well mushrooms used in the current study were grown in Canada, while those used by Woldegiorgis and his colleagues grew in the Horn of Africa (Ethiopia).

6.3 Antioxidant Activity in Mushrooms

Four antioxidant properties were assessed in the current study, which were DPPH radical-scavenging activity, FRAP assay, ferrous-ion-chelating activity, and TAC assay. In these experiments, fresh mushrooms exhibited a DPPH radical scavenging activity ranging from 12.48% to 67.91% when extracted with 70% methanol, and from 16.98% to 71.03% for 100% methanol extracts. These results align with earlier studies, such as Kim et al. (2008), which reported DPPH values in 10 fresh mushroom species ranging from 10% to 72%. In our study, Chaga mushrooms had the highest antioxidant properties, with a DPPH radical scavenging value

of 71.03 ± 1.39% in 100% methanol, indicating their strong antioxidant capacity, which was comparable to the value of 72% reported by Kim and colleagues for *Ganoderma lucidum* found in. In contrast, white button mushrooms had the lowest DPPH radical scavenging value at 12.48 ± 2.78% for the 70% methanol extract, demonstrating a lower antioxidant activity compared to other mushroom species, and similar to the value reported for *Pleurotus eryngii* in Kim's study (2008). Both the current study and Kim's study (2008) showed that different species of mushrooms have different DPPH radical scavenging values, which implies that different species of mushrooms have different antioxidant activities.

Consistent with our results, Chaga has been widely reported in the literature as having exceptionally high antioxidant capacities, primarily attributed to the dense concentration of polyphenolic compounds (Bristy et al., 2022; Nakajima et al., 2009; Abu-Reidah et al., 2021). Amongst the five fresh mushroom types in this study, for antioxidant activity, Chaga not only displayed the highest DPPH radical scavenging activity but also FRAP value. In the previous study, the FRAP activity level of *I. hispidus* extract was reported to be 0.69 ± 0.03 mM TE/g DW when extracted with 80% methanol (Machado-Carvalho et al., 2023). In contrast, the current study showed that fresh Chaga mushroom had a FRAP value of 62.41 ± 0.00 M TE/g DW for the 70% methanol extract, which is equivalent to 0.06 ± 0.00 mM TE/g DW. Additionally, the 100% methanol extract of Chaga mushrooms had a FRAP value of 55.39 ± 0.00 M TE/g DW, equivalent to 0.05 ± 0.00 mM TE/g DW. These findings highlight the significant antioxidant potential of Chaga mushrooms, although the values are lower compared to those of *I. hispidus* in terms of mM TE/g DW. The differences in FRAP values between these studies could be attributed to variations in the extraction solvents and conditions used, which can influence the efficiency of extracting antioxidant compounds from the mushrooms.

Islam et al. (2016) demonstrated that the ferrous-ion-chelating activity in 43 mushroom samples varied significantly, with the highest chelating ability being 41.57 μmol EDTA E/g and the lowest at 1.33 μmol EDTA E/g. Within their study, shiitake mushrooms exhibited a chelating activity of 11.31 ± 0.61 μmol EDTA E/g, while chanterelle mushrooms showed a value of 24.30 ± 1.88 μmol EDTA E/g. In the current study, the chelating activity of mushroom extracts ranged from 0.01 to 0.12 mM EDTA E/g DW, which translates to 10 to 120 μmol EDTA E/g DW. The current study also showed that the ferrous-ion-chelating activity varied greatly among different mushroom samples.

Interestingly, both shiitake and chanterelle mushrooms in the current study exhibited the same chelating activity, measured at 10 μmol EDTA E/g DW. This suggests that the extraction conditions or mushroom samples may have resulted in lower observed chelating activities when compared to those reported by Islam et al. (2016). The discrepancies between results from the current study and those of Islam et al. (2016) could be due to differences in extraction methods, solvent types, mushroom growing conditions, or even the specific strains of mushrooms used in each study. Further research and standardization of methods may be needed to fully understand these variations.

The current study also assessed the total antioxidant capacity using $ABTS^+$ scavenging activity. We found that Chaga mushrooms exhibited the highest $ABTS^+$ radical scavenging activity, measured at 521.05 ± 4.19 mM TE/g. Comparatively, Islam et al. (2016) reported that the highest $ABTS^+$ radical scavenging activity among their samples was 109.19 μmol TE/g, equivalent to 0.11 mM TE/g. In their study, the $ABTS^+$ radical activity for chanterelle mushrooms was 16.31 ± 1.10 μmol TE/g, while shiitake mushrooms had an activity of 8.23 ± 0.45 μmol TE/g, which was 0.02 ± 0.00 mM TE/g, and 0.08 ± 0.00 mM TE/g. In the current

study, the $ABTS^+$ radical scavenging activity of shiitake mushrooms was measured at 126.93 ± 1.87 mM TE/g for the 100% methanol extract, and chanterelle mushrooms showed an activity of 24.72 ± 0.00 mM TE/g in the same solvent. These comparisons highlight the significant differences in antioxidant activities among various mushroom species and extraction methods. The results from our study suggest that both Chaga and shiitake mushrooms exhibit exceptionally high antioxidant capacities, particularly for the 100% methanol extract, indicating their potential as strong sources of antioxidants. The variations between our findings and those of Islam et al. (2016) may be due to differences in extraction methods, solvents, and mushroom species or strains used in the study. Four parameters showed the antioxidant activity of mushrooms with Chaga being the most active.

The antioxidant activity of fresh mushrooms was compared with that of the corresponding capsulated mushrooms (white button, shiitake, and Chaga). The fresh white button, shiitake and Chaga mushrooms and their capsules had significant differences in DPPH, FRAP, ferrous-ion-chelating activity, and $ABTS^+$ scavenging activities ($p < 0.05$.) To the best of the our knowledge, there is no published study that have presented a comparison between the antioxidant properties of fresh food and its capsuled form, or which analyzed the influence of the capsulation process on the antioxidant properties of foods. However, there is a high demand from consumers for antioxidant supplements extracted from natural foods (Embuscado, 2015). Thus, a thorough study of the influence of the dehydration and capsulation process on the antioxidant properties of foods would be beneficial. Our findings suggest that encapsulating mushrooms increases the antioxidant activity of fresh mushrooms on some specific mushrooms such as white button mushroom. But it also decreases the antioxidant activity of fresh mushrooms on some specific mushrooms such as shiitake and Chaga mushrooms.

6.4 *In Vitro* Inhibition of Lipid Peroxidation and Angiotensin Converting Enzyme

Phenolic compounds can scavenge free radicals, thereby inhibiting the lipid peroxidation process and subsequently lowering TBARS levels. Previous studies have demonstrated that fresh Chaga extracts can significantly reduce TBARS formation. This is different from our study result. For example, Lee et al. (2007) identified new antioxidant polyphenols in Chaga that exhibited strong activity against lipid peroxidation. Mau et al. (2002) had shown that extracts from mushrooms can reduce TBARS levels. They demonstrated that several edible mushrooms possess significant antioxidant properties that can decrease TBARS formation in lipid peroxidation assays. In our assay, Chaga capsulated mushrooms had the lowest TBARS level. So, the significant antioxidant properties showed in commercial mushrooms.

The potent inhibitory effect of mushroom polyphenols, on angiotensin-converting enzyme (ACE) activity emphasizes potential therapeutic applications in the control of HTN and cardiovascular health. This result is consistent with the findings of Suzuki et al. (2008) that the ACE inhibitory activity of chlorogenic acid improved vasodilatation in hypertensive rats, and that chlorogenic acid in coffee lowered blood pressure in mildly hypertensive rats. This inhibitory effect combined with high antioxidant capacity suggests the value of Chaga and other mushrooms in the comprehensive treatment of chronic diseases.

6.5 Chaga Storage Time and Its Total Phenolic and Flavonoid Contents

The results of our study indicate a trend whereby fresher Chaga mushrooms exhibit significantly higher TPC and TFC. Specifically, fresh Chaga mushrooms exhibited the highest TPC and TFC, which decreased significantly with storage time. This trend may indicate that as Chaga mushrooms storage time, they gradually reduce bioactive compounds that contribute to

their antioxidant properties. Due to the high water content of edible mushrooms, the rate of respiration and metabolism is rapid, resulting in a loss of quality, such as the antioxidant properties of mushrooms (Marçal et al., 2021; Lin et al., 2019; Abdelshafy et al., 2023). In addition, the antioxidant capacity of mushrooms can be influenced by several factors, including species, environmental conditions (Zargoosh et al., 2019), and notably, the storage time or developmental stage of the mushrooms (Chaipoot et al., 2023). Research indicates that the concentration and efficacy of phenolic compounds can vary significantly as mushrooms grow and mature (Moradi et al., 2020). The antioxidant assays (DPPH, ABTS, and FRAP) revealed a corresponding decrease in activity with increasing period time of the Chaga mushrooms in our study.

Chapter 7 Conclusion, limitations, and future directions

7.1 Conclusion

The current study explored the antioxidant properties of mushrooms in detail, focusing on TPC and TFC as well as various antioxidant assays including DPPH free radical scavenging, FRAP, ferric ion chelating activity and TAC assay. Although methanol concentration affects TPC, TFC and antioxidant activity of mushrooms, the results of the present study provide convincing evidence for the great antioxidant potential contained in mushroom extracts. However, the antihypertensive effect was not related to the phenolic content. Different species of mushrooms showed different antihypertensive effects.

The analyses showed that Chaga had the highest TPC and TFC levels among the five species of mushrooms studied, which were significantly better than the other species studied. However, the data were low for all samples. This observation suggests that the antioxidant activity may derive from other compounds than just phenolic compounds.

Moreover, the current study extends beyond the comparison of fresh mushroom extracts to include commercially produced mushroom extracts, revealing interesting contrasts. It is worth noting that there is a significant difference between fresh and capsule mushrooms, with fresh Chaga mushrooms having a TPC value about four times higher than the corresponding capsule mushrooms.

Additionally, the application of HPLC facilitated the identification of specific phenolic compounds such as chlorogenic acid, isoquercetin, and Kampferol in Chaga, further elucidating the biochemical foundation of its antioxidant properties. However, the encounter with unidentified phenolic compounds due to limitations in the analytical approach underscores the complexity of mushroom biochemistry and highlights the necessity for advanced analytical techniques in future studies.

The study's insights into the in vitro antioxidant properties of mushrooms, through assessments like TBARS and ACE inhibition activity, reinforce the health-promoting potential of mushroom extracts. The various mushrooms' significant ACE inhibition activities highlight mushrooms' role not only in antioxidant defense but also in potential therapeutic applications for managing conditions linked to oxidative stress and HTN.

Our findings from the current work examining the age-related differences in TPC and TFC levels among Chaga mushrooms suggest that age significantly influences the concentration of these antioxidant compounds. Fresh Chaga mushrooms displayed the highest concentrations, with a discernible decrease observed in storage time. This age-related variation in bioactive compound levels presents an intriguing avenue for further research, particularly in the context of optimizing harvest times for maximal antioxidant benefits.

In conclusion, this comprehensive investigation into the antioxidant properties of mushrooms obtained in Manitoba, reinforces the valuable role of mushrooms as a source of potent bioactive compounds. These findings advocate for the inclusion of various mushrooms, in dietary regimens aimed at enhancing antioxidant intake. Nonetheless, the study also opens up new questions regarding the impact of mushroom storage time on bioactive compound concentrations and the potential of unidentified phenolic compounds, setting the stage for future research to build upon the current foundational work.

7.2 Strength

The study had several strengths:

1. The current study is the first to examine the phenolic content and antioxidant properties among various mushrooms commonly found in Manitoba.
2. Showed the effect of methanol solubility on the extraction of phenolic compounds in the same mushrooms.

7.3Limitations

The study had several limitations:

1. The current study focused on a limited number of edible mushrooms in Manitoba, Canada. Although the antioxidant properties of these five mushroom species were studied in detail, there are about 2,200 edible mushroom species globally. Future studies should examine a wider range of mushroom species in order to gain a more comprehensive understanding of their antioxidant potential.
2. Low Phenolic Contents: Although we calculated the polyphenol content, the antioxidant activity may be found from other compounds in the samples as the amount of polyphenols contained in the samples were found to be quite low. This may be due to the

analytical process within our study, where all results were reported based on the gross weight of the samples: The study did not standardized polyphenols across samples (i.e., use the same amount of polyphenols per sample), and therefore could not show the antioxidant properties of the same amount of polyphenols.

3. Sample Preservation Consistency: While efforts were made to standardize sample preservation and handling procedures to minimize variation, as the samples were grown by different producers and purchased from different vendors, there existed potential inconsistencies in the growing conditions and the storage condition within the samples from different sources, which may have introduced variability in their chemical properties. Variations in the growing and storage conditions could potentially impact the stability and composition of the mushroom extracts, thereby influencing the observed antioxidant activity.
4. HPLC Column Consistency: The use of the same HPLC column as in a previous experiment with vegetables (Abellán et al., 2021) may have restricted the identification of every polyphenol component in the mushroom extracts. Different matrices may require specific column selections or adjustments for optimal compound identification. The preferred column used by Çayan et al. (2020) was an Intertsil ODS-3 reverse phase C18 column (5 μm, 250 mm × 4.6 mm) for mushroom phenolics, but a Kinetex® Biphenyl C18 100 RP column (2.6 mm, 150 × 4.6 mm) was used in the current study. As a result, some phenolic compounds present in the mushroom extracts may not have been fully characterized.
5. In Vitro Testing: It is important to recognize that the current study relied on *in vitro* testing to assess antioxidant activity. While these assays provide valuable insights into

the potential health and commercial benefits of mushroom extracts, extrapolating the findings to real health outcomes should be done with caution. *In vitro* conditions may not fully replicate the complexities of *in vivo* environments, and further research is needed to validate our findings in clinical settings.

7.4Future Directions

1. Exploration of Additional Mushroom Species: In this study, we focused on only five edible mushrooms from Manitoba. In the future, we can also explore *Pleurotus ostreatus* (Oyster Mushroom), *Hypomyces lactifluorum* (Lobster Mushroom), and *Laetiporus* (Chicken of the Woods) as edible mushrooms throughout Canada. Future research could explore the antioxidant properties of a broader range of mushroom species.
2. Characterization of Phenolic Compounds: Future research could utilize advanced analytical techniques such as high-resolution mass spectrometry to facilitate the identification and quantification of individual phenolic compounds, providing deeper insights into their antioxidant properties.
3. *In vivo* studies: It would be valuable to conduct *in vivo* studies to assess the antioxidant and health-promoting effects of mushroom extracts in animal models or human clinical trials. The current study used only *in vitro* assays, but the lack of biokinetics may lead to misinterpretation of the data. Therefore, *in vivo* studies are more reliable than *in vitro* tests (Saeidnia et al., 2015). Assessing the metabolism, and physiological effects of mushroom-derived antioxidants in animal and human bodies could validate their potential therapeutic applications.
4. Exploring synergistic effects: There is a lack of information on the interactions and potential synergistic effects between phenolic substances and other substances in

mushrooms. Investigating potential synergistic effects between antioxidant compounds in mushroom extracts, such as those between polyphenolic substances as well as polysaccharide substances, could reveal more detailed antioxidant mechanisms and improve the overall antioxidant capacity of dietary interventions.

5. Assessment of Storage and Processing Effects: Examining the impact of storage conditions, processing methods on the antioxidant properties of mushrooms could provide practical insights for optimizing their nutritional value and health benefits.

Overall, findings from the current study highlight the significance of mushrooms as rich sources of natural antioxidants and underscore their potential role in promoting human health and well-being. By expanding our understanding of the antioxidant properties of mushrooms, this research contributes to the growing body of evidence supporting the inclusion of mushrooms in a healthy diet and underscores the importance of further exploration in this area. Future research endeavors aimed at addressing these directions could contribute to advancing knowledge on the antioxidant properties of mushrooms and their role in promoting human health and well-being.

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