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Extraction and role of mushroom polyphenols in starch digestion inhibition

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A Dissertation

submitted in partial fulfilment

of the requirements for the Degree of

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Parvinder Kaur

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Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Master of Science in Food Innovation

Extraction and role of mushroom polyphenols in starch digestion inhibition

by

Parvinder Kaur

Mushrooms are becoming attractive as a functional food and good sources of vitamins, minerals, especially bioactive compounds. Mushroom are used as medicines due to their antioxidant, antitumor, and antimicrobial properties. These are becoming very popular due to their high nutritional value, high protein, and low fat content. Mushroom proteins are rich in all the nine amino acids necessary for human body. They are utilized in preventing several diseases like hypertension, diabetes, hypercholesterolemia, and cancer. These are rich sources of bioactive substances like polyphenols, glycoproteins, and polysaccharides. The phenolic compounds are used as supplements in the isolated forms and have positive impact on the human health. The extraction of polyphenols is very complex due to existence of polyphenols in the form of insoluble complexes. There are several methods utilized for polyphenol extraction. Conventional solvent extraction is one of the most significant method for extracting bioactive substances. In this study, three different kinds of mushrooms (*Lentinula edodes, Auricularia auricula,* and *Tremella fuciformis*) were used for extracting polyphenols. Extraction was performed by using water and different concentrations of methanol at different temperature and pH. The antioxidant activity and protein content was determined. The results showed that water mushroom extracts showed higher antioxidant activity and higher protein content than methanol mushroom extracts. Nowadays, cereal products with mushroom powder inclusion are trending because mushroom powder enhances the nutritional value of the product. The 1% concentrated mushroom extracts were incorporated in the white sorghum flour to determine the effect on pasting properties and release of reducing sugars as compared to control group. The results revealed that, addition of mushroom extracts showed negative effect on the pasting properties of white sorghum flour than control group. The release of reducing sugars also decreased due to incorporation of mushroom extracts in the white sorghum flour than control group during *in vitro* starch digestion. The area under curve (AUC) illustrated that mushroom extracts decreased the predicted glycaemic response of the white sorghum flour. Mushroom extracts also elevated the level

of antioxidants (Total phenolic contents and Ferric reducing antioxidant power) in the white sorghum flour as compared to that of control. Moreover, the incorporation of mushroom extracts also increased the protein digestibility during *in vitro* starch digestion. These results suggested that mushroom extracts can be incorporated into the white sorghum flour to provide health benefits to consumers.

Keywords: Shiitake mushroom (*Lentinula edodes*), Black ear (*A. auricula*), and Silver ear (*Trimella fuciformis*), white sorghum flour, extraction, yield, antioxidant capacities, protein content, pasting properties, starch gelatinisation, glycaemic index, protein digestibility.

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Chapter 1

Introduction

Mushrooms are widely accepted food which provides balanced nutritional and health benefits to human body. The global consumption of cultivated edible mushrooms has increased from 1 kg to 4.7 kg per capita from 1997 to 2013. The production of mushrooms reached to 11 million tons in 2016 (Xiaokang et al., 2020). In 2018, the consumption of mushrooms was 12.78 million tons and still the demand is elevating across the world (Neergheen et al., 2020). These are highly rich source of minerals, proteins, carbohydrates, fibers, and along with contains substantial amounts of vitamins such as vitamin B1, B2, B12, C, D, and E (Gogoi, Chutia, Singh, & Mahanta, 2019). Moreover, mushrooms are magnificent sources of the biologically active substances like carotenoids, unsaturated fatty acids, tocopherols, flavonoids, ascorbic acid, indole, and phenolic compounds (Ma, Chen, Dong, & Lu, 2013). Mushrooms are considered as effective anti-inflammatory, anti-cancerous, antioxidant, and antibacterial agents (Gogoi, Chutia, Singh, & Mahanta, 2018). Mushrooms are an important source of secondary metabolites which are responsible for the inhibition activities of alpha-amylase and alpha-glucosidase. The inhibition of alpha amylase and alpha glucosidase decreases the rate of absorption of blood sugar which results in maintaining glucose levels in the body (Papoutsis et al., 2020). Therefore, by products of mushrooms are utilized in pharmaceutical industries which in turn add value to the horticulture sector (Xiaokang et al., 2020). Water and solvents are used for extracting the nutritionally valuable substances from the mushrooms (Rosello-Soto et al., 2016). Extraction of the phenolic compounds is affected by several parameters such as temperature, pH, concentration of solvent, and solvent to solid ratio. The combination of these factors and the determination of optimal conditions are important in order to obtain a maximum extraction potential (Bach et al., 2019). In recent years, gut microbiota is considered to have significant link in preventing and treating human diseases. Mushrooms are rich source of prebiotics as they contain bioactive compounds. Mushrooms can change the composition of gut microbiota and enhance the secretion of beneficial metabolites (Liu et al., 2021). However, the composition and technological properties of mushrooms offer several opportunities in the food industry. This study includes the extraction of polyphenols from three kinds of mushrooms studying the effect of polyphenols on potential glycaemic response and digestibility using white sorghum flour.

1.1. Aims

The aim of this study was to use three different kinds of mushrooms (Shiitake mushroom, Black ear mushroom, and Silver ear) to extract the polyphenolic contents. The mushroom powders were

utilized to extract the polyphenolic compounds. The extraction was performed by using RO water, 50% Methanol, and 80% Methanol. The effect of adding extracts of mushrooms in white Sorghum flour was analyzed. Additionally, the bioactive compounds in mushrooms and digested samples were evaluated. The main aims of the research can be summarized into key points.

1. To analyze the total phenolic contents and antioxidant properties of three kinds of mushrooms.

2. To analyze the effect of mushroom aqueous extracts on *in-vitro* digestion of white sorghum flour.

3. To analyze the total phenolic contents, antioxidant properties, and protein digestibility of *in vitro* digested white sorghum paste with mushroom extracts.

1.2. Hypothesis

1. Mushrooms polyphenols will be affected by changing extraction conditions (temperature, pH, and concentration of solvent) or not.

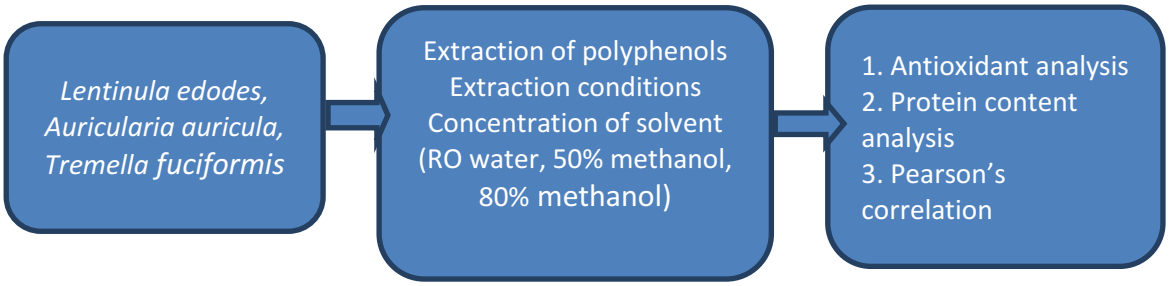
2. Mushroom polyphenols will inhibit starch degrading enzymes or not.

2. Mushroom polyphenols will be affected during *in vitro* digestion (with or without enzyme) or not.

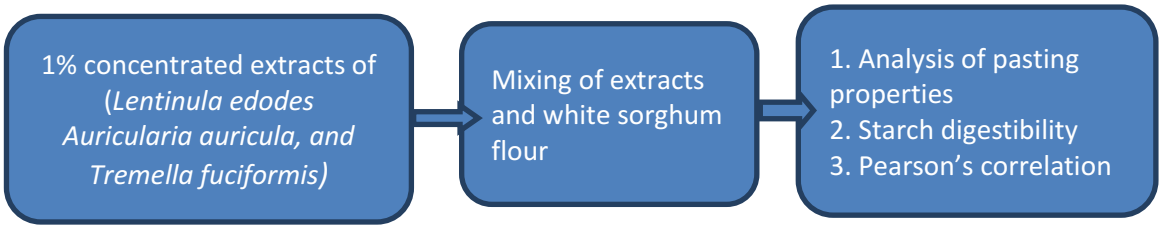
1.3. Thesis Outline

- **Project Title: The role of mushroom polyphenols in starch digestion inhibition**

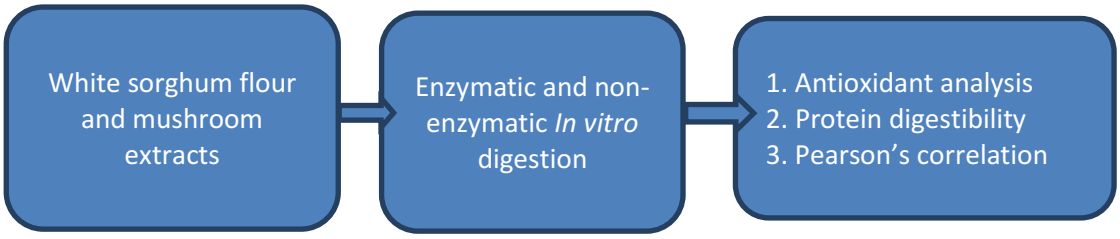
- **Chapter 1:** Introduction and thesis outline
- **Chapter 2:** Literature review
- **Chapter 3:** Materials and methods
- **Chapter 4:** Extraction of polyphenol compounds from mushroom samples



- **Chapter 5: In vitro digestion of White Sorghum flour with addition of mushroom aqueous-solvent extracts**



- **Chapter 6: In vitro phenolic compounds release of white sorghum flour with mushroom extracts**



- **Chapter 7: General discussions and conclusions**
- **References**

Chapter 2

Literature review

2.1. Mushrooms

Mushrooms are the macroscopic fungi which are found in several shapes, color, sizes, appearance, and edible, however the main characteristic feature of mushrooms is a fleshy sponge which is umbrella shaped. Mushrooms have been utilized since long time, they are very popular nowadays and available commercially worldwide. The total world production of mushrooms was six million metric tons in 2010 according to FAOSTAT data (Kalac, 2013). Mushrooms have not only good taste, aroma, and textural properties but also known for their nutritional value. The relationship of humans with mushrooms is interesting as mushrooms are being utilized both as food and medicine from long time. Mushrooms are inseparable part of human diet since the time when the humans were food hoarders and hunters revealed by the studies on Cutural Revolution (Rathore, Prasad, & Sharma, 2017). Moreover, mushrooms possess complex flavor and good nutrition value like proteins, minerals, vitamins, and bioactive components because of this these are used as raw and functional food (Poojary, Orlien, Passamonti, & Olsen, 2017). There are nearly twenty cultivated species of mushrooms, white button mushroom (*Agaricus bisporus*) is the leading cultivated mushroom worldwide, followed by shiitake (*Lentinula edodes*), oyester mushroom (*Pleurotus spp.*), and golden needle mushroom (*Flammulina velutipes*) (Kalac, 2013).

2.1.1. Composition and nutritional values

Mushrooms are of great importance in human diet. Wild and cultivated mushrooms comprised of proteins, carbohydrates, fibres, minerals, and vitamins (Aida, Shuhaimi, Yazid, & Maaruf., 2009). Moreover, mushrooms have less amounts of calories, sodium fats, and cholesterol (Barros, Baptista, & Ferreira, 2007). The gross composition of mushrooms is water (90%), and 10% comprised of proteins and amino acids, fats (2-8%), carbohydrates (3-32%), and ash (8-10%) (Motta, Greshwin, & Selmi, 2021). Mushrooms were considered as richer source of proteins and contains substantial amount of non-essential amino acids like alanine, alginine, glycine, glutamic acid, aspartic acid, proline, and serine. They provide structure to cells, tissues, and organs and hence important for the growth and repair of cells (Teklit, 2015). Mushrooms are regarded as the protein of poor man due to the higher amount of protein, vitamin, and *Calocybe* indica *Russula delica Lyophyllum* mineral (Braaksma & Schaap, 1996). These can be used to overcome the problem of malnutrition as they have good nutritional value especially as a valuable source of protein in developing countries where

the animal protein is very expensive (Guillamon et al., 2010). In addition to it, the consumption of mushrooms is marginal in many developed countries due to limited knowledge on the composition and nutritional value of mushrooms as compared to vegetables. Providentially, the situation has been changing continuously, the number of research papers about the mushrooms is increasing day by day and several times higher than 10 – 15 years ago (Kalac, 2009).

It has been investigated that fresh mushrooms contain higher amounts of proteins than vegetables (Bora & Kawatra, 2014). The mushroom protein content ranges between 15 – 35% of dry weight (Cheung, 2010). According to the Food and Agriculture Organization (FAO) standard, the quality of mushroom proteins is far better than most of the plant proteins (Cheung, 2010). The amount of essential amino acids in the proteins of mushrooms is within range from 30 – 50 g/100 g protein dry weight (Manzi, Gambelli, Marconi, Vivanti, & Pizzoferrato, 1999). Mushroom proteins are rich source of amino acids like threonine (41-95 mg/g protein dry weight), valine (36-89 mg/g protein dry weight), glutamic acid (130-240 mg/g protein dry weight), aspartic acid (91-120 mg/g protein dry weight), and arginine (37-140 mg/g protein dry weight). Although, mushrooms are poor sources of methionine (1.2-22 mg/g protein dry weight) and cysteine (16-19 mg/g protein dry weight). It has also been investigated that edible mushrooms contain lesser amount of lysine, leucine, isoleucine, and tryptophan (Cheung, 2010). Mushrooms contain free amino acids such as glutamic acid and alanine like monosodium glutamate which contributes to the unique taste of mushrooms (Mau, Lin, & Chen, 2001).

For maintaining good health, carbohydrates, fats, vitamins, and minerals are also important. Mushrooms have been appreciated continuously because of the low calories, high vitamin and mineral content present in mushrooms. The moisture content of fresh mushrooms is about 70 – 95 % but it decreases to 10 -13 %, depending upon the several conditions such as type of specie, harvesting time, and environment conditions (Breene, 1990). The amount of carbohydrates and fibre contents of fresh mushrooms are 3-21% and 3-35% respectively (Breene, 1990). Generally, edible mushrooms contain lipids less than 5% dry weight. The most common lipids present in mushrooms are unsaturated fatty acids specifically linoleic acids (Yang, Lin, & Mau, 2002). Despite, mushrooms have low level of linolenic acid and linolenic acid is directly related to the flavor of mushrooms. Moreover, linolenic acid acts as a precursor to 1-octen-3-ol (aromatic substance) called as the fungal alcohol in mushrooms (Cheung, 2010). Edible mushrooms are also good source of minerals and trace elements such as potassium, phosphorus, magnesium, and copper (Vetter, 1990). Riboflavin, niacin, and folates are present in mushrooms and riboflavin content is higher than vegetables (Mattila, Lampi, Ronkainen, Toivo, & Piironen, 2002). Additionally, mushrooms comprised a substantial number of vitamins such as B1, B2, B12, C, D, and E) (Heleno, Barros, Sousa, Martins, & Ferreira, 2010), however mushrooms contain less amount of calcium (Barros, Falcao, Baptista, Freire, Vilas-

Boas, & Ferreira, 2008). Mushrooms are the only natural and non-animal source of vitamin D and only natural vitamin D for vegetarians. Wild mushrooms are the excellent source of vitamin D, but the cultivated mushrooms are grown in dark, so they don't have vitamin D (Valverde, Hernandez-Perez, & Paredes-Lopez, 2015).

2.1.2. Medicinal properties

Mushrooms are considered as ingredient of epicure cuisine world-wide specifically for the unique flavor and regarded as a dietary wonder (Valverde, Hernandez-Perez, & Paredes-Lopez, 2015). Mushrooms are considered as delicacy with higher nutritional and functional value and accepted as nutraceutical foods. They are of great interest because of medicinal properties, organoleptic properties, and economic importance (Ergonul, Akata, Klyoncu, & Ergonul, 2013). Although, there is not much difference within the edible and medicinal mushrooms due to many edible species have medicinal properties and medicinal mushrooms are also edible (Guillamon et al., 2010). Mushrooms could be regarded as alternative source of new antimicrobial substances, especially secondary metabolites (terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolones) as well as primary metabolites (oxalic acid, peptides, and proteins) (Valverde, Hernandez-Perez, & Paredes-Lopez, 2015). Mushrooms are considered as mini-pharmaceutical factories because of the substances produced by them having phenomenal biological properties (Patel & Goyal, 2012; Pereira, Barros, Martins, & Ferreira, 2012).

Mushrooms produce more than 100 medicinal function and these includes antioxidants, anticancer, antidiabetic, antiallergic, immunomodulating, cardiovascular protector, anticholesterolemic, antiviral, antibacterial, antiparasitic, antifungal, detoxification, hepatoprotective effects, and act against tumour and inflammatory processes (Valverde, Hernandez-Perez, & Paredes-Lopez, 2015). Additionally, several bioactive molecules are produced by macro-fungi and these are polysaccharides, proteins, fats, minerals, glycosides, alkaloids, volatile oils, terpenoids, tocopherols, phenolics, flavonoids, carotenoids, folates, lectins, enzymes, ascorbic acid and organic acids (Zaidman, Yassin, Mahajna, & Wasser, 2005; Chang & Wasser, 2012; Finimundy et al., 2013; and Chen & Serviour, 2007). Polysaccharides are most significant and mainly β-glucan is the most adaptable metabolite with a broad range of biological action (Valverde, Hernandez-Perez, & Paredes-Lopez, 2015). Mushrooms are utilized from many years for treating several diseases and mushroom extracts are being used as dietary supplements for enhancing the function of immune system and antitumor activity (Guillamon et al., 2010 & Finimundy et al., 2013).

Edible mushrooms are rich in dietary fiber with several health benefits. Polysaccharides, lignin, oligosaccharides, and related plant compounds are the common dietary fibres. Dietary fibres are of

two types water soluble and water insoluble (Devi et al., 2014). Dietary fibre is mainly present in the cell walls of mushrooms. The mushroom cell wall contains fibrillar and matrix substances which are composed of chitin and the polysaccharides like beta glucans and mannans (Kumari, 2020). Beta glucans are present in higher amounts (more than 80%) in mushroom sclerotium (Wong, Lai, & Cheung, 2011). It has been reported that beta glucan increases the immunity in humans by producing cytokine and directly inhibiting the growth of cancerous cells (Kumari, 2020). Moreover, increased consumption of dietary fiber prevents diabetes, hypertension, obesity, and dyslipidemia in humans (Lairon et al., 2005).

The antioxidant substances of mushrooms act as protective factors to assist the endogenous defence system to decrease the oxidative harm (Barros et al., 2007). Oxidation is significant in producing energy to charge biological processes which keep the organisms alive, like that free radical production is certain in case of normal as well as pathological metabolism of cell (Elmastas et al., 2007). The unlimited production of oxygen derived free radicals is associated with several diseases like rheumatoid arthritis, cancer, arteriosclerosis, and ageing (Elmastas et al., 2007). Edible mushrooms contain antioxidants which assist to fight oxidative stress, whereas inedible mushrooms are sources of extracted phenolic substances and utilized as food additives or constituents of pharmaceutical and cosmetic products (Vaz et al., 2011). For example, *Agaricus bisporus, Pleurotus sp.,* and *Lentinula edodes* are consumed and cultivated at large scale and comprised of powerful antioxidants (Boa, 2004).

2.1.2.1. Mushroom and cancer

Mushrooms are becoming major part of human diet because of nutritional, organoleptic, and medicinal attributes. From about twenty thousand known mushroom species, various genera such as Ganoderma, Pleurotus, Agaricus, Trametes, Calvatia, Innonotus, Funlia, Lactarius, Russula, Flammulina, Suillus, Xerocomus, Cordyceps, and Antrodia are analyzed for anti-cancerous and medicinal properties (Neergheen et al., 2020). Presently, medicines from various natural sources are utilized as powerful therapeutic agents in suppressing and treating cancer due to having low toxicity and very few side effects. According to Global Burden of Disease Cancer Collaboration, cancer is the second largest cause of deaths in industrialized countries. Recently, a study conducted on bioactive compounds of medicinal mushrooms investigated that medicinal mushrooms have substances which inhibits the proliferation of cancerous cells and are utilized for the development of anti-cancerous drugs (Kumar et al., 2020). They have several substances that interfere in the signaling passageways of tumor expansion, apoptosis control, cancer-specific metabolism, metastasis, angiogenesis, and specific activities of immune system (Jeitler et al., 2020). These main features are related with mainly two mushrooms *Ganoderma lucidium* and *Ganoderma frondosa.* Medicinal mushrooms have high molecular weight substances like beta-glucans (polysaccharides), glycoproteins, and low molecular

weight compounds such as sesquiterpenes, isoflavones, catechols, quinones, and steroids which are linked to antitumor function (Rossi et al., 2018). A study conducted on importance of medicinal mushrooms investigated that extracts of several mushrooms are utilized for the treatment of various kinds of cancer. For example, *Agaricus sylvaticus* extract helps in treating breast cancer and colorectal cancer, *Agaricus blazei* for treating multiple myeloma, cervical, ovarian, and endometrial cancer, *Coriolus versicolor* for advanced hepatocellular carcinoma, and *Antrodia cinnamomea* for the treatment of advanced and metastatic adenocarcinoma (breast, gastric, lung, liver, and colorectal) (Jeitler et al., 2020). Meanwhile, study conducted on the medicinal plants and mushrooms showed that natural sources modify the gut microbiota for the improvement of gut dysbiosis and helps in treating cancer (Cheung, Yue, Chiu, & Lau, 2020). However, several clinical studies conducted on mushrooms represented the efficiency of mushrooms in treating cancers and the multitargeted behavior of mushroom substances in preventing growth of cells (Neergheen et al., 2020).

2.1.2.2. Mushroom and metabolic disorders

Metabolism is an important process to get energy for humans. It is defined as the sum of reactions occurring in the body during digestion. These reactions include the breakdown of food components into amino acids, sugars, fatty acids, vitamins, and minerals. Imbalance in diet like overeating or malnutrition which lead to metabolic disorders such as diabetes, obesity, and cardiovascular diseases (Whelan et al., 2013). Obesity is a disorder which is increasing worldwide and lead to several serious complications like diabetes, cardiovascular diseases, and pulmonary diseases (Ganesan & Xu, 2018). Therefore, more efforts are needed to prevent obesity. Mushrooms are highly nutritious with several bioactive substances having well known effects on cardiac markers (Ganesan & Xu, 2018). Previous study showed that mushrooms have capability to decrease the triglycerides, total cholesterol, plasma glucose, and hypertension in rats suffering from diabetes. These studies revealed that mushroom intake provides health benefits by reacting on atherogenic profile under hyper and normocholesterolemic conditions in rats (Wang et al., 2015). Moreover, the clinical studies conducted on obese people by substituting 20% of high energy beef with 20% of low energy *Agaricus bisporus* mushroom in their diet. The results from this study represented that individuals consuming mushrooms have lower body mass index, reduced belly circumference, and elevated satisfaction without affecting appetite (Cheskin et al., 2008; Poddar et al., 2013). Similarly, *Hericium erinaceus* and *Lentinus edodes* has anti-obesity and antidiabetic potential (Ganesan & Xu, 2018). In addition to it, mushrooms have the power to lower the increased blood sugar levels. For example, *Agaricus brasiliensis* has α-glucan and β-glucan polysaccharides which elevates plasma insulin and reduce pancreatin glucagon, elevates the insulin sensitivity, restraining of α-glycosidase enzymes in bowel and decreases the decomposition and absorption of carbohydrates, inhibits sugar dysplasia, and lipid peroxidation (Fang et al., 2016). Another study showed that *Agaricus brasiliensis* extracts decreased

the levels of blood glucose in rats suffering from diabetic rats and clinical studies reported that *A. brasiliensis* when combined with antidiabetic drugs improved the insulin resistance in Type 2 diabetic rats (Vitak et al., 2017). It has also been reported that β-glucans and oligosaccharides of *A. brasiliensis* showed anti-hyperglycemic, anti-hypercholesterolemic, anti-hypotriglyceridemic, and anti-arteriosclerotic activity in rats suffering from diabetes (Vitak et al., 2017).

2.2. Polyphenolic compounds of mushroom

Polyphenolic substances are broadly studied because of their antioxidant characteristics (Perron & Brumaghim, 2009) due to which they have received much attention in the field of pharmaceutical, biochemical, food, and cosmetic industry (Yang et al., 2009). Phenolic substances are aromatic hydroxylated compounds mainly found in several food sources (Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012). The main structural features of most of the polyphenols include three-membered flavan ring system and there are thousands of different polyphenolic compounds. All the polyphenolic compounds are divided into seven subclasses which are catechins, flavonols, flavones, anthocyanins, proanthocyanins, and phenolic acids. These are found in coffee, fruits, tea, wine, chocolates, and vegetables. People with good intake of fruits and vegetables generally consume one or more grams of polyphenolic substances (Perron & Brumaghim, 2009). Polyphenolic compounds are very essential substances that assist the endogenous defence network in decreasing the oxidative harm (Barros et al., 2008). The primary phenolic substances present in mushrooms are phenolic acids such as hydroxybenzoic and hydroxycinnamic acids derived from non-phenolic compounds (benzoic and cinnamic acid) (Vaz, Almeida, Ferreira, Martins, & Vasconcelos, 2012). Polyphenols are best known for their antioxidant features. The activity of polyphenols depends on the scavenging free radicals and reactive oxygen/nitrogen species, diminishing of oxidized intermediates, iron and copper binding, inhibiting the enzymes forming free radicals, activating antioxidant enzymes, and preventing oxidation of ascorbic acid and vitamin E (Efenberger-Szmechtyk, Nowak, & Czyzowska, 2021). The polyphenol content varies in plant and plant extracts. In plant extracts, the extraction method affects the phenolic content and antioxidant activity and efficiency of extraction depends upon the solvent, time, and temperature (Efenberger-Szmechtyk, Nowak, & Czyzowska, 2021).

Mushrooms contain higher levels of phenolic substance, especially sulfur-containing amino acid ergothioneine (ERGO) (Kalras, Richie, Calcagnotto, & Beelman, 2017). Ergothioneine was first identified in Ergot fungus (*Claviceps purpurea*) in 1909 (Weigand-Heller, Kris-Etherton, & Beelman, 2011).). Mushrooms are the primary source of ERGO comprising 0.4 to 2.0 mg/g dry weight (Weigand-Heller, Kris-Etherton, & Beelman, 2011). ERGO protects from the oxidative stress by having ability to act with other antioxidants in the mitochondria (Paul & Snyder, 2010). Earlier studies investigated that mushrooms contain higher levels of ERGO (Kalras, Richie, Calcagnotto, & Beelman,

2017). It has been reported that after consumption of mushrooms, ERGO is bioavailable and its consumption is related to attenuated postprandial triglycerides response (Weigand-Heller, Kris-Etherton, & Beelman, 2011). Moreover, ERGO is associated with other sulfur containing antioxidant namely, glutathione (GSH). GSH is an ubiquitous tripeptide (Y-glutamyl cysteinyl glycine) and regarded as the major intracellular antioxidant in all the organisms with several functions such as detoxification, post translational regulation of protein function, and maintaining immune function (Kalras, Richie, Calcagnotto, & Beelman, 2017). A study conducted on ERGO and GSH content of mushrooms showed that mushrooms have higher level of GSH ranging up to 7.8 mg/g DW. In addition to it, yellow oyster and porcini contains highest levels of ERGO within range 0.2 to 7.3 mg/g DW (Kalras, Richie, Calcagnotto, & Beelman, 2017). Therefore, mushrooms are important sources of GSH and ERGO in human diet. For example, *Agaricus bisporus, Pleurotus sp., Lentinula edodes* are important cultivated species and sources of antioxidants (ERGO) (Boa, 2004). Several wild species such as *Boletus edulis, Agavicus blazei Murill, Agrocybe cylindracea Gillet,* and *Amanita caesarea* are also identified possessing radical scavenging activity (Caglarirmak, 2007).

2.3. Extraction of polyphenols

Due to the positive impacts on human health, phenolic substances in the isolated form can be utilized as supplements in food or nutraceuticals. For the isolation of phenolic compounds, a best technique is required which should be cost-effective for the separation of polyphenolic compounds from plants. The extraction and isolation of polyphenolic compounds is very challenging because of the complex structure and instability (decomposition and reaction throughout the process) (Suwal & Marciniak, 2018). Consequently, the effectiveness of the phenolic substances extraction from plant material is enhanced by various constants like the chemical nature and site within the plant matrix of phenolic compounds, type of extraction technique, the particle size of sample, existence of obstructive compounds, and subsequent biochemical and chemical reactions (Naczk & Shahidi, 2004). The chemical nature of plant phenolics vary widely from simple to complex (highly polymerized compounds) comprising different amount of phenolic acids, phenylpropanoids, tannins, and anthocyanins (Naczk & Shahidi, 2004). Moreover, the polyphenols also exist in the form of complexes with several high molecular weight phenolics, carbohydrates, and proteins and these complexes are insoluble (Naczk & Shahidi, 2004). Therefore, the polyphenol extracts are mixture of various classes of phenolics which are soluble in the specific solvent system utilized (Mustafa & Turner, 2011). Phenolic substances are significantly present in cell walls, leaves, and vacuoles (Suwal & Marciniak, 2018). Additionally, various phenolic components in plants are attached to plant materials by covalent bonds which is very challenging to release them into isolated form (Xu, Ye, Chen, & Liu, 2007). Hence, distinctive extraction techniques have been expanded for the extraction of polyphenols from plants. The rate limiting step for the isolation of polyphenols from plant part

includes solubility, diffusibility of phenols through the cell walls, and leaching out of the cell wall (Mustafa & Turner, 2011; Xu, Ye, Chen, & Liu, 2007). There are many techniques utilized for the extraction of polyphenols from the plants. Traditional solvent extraction is one of the important extraction methods of bioactive compounds from plants, but this method is still used widely for extracting phenolic compounds from several sources (Gil-Chavez et al., 2013). The pre-treated (dried or washed) plant material is mixed with solvents such as water, hexane, ether, chloroform, benzene, methanol, and ethanol which absorbs the molecules of polyphenols. Thus, the capability of solvent extraction techniques is influenced by the solvent utilized (solubility of polyphenols depends upon the type of solvent and polarity of solvent), and the degree of polymerization of phenolics, and the association of phenolics with other plant or food materials (Suwal & Marciniak, 2018). The option of extraction methods depends upon the site of polyphenols in the plants, the phenols which are stored in the vacuoles are only extracted by using alcoholic or organic solvents (Robbins, 2003). The solid-liquid extraction was the first method used for extracting polyphenolic compounds also known as Soxhlet extraction (Suwal & Marciniak, 2018). This method is usually regarded as leaching of compounds. This is technique is very easy and proper training is not required and it gives more extraction productivity. Moreover, this method consumes a lot of time and usage of large amounts of organic solvents which is not good from environmental point of view. In addition to it, the cost is very high and the long treatment time and thermal decomposition of extracted of the extracted molecules (Suwal & Marciniak, 2018). The long exposure time to light and oxygen results in degrading the sensitive substances (Denery, Dragull, Tang, & Li, 2004). Therefore, several improved techniques of solvent extraction are increasingly being developed which focuses on the use of less quantity of solvent, lesser treatment time period and energy, and enhance the yield of extracts. In present study, solvent extraction method on the stirrer is used for polyphenol extraction.

2.4. Shiitake mushroom (*Lentinula edodes*)

Shiitake mushrooms (*Lentinula edodes*) are the most common edible mushrooms having nutritional and flavor characteristics. In Japan, it is historically called as shiitake and “fragrant mushroom” (Gukov & Komin, 2020). It is the world’s second largest mushroom and considered as “the Queen of mushrooms” (Qin et al., 2020). It is utilized in Asian dishes and traditional medicine for hundreds of years (Frey, Durmus, Sillls, Isik, & Corner, 2020). It is a ligninous mushroom which is most cultivated mushroom strains globally (Popa-Vecerdea & Oancea, 2020). The nutritional compounds include bio-active polysaccharides like β-D-glucan, hetroglucan, xylomannan, lentinan and eritadenine; free sugars (arabinose, arabitol, mannose, mannitol, tetrahalose, and glycerol); vitamins (B2, B12, D2) and dietary fibre (Rahman, Abdullah, & Aminudin, 2018). Bioactive compounds of shiitake with bioactivities are adenine derivatives (antibacterial), agaritine (antifungal), ergothioneine (vitamin D precursor and anti-hypercholesterolemic), eritadenine (anti-inflammatory), formaldehyde

(antilipidemic antioxidant), galactose (antioxidant enzyme activity), glucose (antitumor), lectins (antiviral like influenza infections), lentinamuicin (arterosclerosis), lentinan (headache), mannose (hepatocirrhosis), polyisoprenoid alcohols (immunopotentiator), RNA from spores (vertigo), and statins (Gonzalez-Quero & Martinez, 2020). In addition to it, dried shiitake mushrooms fruiting bodies were detected with higher levels of vitamin B12 and was approximately equal to 5.61 µg/100g dry weight (Watanabe, Yabuta, Bito, & Teng, 2014). Shiitake mushrooms are very famous in dried as well as fresh form in several dishes. Shiitake mushrooms are unable to synthesize vitamin B12 and it has been observed that the vitamin level in the fruiting bodies is similar to level of vitamin B12 present in the bed logs of shiitake (Bito et al., 2014). Therefore, dried shiitake fruiting bodies are considered as rich source of plant-based vitamin B12. There are many applications of shiitake mushroom in the food industry due to its antioxidant properties. These are widely utilized in the food products to enhance the health benefits of the products. Many studies are conducted on the incorporation of mushroom powder into several food products to make them healthier (Wang et al., 2020; Lu et al., 2018; and Aguilera, 2018).

2.5. Black ear mushroom (*Auricularia auricula*)

Auricularia auricula belongs to heterobasidiae of basidiomycetes and also known as Jew's ear, wood ear, red ear, black ear fungus, is a non-toxic edible fungus (Fan, Chen, Wei, He, & Yan, 2015). Black ear mushroom (*Auricularia auricula*) is a significant genus of cultivated mushrooms with lots of health benefits (Vallee et al., 2017). The black ear mushrooms are categorized as jelly fungi. The term jelly fungi applied to that fungal species with jelly like consistency (Priya, Geetha, & Darshan, 2016). Ear mushrooms are considered as the traditional medicines in China and often utilized as food. Generally, the cultivation of black ear mushroom is like shiitake mushrooms on the bed logs (Mau, Chao, & Wu, 2001). Black ear mushroom is widely cultivated in China, Taiwan, Thailand, Philippines, Indonesia, and Malaysia. The total annual production of black ear mushrooms in china was 3.6 million ton in 2010 (Priya, Geetha, & Darshan, 2016). Mushroom compounds have been investigated recently in relation to health benefits, for example extracts of *A. auricula* inhibits the lipid peroxidation and reduces the chances of liver damage in mice treated with benzo [α] pyrene. In addition to it, the antioxidant and hypolipidemic properties of an *A. auricula* have been demonstrated with polysaccharides of *A. auricula* being dynamic antioxidants against hydroxyl and superoxide radicals (Vallee et al., 2017). *A. auricula* have several medicinal properties such as anti-inflammatory, anti-tumor, antioxidant activity, anti-coagulant, hypo-cholesterolemic activity, hypolipidemic activity, immunity booster, and lowering of blood glucose (Fan, Chen, Wei, He, & Yan, 2014). Moreover, the methanolic extracts from *A. auricula* showed excellent antioxidant activities (Mau, Chao, & Wu, 2001). Another study conducted on bioactive substances of black ear mushroom

also showed that it has anti-tumor, anti-viral, anti-bacterial, and anti-parasitic effects (Onyango et al., 2011).

2.6. Silver ear mushroom (Tremella fuciformis)

Tremella fuciformis is commonly called as white jelly mushroom with medicinal properties (Deng et al., 2016). It is cultivated artificially in Taiwan (Tsai, Huang, Juan, & Lin, 2018). It is a translucent white species of jelly fungus (Tseng, Yang, Li, & Mau, 2010). It is generally utilized as a food in oriental countries and in china is used as a traditional medicine (Tseng, Yang, Li, & Mau, 2010). It is considered as a masterpiece of Asian cooking. Moreover, silver ear possesses several medicinal properties such as anti-inflammatory, anti-tumor, blood pressure regulation, hypercholesterolemia, hyperlipidemia, cardiovascular disorders, and chronic bronchitis (Tseng, Yang, Li, & Mau, 2010). Silver ear is a popular food and medicine because of having low lipid content and higher antioxidant activity (Zhang et al., 2015). Mushroom can be added to food products as a supplement to increase its consumption to deliver several health benefits through food products. A study conducted on bread showed that incorporation of silver ear in bread increases the nutritional value of the bread (Tseng, Yang, Li, & Mau, 2010). In addition to it, silver ear powder was added to buns to enhance the health benefits of buns (Tsai, Yang, Tseng, Lee, & Mau, 2010). Silver ear powder can be utilized as a substitute of meat to enhance the sensory and physico-chemical properties (Cha, Heo, Lee, Lo, & Moon, 2014). It has been investigated the particles of silver ear powder has highest water holding capacity and can be used for the manufacturing of instant foods (Tsai, Tsay, Li, Hung, & Lin, 2020).

2.7. White Sorghum flour (Sorghum bicolor)

White sorghum (*Sorghum bicolor*) is a nonceliac cereal grain cultivated in Asia and Africa. Grain is utilized as a food in many developing nations and food for animals in developed nations (Ali & Hasnain, 2011). Granules contains linear polysaccharide known as amylose and highly branched polysaccharide called amylopectin (Olayinka, Adebowale, & Olu-Owolabi, 2008). The main component of sorghum grain is starch. Starch is a biopolymer commonly used in food and other industries. Thus, starch have restricted uses due to retrogradation, sensitivity to pH, shear, and heat, and low paste clarity (Sajilata & Singhal, 2005). Consequently, starches are altered through physical and chemical modifications for the improvement of functional properties and elevating its application in food industries (Ali & Hasnain, 2011). Moreover, the starches in the flour releases higher amount of reducing sugar during digestion and more sugars results in causing diabetes mellitus (Wolter, Hager, Zannini, & Arendt, 2013). Therefore, maintaining glycaemic control is very important to reduce the risk of diabetes (Wolter, Hager, Zannini, & Arendt, 2013). The postprandial glycaemic effect of food is associated with the degree of carbohydrate digestion and specified by glycaemic index (GI). The GI is explained as the marginal area under the curve (AUC) of the blood

glucose concentration on ingesting of carbohydrate rich foods (Wolter, Hager, Zannini, & Arendt, 2013). White sorghum is a cereal and it comes the category with high GI (Atkinson et al., 2008). Food products with higher GI cause fast and higher amount of release of blood glucose, although food products with low GI and comprised slowly digested carbohydrates and lead to slower elevation of blood glucose level (Brand-Miller et al., 2009). The glycaemic response is linked with the degree of digestion and immersion of carbohydrate enriched foods with the assistance of *in vitro* methods, which are imitating the *in vivo* digestion processes (Singh et al, 2010). The glycaemic response is dependent upon the endogenous factors of the food medium such as starch susceptibility, protein and lipid content. Starch susceptibility is analyzed by its structure, crystallization, physical encapsulation, gelatinization degree, and retrogradation of starch (Fardet et al., 2006). To decrease the release of reducing sugars and starch digestibility, mushrooms are incorporated in the cereal products to enhance their nutritional value. Many studies are conducted on the incorporation of mushroom powder into the cereal products to decrease the release of reducing sugars and deliver the health benefits to individuals (Lu et al., 2018; Majeed et al., 2019; and Lu et al., 2020).

Chapter 3

Materials and Methods

3.1. Materials

3.1.1. Mushroom powder

Dried Shiitake mushrooms (*Lentinula edodes*) (Jade Phoenix, China) were obtained fom the local New World Supermarket (Foodstuffs, New Zealand), dried black ear and silver ear mushroom was obtained from the local Chinese supermarket (Sunson, New Zealand).

Dried shiitake mushroom, black ear mushroom, and silver ear mushroom slices were ground into powder by a mill (model: BCG200; Coffee Grinder, Breville, Sydney, Australia) and the powder was put in sealed bag and stored at room temperature until required.

3.1.2. Other materials

White Sorghum flour (Woods Foods) made in Australia from locally grown sorghum free from any genetic modification was obtained from local New World Supermarket (Christchurch, New Zealand).

3.2. Extraction of Samples

0.5 g of ground sample of mushroom was mixed with 20 mL of RO water, 50% Methanol, and 80% of Methanol and stirred for three hours on a magnetic agitator at different temperature (25^{o} C, 50^{o} C, and 80^{o} C) and pH (pH 2 and pH 7) conditions. After maceration, the solvent was evaporated to 1% (10 mg/mL) concentrates under reduced pressure by using a rotary evapoartor at 50^{o} C.Then sample extractions were utilized for total phenolic content (TPC), DPPH radical scavenging activity, and Ferric reducing antioxidant power (FRAP) analysis.

3.3. Measurement of the extraction yield

The weight of dried extract of each mushroom sample was measured, and the yield percentage was calculated using the formula:

Extract yield (%) = Weight of dried extract/Weight of dried mushroom samples*100

3.4. Antioxidant Analysis

TPC of all samples was measured using 0.2 N Folin-Cioalteu reagent (Sigma, St Louis, USA) according to the method reported by (Singleton and Rossi, 1965) with some modifications. The shiitake (*L.*

edodes) sample extracts were diluted two times with RO water. The 1% concentrated sample extracts were diluted 5 times with RO water. Sample extract (0.5 mL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent and 2 mL of 7.5% sodium carbonate. The samples were incubated at room temperature for 2 hours in an dark place and then absorbance was read at 760 nm (V-1200 spectrophotometer, Global Science). Gallic acid (25-200 µg) was used for standard curve (Sigma-Aldrich, Steinheim. Germany). The results were expressed as gallic equivalent per gram dry weight.

The Ferric reducing/antioxidant power assay was performed using the method given by (Liu et al., 2019) with some modifications. The Shiitake samples extracts (at 25^{o} C and pH 2 and pH 7) were diluted two times with RO water. The shiitake sample extracts (at 50^{o} C and 80^{o} C, pH 7) were diluted three times with RO water. The 1% concentrated extracts of three mushrooms were diluted five times with RO water. FRAP reagent was prepared by mixing 300 µM Acetate buffer ph 3.6, 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) in 40 mM HCL and 20 mM $FeCl_3$ at a ratio of 10:1:1. To 250 µL of sample extract, 2.5 mL of the FRAP reagent was added and mixture was incubated at 37^{o}C for two hours. The absorbance was read at 593 nm (V-1200 spectrophotometer, Global Science). A standard calibration curve was prepared using iron (III) sulphate (0-1000 µmol). The results were expressed as micromoles of Fe^{2+}/g of dry weight.

The ability of all the samples to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) radical was determined by the method adapted by (Floegel et al., 2011) with some modifications. Dilutions were performed with RO water and described in Table 1. A 0.1 mM of DPPH stock solution was prepared with 100% methanol. To 0.5 mL of sample extract, 1 mL of DPPH and 1.5 mL of 100% methanol were added and the mixture was incubated for 30 min in the dark. The absorbance was read at 517 nm (V-1200 spectrophotometer, Global Science). A standard calibration curve was prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (0-100 µmol). The results were expressed as micromoles of Trolox per gram of dry weight.

3.5. Pasting properties of starch

Pasting properties of starch in the white sorghum flour blend with 1% mushroom extracts were measured on a Rapid Visco Analyser (Perten RVA 4500, Australia). A sample of 2.5 g of white sorghum flour was transferred into canister and 25 mL of 1% (10 mg/mL) concentrated mushroom extracts were added. The slurry was heated to 50^{o} C at the rate of 10 C/min and stirred at 160 ppm for 10 s for thorough dispersion. The slurry was held at 50^{o} C for up to 1 min, and after that heated to 95^{o} C for 5min, and in the end cooled to 50^{o} C over 6.18 min. The pasting temperature (the temperature where viscosity first increases by at least 25 cp over a 20s period), peak time (the time at which peak viscosity occured), peak viscosity (the maximum hot paste viscosity), holding strength or trough viscosity (the trough at the minimum hot paste viscosity), final viscosity (the viscosity at the

end of test after cooling to 50^{o} C and holding at this temperature), breakdown (peak viscosity - holding strength or trough viscosity) and setback (final viscosity - holding strength) were calculated from the pasting curve, using Thermocline windows software (Perten RVA 4500, Australia).

Table 1 Detail of dilutions performed with RO water for DPPH radical scavenging activity assay

Dilutions with RO water							
		25 ^{o} C		50^{o} C	80^{o} C	1% concentrated extracts	Digested samples
Sample	Concentration of solvent	pH 7	pH 2	pH 7	pH 7	pH 7	
L. edodes	0%	8x	8x	7x	7x	5x	5x
	50%	5x	5x	5x	5x	5x	5x
	80%	4x	4x				
A. auricula	0%	1x	2x	2x	2x	5x	5x
	50%	1x	2x	1x	1x	5x	5x
	80%	1x	2x				
T. fuciformis	0%	1x	1x	2x	2x	5x	5x
	50%	1x	1x	1x	1x	5x	5x
	80%	1x	2x				

3.6. *In vitro* starch digestion

An *in vitro* digestion process was used to evaluate the sugar release over period of 120 min as described by (Foschia et al., 2015).

3.6.1 *In vitro* Starch Analysis

8.25 g of each sample was weighed in plastic biopsy pots in triplicates. Two sample empty containers was placed as reagent blanks. To 8.25 g of samples, 7.5 mL of RO water was added using Fortuna Optifix bottle top dispenser. The plastic biopsy pots were placed on pre-heated 15 place magnetic heated stiiring block as shown in figure (IKAMAG® RT15, IKA®-WERKE Gmblt & Co., Staufen, Germany). A small magnetic flea was placed in each sample container and the stirrer and heat was turned on. To each sample container, 0.4 mL of 1 M HCl was added. After that, the sample containers were covered with the large tinfoils sheets and towel. The temperature of the multistirrer was set up to 37^{o}C and maintained between 35 and 40^{o} C throughout the digestion. The next step was the addition of pepsin after setting the temperature to 37 ^{o}C. A 0.5 mL of 10% of pepesin solution in 0.05 M HCl was added to each sample pots. The tinfoil sheets and towel was replaced and left for 30 minutes at 37 ^{o} C with steady constant stirring. To each container 1 mL of 1 M $NaHCO_3$ and 2.5 mL of 0.1 Sodium maleate buffer ph 6 was added. A rack of 15 mL falcon tubes were set and labelled in the same configuration as the set up of the sample containers on the multisirrer. To each falcon tube, 1

mL of 100% ethanol and 0.25 mL of samples from each sample container and these collected samples were time 0 aliquots. After that 50 µL of amyloglucosidase was added to each sample container as amyloglucosidase prevents end product inhibition of pancreatic α-amylase. Then, 2.5 mL of 2.5% pancreating solution in 0.1 M sodium maleate buffer ph 6 was added to each container and 5 mL of RO water was added to make the digest reaction volume 26.25 mL accurately. Again the sample pots were covered with tin foil sheets and towel, and incubateda at 37 ^{o}C for 120 minutes with steady constant stirring. The samples were collected in the falcon tubes at an interval of 20 min, 60 min, and 120 min and these samples were time 20, time 60, and time 120 aliquots respectively. At the end of the process, the samples were centrifuges at 12000 G for 10 min (4^{o} C) to get the bioaccessible fractions and stored in the fridge at 4 degree C until analysis of reducing sugar.

3.6.2. Measurement of Reducing Sugars produced during *In vitro* digestion

The samples stored in Fridge at 4^{o} C were taken out and centrifuged at 1000 rpm for 5 minutes. To each test tube, 50 uL of aliquot from in vitro digestion was added. To this aliquot 0.25 mL of enzyme solution (1% invertase and 1% amyloglucosidase in actate buffer ph 5.2). The mixture was incubated for 20 minutes at room temperature. To this mixture, 0.75 mL of DNS mixture (0.5 mg/mL glucose : 4 M NaOH : DNS reagent) was added and the test tubes were covered with tinfoil in metal racks and placed in an electric frying pan filled with boiling water (95-100^{o} C) for 15 minutes. The absorbance was read at 530 nm (V-1200 spectrophotometer, Global Science). A standard calibration curve was prepared using glucose standard solution (5 mg/mL and 10 mg/mL). Glucose release was plotted against time and areas under the glucose release curves were calculated using the trapezoid rule.

3.7. Protein Content and Protein Digestibility

Protein content was determined by Thermofischer BCA (Bicinchoninic acid) Protein assay kit. The sample aliquots during digestion was collected at time 0 min, time 20 min, time 60 min, and time 120 min was heated for 5 minutes at 95^{o} C for BCA assay. This was the gastric and intestinal protein digestibility. A standard calibration curve was prepared by using BSA (Bovine Serum Albumin) (0 - 2000 µg/mL). The absorbance was read at 562 nm . The results were expressed as protein content (g/g dry weight) and protein digestibility (%age).

3.8. Statistical Analysis

The experiments were performed in triplicates. Statistical differences were analysed by one-way analysis of variance (ANNOVA) and Tukey's comparison test ($p < 0.05$). Pearson's correlations were also carried out to analyse the significant correlations at $p \leq 0.05$, $p \leq 0.01$,and $p \leq 0.001$, respectively.

Chapter 4

Extraction of poly-phenol compounds from Mushroom samples

Abstract

Extraction of polyphenol compounds from L. edodes, A. auricula, and T. fuciformis was performed with water and concentrations of methanol (50% and 80%), pH 2 and pH 7, temperature rate of 25^{o}C, 50^{o}C, and 80^{o}C. Total phenolic contents, ferric reducing antioxidant power, and DPPH radical scavenging activity were analysed. The mushroom extracts were concentrated to 1% and further total phenolic contents, ferric reducing power, DPPH radical scavenging activity, protein content, and yield of extracts was determined. The water extracts of mushroom samples showed significantly higher antioxidant activity, protein content, and yield percentage than methanol extracts at different conditions of extraction. L. edodes investigated with higher total phenolic contents, ferric reducing power, DPPH radical scavenging activity, protein content, and yield as compared to *A*. *auricula* and *T*. *fuciformis*.

4.1. Introduction

The hunt of new products with antioxidant properties is very active state of research. Mushrooms are becoming very attractive as functional foods and rich source of bioactive compounds like polyphenols and polysaccharides (Sarikurkcu, Tepe, & Yamac, 2008). Polyphenols are the metabolites comprised of one or more aromatic rings with one or more hydroxyl groups. These can reduce the ability of reactive oxygen species and are known for their antioxidant capacity (Xiaokang et al., 2020). Mushroom polyphenols are linked to phenolic acids and flavonoids with different amount of content within different species (Cayan, Deveci, Tel-Cayan, & Duru, 2020). Both caffeic and chlorogenic acids have investigated in Shiitake and many other mushroom species (Xiaokang et al., 2020). For the recovery of polyphenolic compounds from mushroom samples, extraction is the first step. Extraction can be performed by using conventional and non-conventional techniques. Conventional techniques include the use of water and several organic solvents such as methanol, ethanol, etc. Non-conventional techniques are microwave-assisted extraction, ultrasound assisted extraction, enzyme-assisted extraction, subcritical and supercritical fluid extraction (Xiaokang et al., 2020). However, solvent extraction is widely utilized for the isolation of phenolic contents from several plant materials. There are various factors which enhance the efficiency of extraction steps such as sample preparation, temperature and pH conditions, ratio of solvent and solid, and particle size of the solid contents (Zhang, Lv, pan, Wu, & Fan, 2009). The utilization of several solvents such as methanol, ethanol, acetone, and combination of these solvents with water has been widely reported in the

literature (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). The use of finely powdered plant material increases the extraction of polyphenols by enhancing the surface area of the sample and promoting breakdown of cell wall (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). The current study aims at analysing the effects of different temperatures, different ph, different concentrations of methanol, and RO water on the total phenolic content and antioxidant properties of *Lentinula edodes*, *Auricularia auricula*, and *Tremella fuciformis* samples.

4.2. Materials and methods

4.2.1. Materials

As described in 3.1.1.

4.2.2. Extraction of samples

Sample were prepared as described in 3.3.

4.2.3. Antioxidant analysis

Antioxidant analysis was determined as described in 3.4.

4.2.4. Protein analysis

The protein analysis was determined as described in 3.6.

4.2.5. Statistical analysis

Statistical analysis was performed as described in 3.7.

4.3. Results and discussion

4.3.1. Effect of different concentrations of methanol on the extraction of polyphenol compounds from mushroom samples at pH 7 and pH 2.

Mushrooms contain a wide variety of secondary metabolites such as phenolic substances, terpenes, polyketides, and steroids. Many common edible mushrooms are consumed widely in Asia and have been found to own antioxidant power which is linked to phenolic compounds (Sarikurkcu, Tepe, & Yamac, 2008). For the extraction of polyphenol compounds, different extraction methods were used and it is very difficult to develop a single efficient technique for the extraction of all the phenolic components which have different hydroxyl groups such as alkyl groups, sugars or acids, and several polarities (Sezer, Sufer, & Sezer, 2017). Methanol is the most suitable solvent for the extraction of polyphenols. It has been found that different concentrations of methanol for extraction positively affected the total phenolic components and antioxidant activity of *Agaricus bisporus* and *Pleurotus*

ostreatus (Sezer, Sufer, & Sezer, 2017). The commonly utilized kinds of solvents for the extraction of polyphenols are methanol, ethanol, acetone, and their aqueous mixtures. Consequently, alcoholic solvents are commonly employed for the extraction of polyphenols from natural sources as they give higher yield of extracts. In particular, the aqueous mixtures of alcohols have proved to be more proficient in phenolic compounds extraction than the comparable mono-component solvent system (Yang et al., 2009). Therefore, in this study, RO water and two different concentrations of methanol (50% and 80%) at different pH (pH 7 and pH 2) were used for the extraction of polyphenols from *L. edodes, A. auricula,* and *T. fuciformis.*

Total phenolic contents (TPC) of three mushroom samples with different methanolic extractions at pH 7 and pH 2 are shown in Table 2. The highest total phenolic contents were 8.52 mg GAE and 8.32 mg GAE in RO water extracts of *L. edodes* at pH 7 and pH 2 respectively, while the lowest total phenolic contents were 4.52 mg GAE and 4.27 mg GAE in 80% methanolic extracts of *L. edodes* at pH 7 and pH 2 respectively (Table 2). Furthermore, in case of *A. auricula* and *T. fuciformis,* the total phenolic content was higher in RO water extracts followed by 50% methanol and 80% methanol (Table 2). The sequence for solubility of total phenolic content of three mushroom samples was RO water extract > methanol extract (50%) > methanol extract (80%) ($p < 0.05$). This happens may be because of the pressure created by water inside the cell wall and results in breakdown of cell wall, hence release of bioactive substances (Xiaokang et al., 2020). The effect of pH was also found to be significantly different ($p < 0.05$) on TPC which decreases on decreasing pH in case of all the three mushroom samples. Although, the different concentrations of methanol had a significant effect ($p < 0.05$) on TPC for *L. edodes, A. auricula,* and *T. fuciformis* (Table 2). These results of total phenolic contents showed that RO water extracts recovered higher total phenolic contents than methanolic extracts. These out comes are not in agreement with other studies which represented that total phenolic contents were higher in 80% methanolic extracts of *Agaricus bisporus* and *Pleurotus ostreatus* (Sezer, Sufer, & Sezer, 2017). On the other hand, the total phenolic contents in water extracts of *L. edodes* was higher (8.52 mg GAE) as compared to *A. auricula* (2.83 mg GAE) *and T. fuciformis* (2.30 mg GAE). A study conducted on mycelial exudates of *L. edodes* strains showed that the total phenolic content was 237.33 and 24.08 mg GAE (Huang, Kim, & Chung, 2010). Meanwhile, the methanolic extracts of *L. edodes* represented the total phenolic contents in the range of 6.27 to 9.11 mg GAE (Yang, Lin, & Mau, 2002). The results of our study are not in accordance with the previous studies conducted because our studies showed that water extracts of mushroom samples have higher total phenolic contents as compared to methanolic extracts. This might be due to the presence of water-soluble phenolic compounds like phenolic acids, phenylpropanoids, flavonoids, and quinones in the mushroom samples (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012).

Table 2 The level of TPC, FRAP, and DPPH of Lentinula edodes, Auricularia auricula, and Tremella fuciformis at pH 7 and pH 2 (25°C).

Sample	Solvent	TPC (GAE conc. (mg/g)		FRAP (µmol Fe^{2+}/g)		DPPH (µmol Trolox equivalents/g)	
		pH 7	pH 2	pH 7	pH 2	pH 7	pH 2
L. edodes	RO Water	8.52 ± 0.12^{aA}	8.32 ± 0.10^{aA}	95.62 ± 0.35^{aA}	79.87 ± 4.2^{aB}	25.32 ± 2.11^{aA}	16.78 ± 0.95^{bA}
	50% Met OH	5.47 ± 0.08^{bA}	5.23 ± 0.13^{bA}	76.31 ± 1.29^{bA}	79.75 ± 4.31^{aA}	15.90 ± 0.52^{bA}	14.09 ± 0.19^{bB}
	80% Met OH	4.52 ± 0.03^{cA}	4.27 ± 0.41^{cA}	62.24 ± 2.74^{cA}	51.16 ± 3.89^{bB}	13.89 ± 0.32^{bA}	11.17 ± 0.43^{cB}
A. auricula	RO Water	2.83 ± 0.14^{dB}	2.21 ± 0.07^{dCD}	30.41 ± 4.95^{dC}	23.77 ± 0.67^{cCD}	3.07 ± 0.17^{dC}	1.65 ± 0.09^{dC}
	50% Met OH	1.56 ± 0.14^{fBC}	1.36 ± 0.04^{eC}	22.48 ± 0.37^{eB}	18.93 ± 0.10^{cdBC}	3.05 ± 0.62^{dD}	1.43 ± 0.04^{dC}
	80% Met OH	1.10 ± 0.05^{gB}	1.15 ± 0.03^{efB}	15.87 ± 0.21^{fgC}	15.88 ± 0.36^{dC}	2.89 ± 0.05^{dC}	1.21 ± 0.27^{dD}
T. fuciformis	RO Water	2.30 ± 0.02^{eC}	2.00 ± 0.06^{dD}	20.60 ± 0.63^{efDE}	14.83 ± 0.30^{dE}	1.62 ± 0.05^{cC}	1.17 ± 0.07^{eC}
	50% Met OH	1.73 ± 0.00^{fB}	0.85 ± 0.02^{FD}	14.44 ± 0.13^{gCD}	12.68 ± 0.55^{dD}	1.20 ± 0.10^{cD}	0.88 ± 0.05^{eD}
	80% Met OH	0.73 ± 0.02^{hB}	0.83 ± 0.07^{fB}	12.65 ± 0.26^{gC}	12.26 ± 0.26^{dC}	1.14 ± 0.18^{cD}	0.67 ± 0.05^{eD}

Mean ± standard deviation. Values within a column followed by the same lowercase letter are not significantly different from each other (p<0.05, n = 3). Values within a row followed by same capital letters are not significantly different from each other (p<0.05, n = 3).

The Ferric reducing antioxidant power of mushroom samples with different methanol extractions at pH 7 and pH 2 is summarized in Table 2. The ferric reducing power of *L. edodes* had highest level (95.62 µmol Fe^{2+}/g) in water extraction followed by 76.31 µmol Fe^{2+}/g and 62.24 µmol Fe^{2+}/g in 50% and 80% methanol extractions respectively at pH 7. But in case of pH 2, the FRAP levels were lower for *L. edodes* as compared to pH 2. Furthermore, ferric reducing antioxidant power of *A. auricula* was 30.41 µmol Fe^{2+}/g and 23 µmol Fe^{2+}/g in water extracts, 22.48 µmol Fe^{2+}/g and 18.93 µmol Fe^{2+}/g in 50% methanolic extracts, and 15.87 µmol Fe^{2+}/g and 15.88 µmol Fe^{2+}/g in 80% methanolic extracts at pH 7 and pH 2 respectively. However, the lowest level of FRAP was shown by *T. fuciformis* as compared to *L. edodes* and *T. fuciformis.* The results explained above revealed that the trend of ferric reducing power is higher in water extracts followed by 50% methanol and 80% methanol extracts in three mushroom samples. The statistical analysis indicated that FRAP of *L. edodes, A. auricula,* and *T. fuciformis* was affected significantly ($p < 0.05$) by using water and different methanol concentrations at pH 7 and pH 2 (Table 2).

The DPPH radical scavenging activity of mushroom samples was much less as compared to ferric reducing power (Table 2). The DPPH radical scavenging activity of *L. edodes* water extracts was highest 25.32 µmol Trolox equivalents/g and 16.78 µmol Trolox equivalents/g) at pH 7 and pH 2 respectively than methanolic extracts. However, the DPPH radical scavenging activity was very less in *A. auricula* and *T. fuciformis* with values ranging from 1.14 µmol Trolox equivalents/g to 3.07 µmol Trolox equivalents/g and 0.67 µmol Trolox equivalents/g to 1.65 µmol Trolox equivalents/g in

different concentration of methanol extracts at pH 7 and pH 2 respectively. The DPPH radical scavenging activity also followed the same trend as total phenolic contents and ferric reducing power highest in water extracts and lowest in 80% methanol extracts. The water and different concentrations of methanol ($p < 0.05$) showed significant effect on DPPH radical scavenging activity at pH7 and pH 2 ($p < 0.05$) (Table 2). These results are not consistent with the previous studies which represented the highest total phenolic contents and antioxidant power in mushroom samples in 80% methanol extracts (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). The study conducted on extraction of phenolic compounds from tiger nuts showed that pH not affected significantly the recovery of phenolic substances (Rosello-Soto et al., 2019). It has been also reported that the decrease in pH assists the extraction of phenolic acids and total phenols along with enhanced antioxidant activity of extracts (Putnik, Bursac Kovacevic, Radojcin, & Dragovic-Uzelac., 2016). Moreover, the stability of phenolic compounds depends upon the pH (Freidman & Jurgens, 2000). But our study showed that the recovery of phenolic compounds decreases on decreasing the pH during extraction process.

4.3.2. Effect of concentration of solvent on the level of polyphenols and protein content of the 1% concentrated aqueous mushroom extracts

The level of total phenolic contents (Fig. 1a) was highest in water extracts of *L. edodes* (24.61 mg GAE/g), followed by *A. auricula* (18.93 mg GAE/g), and *T. fuciformis* (18.37mg GAE/g). In the 50% methanol extracts, the level of TPC detected was lower compared to water extracts of three mushrooms. The statistical analysis revealed that concentration of solvent affected significantly ($p < 0.05$) the total phenolic contents in 1% concentrated mushroom extracts. Similarly, the ferric reducing antioxidant power of 1% concentrated mushroom extracts (Fig 1b) also followed the same tend as TPC, higher level of ferric reducing antioxidant power in water extracts followed by 50% methanol extracts. *L. edodes* has higher antioxidant power followed by *A. auricula* and *T. fuciformis* in both water and methanol extracts. The solvent concentration also showed significant ($p < 0.05$) effect on the ferric reducing antioxidant power of 1% concentrated mushroom extracts. Furthermore, the DPPH radical scavenging activity of mushroom extracts also got affected significantly ($p < 0.05$) by the concentration of solvent (Fig. 1c). The water extracts of *L. edodes* investigated with higher radical scavenging activity, however, in 50% methanol extracts, the radical scavenging activity dropped down four times. These results are not consistent with the previous study conducted on effect of solvent on olive leaf extracts showed that methanol extracts possess highest total phenolic contents and antioxidant activity (Cho, Kim, Lee, Yeon, & Lee, 2020). This might be because of the presence of polyphenols that are more soluble in water than methanol). *L. edodes* extracts (1% concentrated) using water had higher levels of polyphenols and were highly antioxidant. On the other hand, water and 50% methanol extracts of *A. auricula* and *T. fuciformis* was detected

with very low radical scavenging activity as compared to *L. edodes.* But in case of *T. fuciformis,* the methanol extracts showed higher radical scavenging activity than water extracts. The study conducted on effect of solvent on olive leaf extracts also showed that methanol extracts possess highest total phenolic contents and antioxidant activity (Cho, Kim, Lee. Yeon, & Lee, 2020). The statistical analysis revealed that concentration of solvent had significant ($p < 0.05$) effect on the radical scavenging activity of *L. edodes* and *T. fuciformis* but not ($p > 0.05$) on the antioxidant activity of *A. auricula.*

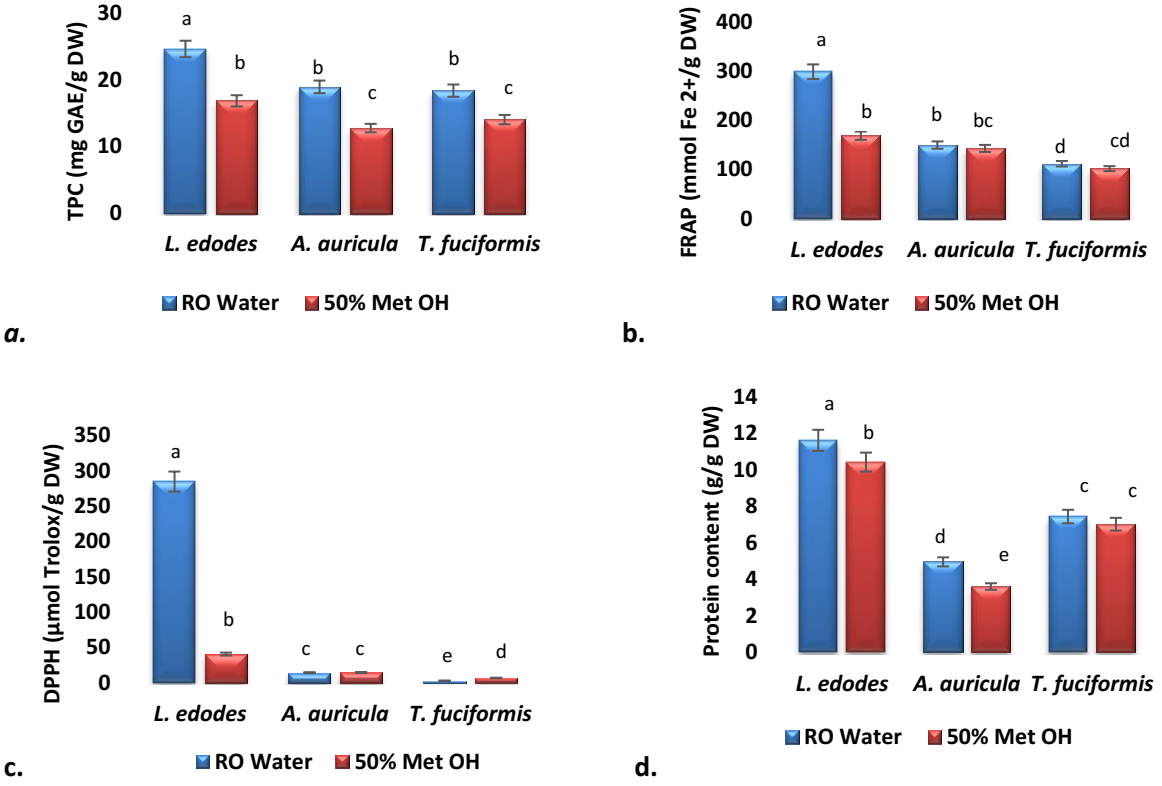


Figure 1 Level of polyphenols and protein contents in 1% concentrated aqueous extracts of mushroom samples a. TPC (mg GAE/g DW), b. FRAP (mmol Fe 2+/g DW), c. DPPH (µmol Trolox equivalents/g DW), and d. Protein content (g/g DW).

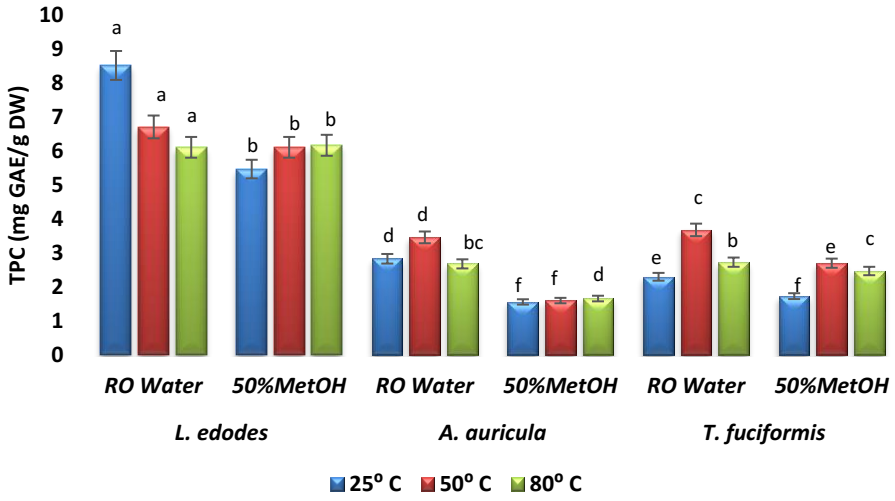
The results obtained from the protein content analysis of 1% concentrated extracts are described in Fig 1d. The results revealed that the highest protein content was detected in water extracts of *L. edodes* (11.61 g/g) followed by *T. fuciformis* (7.44 g/g) and *A. auricula* (4.96 g/g). The 50% methanol extracts of *L. edodes* contained 10.42 g/g of protein higher than *T. fuciformis* and *A. auricula*. However, the water extracts of mushrooms were detected with higher protein content than 50% methanol content. If compared between the species the higher amount of protein content was detected in *L. edodes* both in water and 50% methanol extracts Than *T. fuciformis* and *A. auricula.* The previous study conducted on the wheat shiitake noodles showed that with the incorporation of shiitake powder increases the protein content of noodles (Wang et al., 2020).

4.3.3. Effect of temperature (25^{o}C, 50^{o}C, and 80^{o}C) on the extraction of polyphenol compounds from mushroom samples.

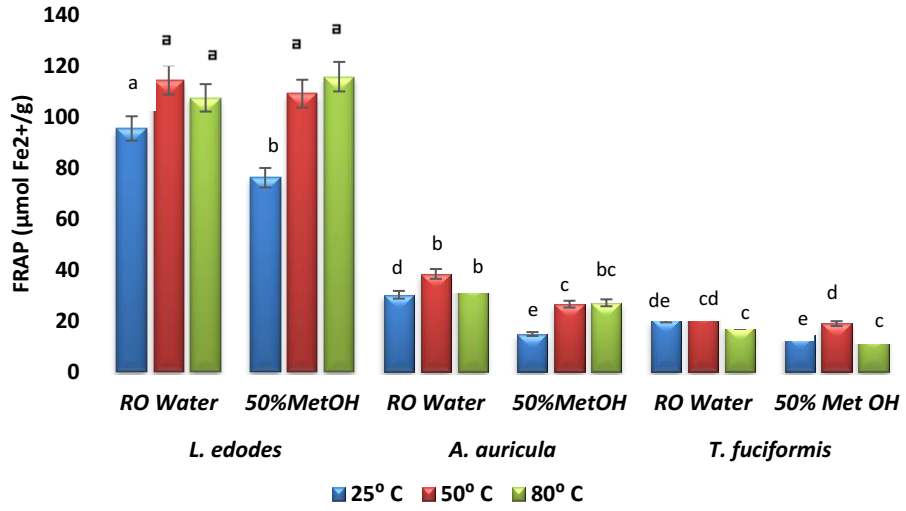
Temperature is one of the significant parameters of the extraction processes for recovering polyphenolic substances from the natural sources. The results of polyphenolic substances extracted at different temperatures are summarized in Fig. 2a, 2b, and 2c which shows the total phenolic contents, ferric reducing power and DPPH radical scavenging activity of *L. edodes, A. auricula,* and *T. fuciformis.* The RO water extracts of *L. edodes* have released higher phenolic contents (8.52 mg GAE/g) than *A. auricula* and *T. fuciformis* at 25^{o} C. The 50% methanol extracts of *L. edodes* released 5.47 mg GAE/g of total phenolic contents followed by *T. fuciformis* (1.73 mg GAE/g) and *A. auricula* (1.56 mg GAE/g) at 25^{o} C. The temperature (25^{o} C) showed significant effect ($p<0.05$) on the total phenolic contents released from water and methanol extracts of three mushroom samples (Fig. 2a). Furthermore, with the increase in temperature to 50^{o} C and 80^{o} C, the phenolic contents ranged between 6.11 mg GAE/g to 6.71 mg GAE/g in RO water and 50% methanol extracts of *L. edodes* which was less compared to release of phenolics at 25^{o} C. On the other hand, in case of *A. auricula* the release of phenolics was only 3.46 mg GAE/g and 1.6 mg GAE/g from RO water and 50% methanol extracts respectively, at 50^{o} C with the existence of significant effect ($p < 0.05$) of temperature on phenolic contents. With the increase in temperature to 80^{o} C, the amount of phenolics decreased to 2.68 mg GAE/g and 1.66 mg GAE/g in water and methanol extracts of *A. auricula* respectively. But the phenolic contents were increased in the water and methanol extracts of *T. fuciformis* with the increase in temperature to 50^{o} C. Consequently, the amounts of phenolic contents were 2.73 mg GAE/g and 2.47 mg GAE/g at 80^{o} C in water and methanol extracts of *T. fuciformis.* Different temperatures showed significant effect ($p <0.05$) on the total phenolic contents released from the water and methanol extracts of *L. edodes, A. auricula,* and *T. fuciformis* (Fig. 2a). Previous studies revealed that the increase in extraction temperature from 50 to 200^{o} C lead to two-fold increase in total antioxidants in deodorized thyme (Vergara-Salinas, Perez-Jimenez, Torres, Agosin, & Perez-Correa, 2021). The results of present study are not in agreement with this study because in present study only small amount of total phenolic contents increased in case of methanolic extracts of *L. edodes* and water extrtacts of *A. auricula* and *T. fuciformis* at 50^{o} C but not at 80^{o} C (Fig.2a). Another study conducted on apricot pomace, the polyphenol recovery had increased with increase in temperature (Cheaib, Darra, Rajha, Maroun, & Louka, 2018). The increase in temperature promotes extraction, elevating the solubility and diffusion coefficient, but beyond a certain limit phenolic substance could be decomposed. The stability of substance might be affected because of chemical and enzymatic degradation or depletion by thermal decomposition. This was the preliminary mechanism causing loss of polyphenol content in *Schizophyllum commune* (Yim et al., 2013).

The results obtained from the FRAP are described in Fig 2b. The Ferric reducing power of water extracts of *L. edodes* was 95.62 µmol Fe^{2+}/g at 25^{o}C and increased to 114.64 µmol Fe^{2+}/g at 50^{o}C and then decreased at 80^{o}C to 107.63. On the contrary, in case of 50% methanol extracts of *L. edodes* and *A. auricula,* the ferric reducing power showed elevation with increase in temperature. These results are in accordance with the study conducted on thyme and apricot pomace in which antioxidant activity increased as the temperature increased (Vergara-Salinas et al., 2021; Cheaib et al., 2018). The increase in antioxidant activity might be due to the enhancement in the solubility of polyphenol compounds as the temperature increases (Vergara-Salinas et al., 2021). The ferric reducing power of water extracts of *A. auricula* and *T. fuciformis* and methanol extracts of *T. fuciformis* followed the same trend as the water extracts of *L. edodes*. The decrease in ferric reducing power could be due to the decomposition of antioxidants due to increase in temperature beyond a certain point (Yim et al., 2013). The statistical analysis showed that different temperatures affected significantly ($p < 0.05$) the antioxidant activity of the water and methanolic extracts of *L. edodes, A. auricula,* and *T. fuciformis.*

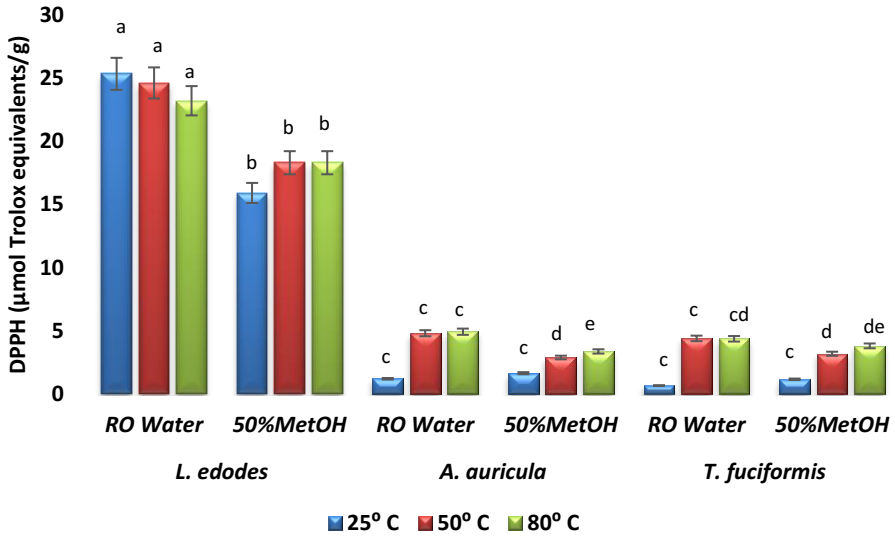
The DPPH radical scavenging activity of three kinds of mushrooms is shown in Fig 2 c. The results showed that the level of DPPH radical scavenging activity was higher in water extracts of *L. edodes* at 25^{o} C and decreased as the temperature increased to 50^{o} C, and 80^{o} C. The decrease in radical scavenging might be due to the thermal decomposition of bioactive compounds (Yim et al., 2013). On the other hand, the radical scavenging activity increased with the increase in temperature in 50% methanol extracts of *L. edodes*. Furthermore, the radical scavenging activity of the water and methanol extracts of *A. auricula* and *T. fuciformis* increased with increase in temperature. The increase in radical scavenging activity may be because of the enhancement in the solubility of polyphenol compounds in the solvent as the temperature increases (Vergara-Salinas et al., 2021). The statistical analysis suggested that different temperatures showed significant effect ($p < 0.05$) on the DPPH radical scavenging activity of the water and methanol extracts of *L. edodes, A. auricula,* and *T. fuciformis* (Fig. 2c).



a)



b)



c)

Figure 2 (a) The level of TPC (mg GAE/g dry weight) (b) FRAP (µmol Fe2+ equivalent/g dry weight) (c) DPPH (µmol Trolox equivalent/g dry weight) of Lentinula edodes, Auricularia auricula, and Tremella fuciformis at temperature (25° C, 50° C, and 80° C) and error bar

4.3.4. Comparison of extraction of polyphenols with RO water and 50% Methanol & yield of extracts.

Efficient extraction of bioactive components requires the utilization of different types of solvents with different polarities because few antioxidants are better soluble in polar solvents like methanol and water (Smolskaite, Venskutonis, & Talou, 2015). The extraction of a given compound from the sample depends upon the solubility of substance as well as polarity of solvent and a study showed that methanol is used as an extraction solvent for extracting polycyclic compounds specifically in a lignin-based substrate such as mushrooms (Ohiri, Ifeanachor, & Preye, 2019). It is obvious (Fig. 3) that the three mushroom samples consisted of various classes of compounds, the solubility of which depends on the solvent used for extraction. Therefore, the highest total yield of all fractions obtained from *L. edodes* was 33.47% in water and 33.3% in 50% methanol. These results are in agreement of the study conducted on different mushroom species which determined higher yield extract in water and methanol (Smolskaite, Venskutonis, & Talou, 2015). The polar solvents have high dielectric constant which gave higher yield of extracts (Smolskaite, Venskutonis, & Talou, 2015). The statistical analysis revealed that no significant differences ($p > 0.05$) existed among the yield percentage of water and 50% methanol extracts of *L. edodes.* Furthermore, the lowest yield extract was observed in *A. auricula* which was 10.81% in water extracts and only 6.4% in 50% methanol extracts. The yield of water extracts of *T. fuciformis* was 16 % and that of 50% methanol was only 10%. The type of solvent significantly ($p<0.05$) affects the yield percentage of extracts of *L. edodes, A. auricula,* and *T. fuciformis.*

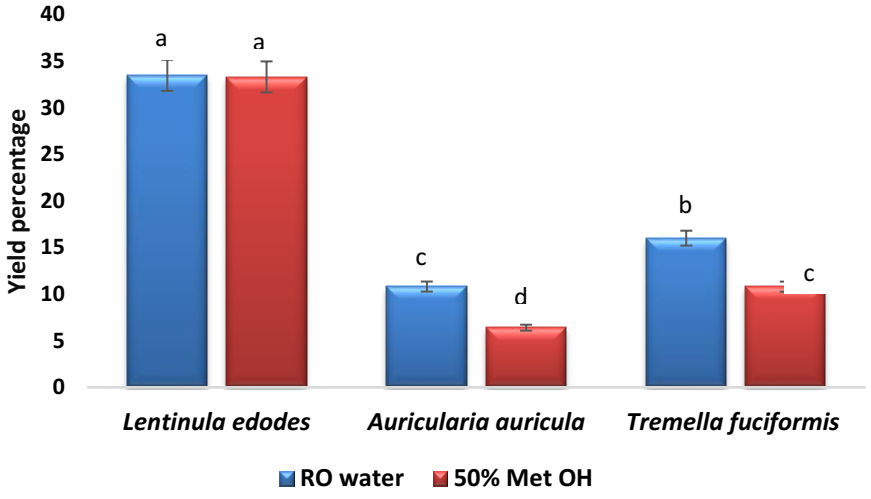


Figure 3 Yield percentage of mushroom extracts extracted with RO water and 50% Methanol.

4.3.5. Pearson's correlation between the antioxidant properties, protein content, and yield of mushroom extracts.

The results shown in Table 2 and Figure 2 and Figure 3 clearly indicated that the quantitative evaluation of TPC, FRAP, and DPPH values influenced by variables that are extracting solvent concentration, extraction temperature, pH, species of mushrooms. Therefore, the correlation analyses between the studied parameters were analysed within the extracts of each variable. The results revealed significant positive correlation between TPC, FRAP, and DPPH. As described in Table 3, the highest linear correlation (r = 0.998, $p \leq 0.001$) was determined within the levels of DPPH radical scavenging activity at 80^{o} C and 50^{o} C (pH 7) of three mushroom species. These findings suggested that strong positive correlations between the level of DPPH radical scavenging activity at 50^{o} C and 80^{o} C. Moreover, a significant positive correlation is observed between FRAP at 25^{o} C and both level of DPPH radical scavenging activity at 50^{o} C and 80^{o} C (r = 0.991, at $p \leq 0.001$) (Table 3). The correlation analysis revealed that the highest correlation existed between the FRAP at 80^{o} C and TPC at 25^{o} C and 50^{o} C (pH 7) with lowest but statistically significant r value of 0.897 and 0.899 ($p \leq 0.001$), respectively. The TPC of 1% concentrated extracts showed statistically significant correlation ($p \leq 0.05$) with TPC (r = 0.645) and FRAP (r = 0.552) at 80^{o} C. Protein content was positively correlated with the TPC and FRAP (1% concentrates) with r value 0.667 and 0.648 respectively showing significant differences ($p \leq 0.01$). The results of correlations demonstrated that the extraction of polyphenols performed at different temperatures and different pH and 1% concentrated extracts exhibited direct significant positive correlations ($r > 0.9000$, $p \leq 0.001$, 0.01, 0.05) between TPC, FRAP, DPPH, protein content, and yield of extracts values. The results of present study are according to the previous study conducted on edible mushrooms which also showed positive and direct correlations of TPC with antioxidant activity of mushroom hydro-ethanol extracts (Bach et al., 2019).

Table 3 Pearson's correlations between the total phenolic contents (mg GAE/g dry weight), ferric reducing power (µmol Fe2+ equivalent/g dry weight), DPPH radical scavenging activity (µmol Trolox equivalents/g dry weight), and yield (%age) of three mushroom samp

	TPC 25° C	TPC 50° C	TPC 80° C	FRAP 25° C	FRAP 50° C	FRAP 80° C	DPPH 25° C	DPPH 50° C	DPPH 80° C	TPC 25° C pH 2	FRAP 25° C pH 2	DPPH 25° C pH 2	Yield of extracts	TPC (1%)	FRAP (1%)	DPPH (1%)
TPC 50° C	0.934***															
TPC 80° C	0.924***	0.976***														
FRAP 25° C	0.976***	0.928***	0.953***													
FRAP 50° C	0.94***	0.931***	0.971***	0.987***												
FRAP 80° C	0.897***	0.899***	0.958***	0.964***	0.989***											
DPPH 25° C	0.978***	0.902***	0.929***	0.98***	0.953***	0.925***										
DPPH 50° C	0.984***	0.946***	0.964***	0.991***	0.977***	0.948***	0.989***									
DPPH 80° C	0.976***	0.944***	0.971***	0.991***	0.981***	0.955***	0.988***	0.998***								
TPC 25° C pH 2	0.996***	0.927***	0.919***	0.98***	0.945***	0.905***	0.979***	0.985***	0.977***							
FRAP 25° C pH 2	0.924***	0.918***	0.968***	0.978***	0.997***	0.991***	0.946***	0.97***	0.976***	0.931***						
DPPH 25° C pH 2	0.954***	0.907***	0.955***	0.988***	0.987***	0.975***	0.98***	0.984***	0.99***	0.958***	0.986***					
Yield of extracts	0.914***	0.978***	0.989***	0.935***	0.95***	0.932***	0.919***	0.956***	0.96***	0.916***	0.948***	0.934***				
TPC (1% extracts)	0.815***	0.773***	0.645*	0.705***	0.628**	0.552*	0.698***	0.722***	0.693***	0.803***	0.588**	0.624**	0.660**			
FRAP (1% extracts)	0.917***	0.729***	0.726***	0.873***	0.806***	0.755***	0.905***	0.868***	0.857***	0.915***	0.784***	0.855***	0.700***	0.768***		
DPPH (1% extracts)	0.904***	0.720***	0.690**	0.823***	0.737***	0.667**	0.882***	0.845***	0.825***	0.899***	0.713***	0.794***	0.688**	0.804***	0.956***	
Protein content (1% extracts)	0.866***	0.939***	0.928***	0.840***	0.838***	0.803***	0.864***	0.892***	0.894***	0.851***	0.834***	0.841***	0.951***	0.667**	0.648**	0.697***

Abbreviations: TPC, Total phenolic contents; FRAP, Ferric reducing antioxidant power; DPPH, radical scavenging activity; 1%, extracts concentrated to 1% (10 mg/g); Values represent the Pearson correlation coefficient (r), Significance: * represents p ≤ 0.001, ** represents p ≤ 0.01, * represents p ≤ 0.05.**

4.4. Conclusions

This study has shown that the water extracts of *L. edodes, A. auricula,* and *T. fuciformis* has higher total phenolic contents and antioxidant activity than 50% methanol mushroom extracts. Moreover, with decrease in pH, the polyphenols also decreased. The effect of temperature was different depending upon the solvent and mushroom species. The water extracts of mushrooms released more polyphenols as compared to methanol extracts with change in temperature. However, *L. edodes* showed higher amount of release of polyphenols in all the conditions such as solvent concentration, temperature, and pH than *A. auricula* and *T. fuciformis.* Similarly, 1% concentrated *L. edodes* extracts also showed higher antioxidant activity and nutritional content (protein) than *A. auricula* and *T. fuciformis.* Furthermore, the yield percentage of *L. edodes* was higher and that of *A. auricula* was lesser. Therefore, *L. edodes* possess good nutritional value as well as antioxidant activity. Further research about the nutritional and antioxidant activity of *A. auricula* and *T. fuciformis* should be done in the future.

Chapter 5

In vitro digestion of white sorghum flour with addition of mushroom aqueous solvent extracts

Abstract

White Sorghum flour with 1% concentrated mushroom extract from three different species of mushrooms (*L. edodes, A. auricula,* and *T. fuciformis*) were investigated in terms of pasting properties and starch characteristics (gelatinisation and digestibility). Mushroom extracts have decreased significant effect on the pasting properties of white sorghum flour compared to control. The reducing sugar release over 120 minutes in an *in vitro* digestion of the control increased more rapidly than the samples containing mushroom extracts. According to area under the curve (AUC) values, the incorporation of mushroom extracts reduced the predicted glycaemic response of the white sorghum flour, which is related to the decreased the degree of starch gelatinisation. Overall, mushroom extracts could be added to food products to provide health benefits to consumers.

5.1. Introduction

Sorghum is a primary food crop in many countries of Africa and Asia for poorest people. Many studies revealed that sorghum will replace wheat in the future (Souilah et al., 2014). *Sorghum vulgare* and *Sorghum bicolor* are the two species of genus Sorghum and members of grass family (Kulamarva, Soslle, & Raghavan, 2009). It is a good source of starch and protein and is gluten free. The grain of Sorghum comprised of phenolic compounds such as flavonoids which interferes with tumour development. The amount of sugar and starch in sorghum released very slowly as compared to other cereals and thus useful to patients suffering from diabetes (Kulamarva, Soslle, & Raghavan, 2009). Despite having several benefits of consuming sorghum, there is major limitation of white sorghum like decreased digestibility (Olojede, Sanni, Banwo, & Adesulu-Dahunsi, 2020). Moreover, mushrooms are good sources of bioactive substances, dietary fibres and unsaturated fatty acids. Particularly, they contain ergothioneine, which is not synthesized in the human body (Lu, Lou, Hu, Liu, & Chen, 2020). Currently, researchers focussing on the utilization of mushroom bioactive compounds in functional foods to increase the nutritional level of the product. For example, the compounds from the *Pleurotus eryngii* were used to decrease the post-prandial cholesterol levels (Jin et al., 2018). But the challenge is how to use the bioactive compounds in food systems and human nutrition (Lu et al., 2020). The recent study conducted on muffins showed the utilization of *L. edodes* in improving the nutritional quality of muffins (Olawuyi & Lee, 2019). The addition of mushroom extracts has a great impact on the gelatinisation and digestibility of starch molecules and quality of

food products. This is associated to the interactions (crosslinking, entanglement, and encapsulation) among starch and mushroom polysaccharides (Tu, Brennan, & Brennan, 2021). Mushroom polysaccharides show hypoglycaemic effect throughout the starch digestion by forming physical barriers to digestive enzymes and inhibiting gelatinisation of starch (Tu, Brennan, & Brennan, 2021). In present study, the potential glycaemic index of white sorghum with addition of three different mushroom aqueous extracts (0% and 50% methanol) were determined using an in vitro digestion model, described by (Gao et al., 2016).

5.2. Materials and methods

5.2.1. Materials

As described in 3.1.1.

5.2.2. In vitro starch digestion analysis

The samples stored in fridge after pasting was defrosted for 10 minutes at room temperature. The digestion was carried out as described in 3.5.

5.2.3. Statistical analysis

Statistical analysis was carried out as described in 3.7.

5.3. Results and discussion

5.3.1. Effect of pasting properties of mushroom aqueous solvent extracts on the white sorghum flour.

The effect of pasting properties on gelatinisation of Sorghum starch by addition of three different mushroom extracts with different solvent concentration is shown in Table 4. The shape of the pasting curve differed depending upon the type of mushroom extract used (Figure 4). The addition of mushroom extracts showed significant ($p < 0.05$) decrease in peak viscosity (PV) compared to control which contains only flour and water. PV is closely linked to the degree of starch destruction with higher the peak viscosity, higher the starch destruction (Adepehin, 2020). The results showed that the PV of control was 219 cp and it decreased to 120 cp and 110.3 cp on addition of water and 50% methanol extracts (1% concentrated) of *L. edodes* respectively. The decreased PV of sorghum flour indicated a lesser ability of starch to swell before breakdown. A study conducted on fermented sorghum sourdough showed an increase in PV by 51.6% which represents the capability of penetration of water into starch granules throughout fermentation and elevated level of amylase leaching (Adepehin, 2020). Moreover, the PV of white sorghum flour containing water and 50%

methanol extracts was 183 cp and 164 cp respectively while it was 169 cp and 129 cp in case of flour containing water and 50% methanol extracts of *T. fuciformis.*

The trough viscosity (TV) also decreased as PV following the same trend as PV. The trend was Control > Flour containing *T. fuciformis* extracts > Flour containing *A. auricula* extracts > Flour containing *L. edodes* extracts. The decrease in trough viscosity might be because of the decreasing effect of mushroom extracts on the hot paste viscosity of white sorghum (Adepehin, 2020). Furthermore, breakdown viscosity indicates the ability to resist heating and shear stress while cooking (Addebowalle, Sanni, & Awonorin, 2005). The less stability of starch paste is generally associated with high breakdown viscosity (Shimelis et al, 2006). The breakdown viscosity of control was higher than the flour with three different mushroom extracts. Among the samples, the flour with *A. auricula* extracts had higher value with 11 cp and 14 cp for water and 50% methanol extracts respectively. The smaller breakdown viscosity was 0.6 in flour with *L. edodes* water extracts. These results are consistent with the study conducted on fermented white sorghum sourdough (Adepehin, 2020).

The final viscosity (FV) indicates the stability of starch during cooking having capacity of starch in forming viscous paste after cooling (Shimelis et al., 2006). The FV of control was higher (646 cp) than flour containing mushroom extracts. Flour with *T. fuciformis* water extracts have higher viscosity than 50% methanol extracts. However, flour containing *A. auricula* 50% methanol extracts have higher final viscosity (424 cp) than flour with water extracts (322 cp). These results indicated that the water binding capacity and the formation of gel has decreased on addition of mushroom extracts. These results are contradictory with the results obtained from study conducted on fermented sorghum sourdough which showed increase in final viscosity (Adepehin, 2020).

Setback viscosity (SV) is the measure of stability of paste throughout cooling. The SV of control was 436 cp which is higher than flour with mushroom extracts. The SV of flour decreased on adding mushroom extracts. This can be ascribed to the breakdown of starch, which affected the primary structure of the cooled starch paste (Enujiugha, 2006). These results revealed that the addition of mushroom extracts lowers the degree of starch syneresis and degradation lead to lower the stale rate. The decrease in SV results in lower rate of starch degradation and syneresis (Gull et al., 2016). The addition of mushroom extracts in white sorghum four showed significant ($p < 0.05$) effect on gelatinisation of white sorghum starch with lower values for PV, TV, FV, BV, and SV than control (Table 4).

The trough viscosity and peak viscosity showed highest signification correlation with $r = 0.993$, $p < 0.001$. Breakdown viscosity is positively correlated with peak viscosity ($r = 0.640$, $p < 0.01$) and trough viscosity ($r = 0.551$, $p < 0.01$). In addition to it, Final viscosity showed positive correlation with peak viscosity ($r = 0.752$, $p < 0.001$) and trough viscosity ($r = 0.745$, $p < 0.001$) with significant effects. Set back viscosity is also positively correlated with peak viscosity, trough viscosity, and final viscosity. Peak time is negatively correlated with peak viscosity, trough viscosity, and final viscosity with no

significant differences among the pasting properties. However, peak time is positively correlated with breakdown viscosity (r = 0.639 p < 0.01) and negatively correlated with setback viscosity (r = -0.463, p < 0.05) with significant differences. Pasting temperature is negatively correlated with peak time with no significant differences.

Table 4 Average RVA starch pasting properties of white sorghum flour that contain 1% different mushroom extracts

Mushroom Extracts	Peak viscosity (cp)	Trough viscosity (cp)	Breakdown viscosity (cp)	Final viscosity (cp)	Setback viscosity (cp)	Peak time (min)	Pasting temp. (°C)
Control	219.3 ± 3.78^{a}	209.6 ± 4.1^{a}	9.6 ±0.5^{b}	646 ± 32.07^{a}	436.3 ± 27.9^{a}	5.6 ± 0.07^{c}	95.3 ± 0.0^{a}
L. edodes							
RO Water	120 ± 1de	119.3 ± 1.1^{e}	0.6 ± 0.5^{c}	354.6 ± 1.5^{de}	235.3 ± 0.5^{cd}	5.9 ± 0.2^{b}	95.2 ± 0.0^{a}
50% Met OH	110.3 ± 2.5^{e}	109.3 ± 3.2^{f}	1 ± 1^{c}	323.3 ± 5.7^{e}	214 ± 2.6^{d}	5.9 ± 0.1^{b}	95.2 ± 0.05^{a}
A. auricula							
RO Water	183 ± 4.3^{b}	175.6 ± 1.5^{b}	11 ± 1^{b}	322 ± 2^{e}	134 ± 1^{e}	6.9 ± 0.0^{a}	95.2 ± 0.0^{a}
50% Met OH	164.3 ± 3.5^{c}	150 ± 2^{d}	14 ± 1^{a}	424.3 ± 12.0^{bc}	261.6 ± 13.3^{bc}	6.9 ± 0.0^{a}	95.2 ± 0.05^{a}
T. fuciformis							
RO Water	169.3 ± 5.6^{c}	160 ± 6.0^{c}	9 ± 1^{b}	448 ± 20^{b}	287.6 ± 14^{b}	6.9 ± 0.0^{a}	95.2 ± 0.0^{a}
50% Met OH	129.3 ± 3.2^{d}	119 ± 2.6^{e}	10.3 ± 0.5^{b}	386 ± 3.6^{cd}	267 ± 3^{bc}	6.9 ± 0.0^{a}	95.2 ± 0.02^{a}

Data was expressed as Mean ± Sd (n=3). Mean values sharing same letters are not significantly different (P>0.05).

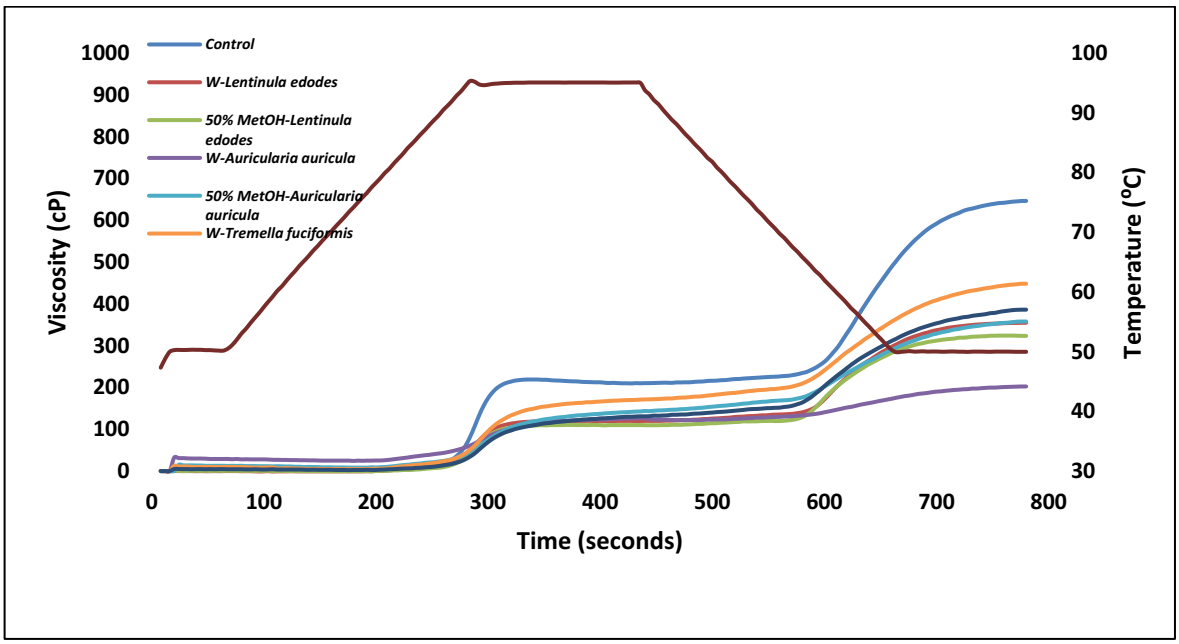
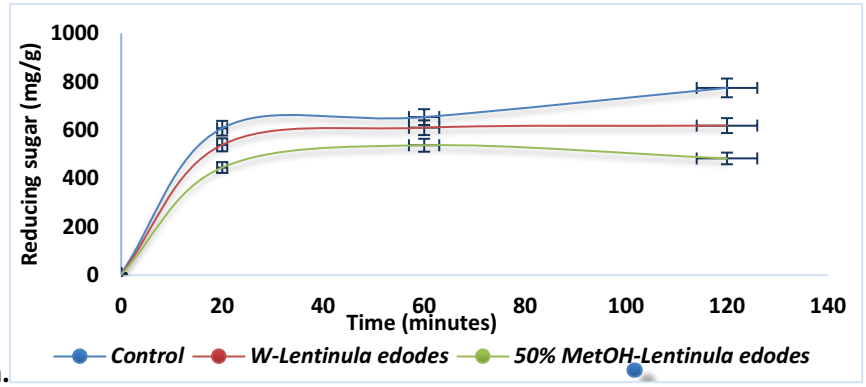


Figure 4 Pasting behaviour of white sorghum with 1% extract of mushroom samples.

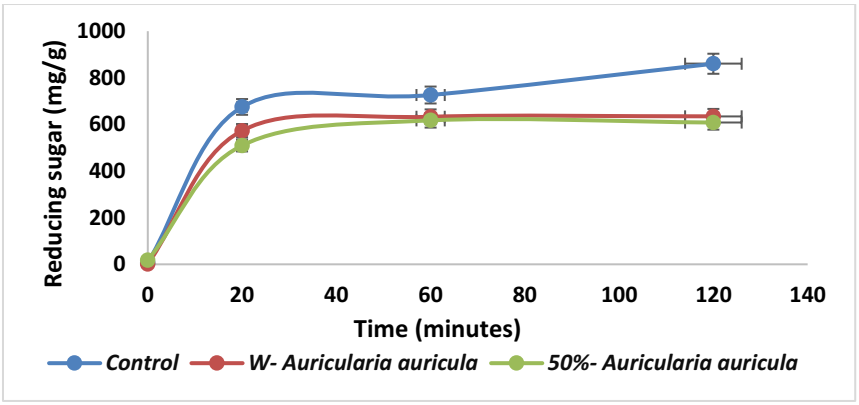
5.3.2. Effect of mushroom aqueous extracts on the digestibility of white sorghum flour

Fig. 5 illustrates the reducing sugars released over a 120 min in vitro white sorghum flour digestion. The levels of reducing sugars in the control sample (white sorghum flour + water) were higher at 20, 60, and 120 min, in the *in vitro* digestion. The decrease in the reducing sugar release was observed in the 50% methanol *L. edodes* extract rich samples (Fig 5a). Moreover, in case of *A. auricula* (Fig 5b) and *T. fuciformis* (Fig 5c) rich samples, the level of reducing sugars decreased when compared with control. The much stronger decrease in reducing sugar release was observed in 50% methanolic mushroom extracts rich sample (Fig 5a, b, and c). In the fastest digested starch throughout the first 20 minutes in the mushroom enriched samples was decreased as compared to the control. The amounts of the slowly digested starch fractions, over 20-120 min, were lower in the mushroom extract rich samples than control. Fig 5d represents the effect of mushroom extracts on the standardised AUC white sorghum flour values when compared to control. The samples with mushroom extracts showed decreased levels of AUC than control. The values of samples enriched with water and 50% methanol *L. edodes* extracts were significantly ($p < 0.05$) lower than the control. This might be because of the polyphenols present in the mushroom extracts. It has been revealed that polyphenols bind onto starch and non-starch polysaccharides and interrupts with *in vitro* starch digestion by inhibiting digestive enzymes and interaction with starch granules (Tu, Brennan, & Brennan, 2021). Moreover, same trend of the AUC values was observed in case of *A. auricula* and *T. fuciformis* extracts enriched samples as observed in *L. edodes.* It showed the significant decrease in AUC values of mushroom enriched samples compared to control. Additionally, the AUC values of samples with 50% methanol mushroom extracts were significantly lower as compared to samples enriched with wate mushroom extracts. The three mushroom aqueous extracts (0% and 50%) utilized in this study comprised of higher protein content than white sorghum flour, which might be the reason for the decreased glycaemic response of the flour enriched with mushroom aqueous extracts (Lu et al., 2018). It has been revealed that the presence of proteins in food products alters the digestibility of starch (Singh, Dartois, & Kaur, 2010). The protein strands showed the encapsulation effect on starch particles, leading in well-formed protein-starch complex, however, decreasing the degradation and release of starch (Cleary and Brennan, 2006). The polyphenolic compounds in the mushroom acts as non-protein amylase inhibitors and reduce the level of hydrolysis of starch by binding with amylase (Singh et al., 2010). Negative correlations (Table 5) were observed between AUC and breakdown viscosity (r = -0.049) and peak time (r = -0.608, $p < 0.05$). AUC is positively correlated with peak viscosity (r = 0.650, $p < 0.001$), Trough viscosity (r = 0.706, $p < 0.001$), Final viscosity (r = 0.656, $p < 0.001$), and set back viscosity (r = 0.527, $p < 0.01$).

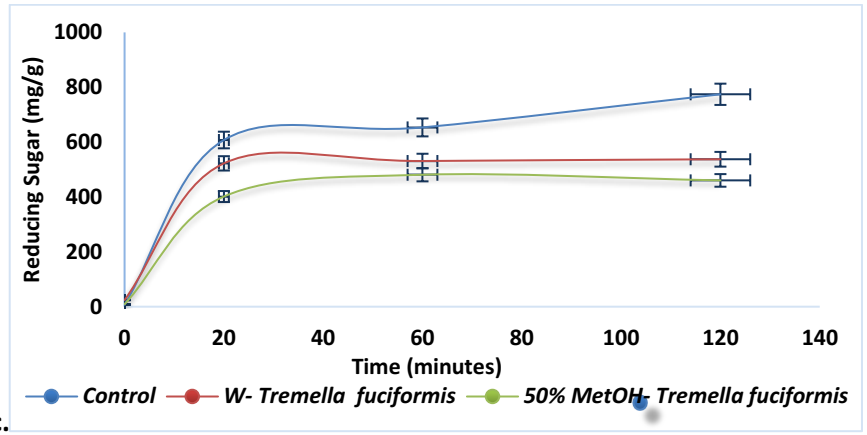
a.



b



c.



d.

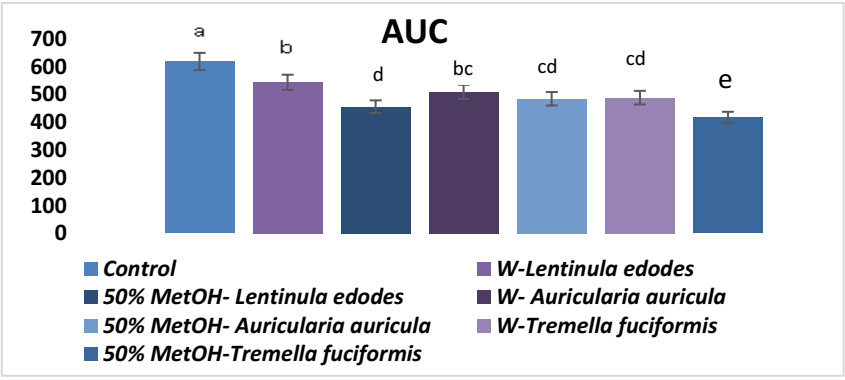


Figure 5 Level of reducing sugars released during in vitro digestion. Comparing the control to (a) water and 50% Met OH L. edodes extracts; (b) water and 50% Met OH A. auricula extracts; (c) water and 50% T. fuciformis extracts; (d) Values of area under curve (AUC)

Table 5 Correlation between pasting properties of white sorghum flour and area under curve of digested samples.

	Peak viscosity	Trough viscosity	Breakdown	Final viscosity	Setback	Peak time	Pasting Temp
Trough viscosity	0.993***						
Breakdown	0.640**	0.551**					
Final viscosity	0.752***	0.745***	0.341				
Setback	0.510*	0.502*	0.158	0.948***			
Peak time	-0.002	-0.082	0.639**	-0.374	-0.463*		
Pasting temp	0.207	0.197	0.080	0.396	0.400	-0.211	
AUC	0.650***	0.706***	-0.049	0.656***	0.527**	-0.608*	0.361

The values denote the coefficient of Pearson correlation coefficient. Significance: ***Significant at $p < 0.001$; **Significant at $p < 0.01$; *Significance at $p < 0.05$.

5.4. Conclusion

This study has shown that mushroom extracts have decreased significant effect on the pasting properties of white sorghum flour and resulted in lower glycaemic index than control.

Chapter 6

In vitro phenolic compounds release of white sorghum flour with mushroom extracts

Abstract

White Sorghum flour with 1% concentrated mushroom extract from three different species of mushrooms (*L. edodes, A. auricula,* and *T. fuciformis*) after pasting digested with and without enzyme. The total phenolic contents and antioxidant activity of enzymatic gastric and intestinal fractions was significant ($p < 0.05$) higher than non-enzymatic fractions. Higher amount of total phenolic contents was released after enzymatic intestinal digestion of samples enriched with *L. edodes* than *T. fuciformis* and *A. auricula.* Mushroom extracts showed increased significant effect on the total phenolic contents and antioxidant activity of white sorghum flour after digestion compared to control. The protein digestibility of the white sorghum flour also increased significantly ($p < 0.05$) on addition of mushroom extracts. Overall, mushroom extracts could be added to food products to provide health benefits to consumers.

6.1. Introduction

Mushrooms have been utilized as a staple food and medicine from centuries. Mushrooms are good source of dietary fibre, vitamins, and minerals (Okafor, Okafor, Ozumba, & Elemo, 2012). Edible mushrooms possess various medicinal properties, for example, anti-tumour, anti-diabetic, anti-cardiovascular, and anti-atherosclerotic effects due to presence of wide range of bioactive components (Weigand-Heller, Kris-Etherton, & Beelman, 2012). White sorghum possesses several health benefits, but this crop has one limitation of decreased digestibility (Olojede, Sanni, Banwo, & Adesulu-Dahunsi, 2020). Therefore, the combination of mushroom extracts and white sorghum flour may possibly enhance the nutritional value, taste, and flavour of the food product. Previous studies have shown the effect of white button (*Agaricus bisporus*), shiitake (*L. edodes*), and porcini mushroom (*Boletus edulis*) on the wheat bread (Lu et al., 2018). In this study, 1% concentrated extracts (water and 50% methanol) of *L. edodes, A. auricula,* and *T. fuciformis* were incorporated into white sorghum flour. The samples were digested with and without enzyme. The effect of mushroom extracts on the antioxidant capacity, total phenolic contents, and protein digestibility of white sorghum flour was determine.

6.2. Materials and methods

6.2.1. Materials

As described in 3.1.

6.2.2. In vitro digestion analysis

As described in 3.5

6.2.3. Antioxidant analysis

As described in 3.3

6.2.4. Protein digestibility

As described in 3.6

6.3. Results and discussion

6.3.1. Effect of adding mushroom extracts on the antioxidant properties of white sorghum flour.

The level of phenolic compounds released during *in vitro* digestion (with and without enzyme) is summarized in Table 6. The digestion was performed with and without enzyme and the level of polyphenolic compounds in samples were determined as compared to control. The level of total phenolic contents in control samples was 0.69 mg GAE/g and 0.92 mg GAE/g in non-enzymatic and enzymatic gastric digestion respectively. After intestinal digestion (with enzyme), the level of total phenolic contents increased to 2.28 mg GAE/g. The highest level of total phenolic content after intestinal digestion (with enzyme) was observed in white sorghum flour with water *L. edodes* extracts and *T. fuciformis* enriched flour extracts (2.77 mg GAE/g) than followed by *A. auricula* enriched sample (2.66 mg GAE/g). The results showed that the total phenolic contents increased significantly ($p < 0.05$) after gastric and intestinal digestion. Overall, the level of phenolic contents after intestinal digestion was higher than gastric digestion. Moreover, the flour enriched with water mushroom

extracts showed higher total phenolic contents than 50% methanol extracts. The total phenolic contents in white sorghum flour increased significantly ($p < 0.05$) on addition of mushroom extracts. The increase in total in total phenolic contents followed the trend as Intestinal fractions > gastric fractions and *L. edodes* > *T. fuciformis* > *A. auricula.* Total phenolic contents decreased after intestinal digestion (without enzyme) and showed significant differences but higher than the control. The increase in total phenolic contents in the flour might be due to the presence of bioactive compounds in mushrooms (Sanchez, 2017). These results are in agreement with the previous study conducted on bread which also showed increase in the phenolic contents and antioxidant activity in the bread (Lu et al., 2018). Significant negative correlation (Table 9) existed between the total phenolic contents released after enzymatic gastric digestion and non-enzymatic gastric digestion and total phenolic contents released after non enzymatic gastric digestion and enzymatic intestinal digestion ($r = -0.525$, $p < 0.05$). In addition to it, total phenolic contents released after enzymatic intestinal digestion and non-enzymatic intestinal digestion were negatively correlated ($r = -0.666$, $p < 0.001$). On the other hand, the total phenolic contents released after non enzymatic intestinal and gastric digestion were significantly positively correlated with values ($r = 0.911$, $p < 0.001$) (Table 9).

Table 6 Level of Total phenolic contents in enzymatic and non-enzymatic digested white sorghum flour with mushroom extracts.

Sample	Solvent	TPC (mg GAE/g DW)			
		Gastric		Intestinal	
		NE	E	NE	E
	Control	0.69 ± 0.03^{f}	0.92 ± 0.03^{d}	0.14 ± 0.00^{e}	2.28 ± 0.13^{b}
L. edodes	RO Water	2.12 ± 0.03^{a}	2.18 ± 0.04^{a}	1.03 ± 0.03^{a}	2.77 ± 0.06^{a}
	50 % Met OH	1.76 ± 0.01^{c}	1.87 ± 0.10^{b}	0.79 ± 0.01^{c}	2.25 ± 0.12^{bc}
A. auricula	RO Water	1.91 ± 0.02^{b}	1.91 ± 0.02^{b}	0.91 ± 0.01^{b}	2.66 ± 0.08^{a}
	50% Met OH	1.41 ± 0.03^{d}	1.77 ± 0.08^{b}	0.57 ± 0.02^{d}	2.00 ± 0.09^{c}
T. fuciformis	RO Water	1.42 ± 0.00^{d}	1.82 ± 0.07^{b}	0.75 ± 0.00^{c}	2.77 ± 0.05^{a}
	50% Met OH	0.89 ± 0.02^{e}	1.37 ± 0.04^{c}	0.61 ± 0.01^{d}	2.11 ± 0.07^{bc}

Abbreviations: NE (No Enzyme), E (with enzyme). Data was expressed as Mean ± Sd (n=3). Mean values sharing same letters are not significantly different (P>0.05).

The level of ferric reducing antioxidant power of white sorghum flour with mushroom extracts is described in Table 7. The results revealed that in case of control after gastric enzymatic digestion the ferric reducing power was 19.70 µmolFe^{2+}E/g and after non enzymatic gastric digestion decreased to 12.10 µmolFe^{2+}E/g. After intestinal enzymatic and non-enzymatic digestion, the ferric reducing power was 13.70 µmolFe^{2}+E/g and 13.16 µmolFe^{2}+E/g respectively. The white sorghum flour with

water *L. edodes* extracts after non-enzymatic gastric digestion observed with ferric reducing power of 54 µmolFe^2+E/g followed by *A. auricula* (29.91 µmolFe^2+E/g) and *T. fuciformis* (23.60 µmolFe^2+E/g). The same trend of reduction in ferric reducing power after non enzymatic gastric digestion observed in white sorghum flour enriched with 50% methanolic mushroom extracts. Furthermore, after enzymatic gastric digestion, the level of ferric reducing power decreased as compared to the non-enzymatic gastric digestion. In case of non-enzymatic intestinal digestion, the ferric reducing antioxidant power was higher (33.78 µmolFe^2+E/g) in flour enriched with water *L. edodes* extracts than sample enriched with 50% methanolic extracts followed by *A. auricula* and *T. fuciformis* enriched flour. The level of ferric reducing power after enzymatic intestinal digestion decreased as compared with non-enzymatic intestinal digestion. The statistical analysis showed that mushroom extracts affect significantly ($p < 0.05$) the ferric reducing power of white sorghum flour after enzymatic and non-enzymatic gastric and intestinal digestion. The ferric reducing antioxidant power after enzymatic intestinal digestion was positively correlated (Table 9) with antioxidant power after enzymatic gastric digestion ($r = 0.959$, $p < 0.001$) and non-enzymatic gastric digestion ($r = 0.945$, $p < 0.001$). The antioxidants released after non-enzymatic intestinal digestion were positively correlated with antioxidant power after non-enzymatic gastric digestion ($r = 0.979$, $p < 0.001$) and enzymatic intestinal digestion ($r = 0.961$, $p < 0.001$). The total phenolic contents released after enzymatic gastric and intestinal digestion were negatively correlated with the antioxidants released after enzymatic and non-enzymatic gastric and intestinal digestion. However, total phenolic contents released after non enzymatic gastric and intestinal digestion were significantly ($p < 0.001$) positively correlated with antioxidants released after enzymatic and non-enzymatic gastric and intestinal digestion.

Table 7 Level of ferric reducing antioxidant power in enzymatic and non-enzymatic digestion of white sorghum flour with mushroom extracts

Sample	Solvent	FRAP (µmolFe^{2+} Equivalent/g DW)			
		Gastric		Intestinal	
		NE	E	NE	E
	Control	19.70 ± 0.86^{e}	12.10 ± 1.43^{c}	13.70 ± 0.97^{d}	13.16 ± 2^{d}
L. edodes	RO Water	54.0 ± 1.19^{a}	20.18 ± 1.82^{a}	33.78 ± 1.11^{a}	30.58 ± 1.48^{a}
	50 % Met OH	39.9 ± 1.13^{b}	23.10 ± 1.03^{b}	23.47 ± 0.84^{b}	20.29 ± 0.31^{b}
A. auricula	RO Water	29.91 ± 0.70^{c}	20.18 ± 1.82^{b}	19.74 ± 0.62^{c}	18.48 ± 2.9^{bc}
	50% Met OH	30.38 ± 1.2^{c}	20.04 ± 2.18^{b}	21.22 ± 1.33^{bc}	16.84 ± 0.93^{bcd}
T. fuciformis	RO Water	23.60 ± 0.49^{d}	11.84 ± 0.96^{c}	15.59 ± 1.2^{d}	14.95 ± 0.30^{cd}
	50% Met OH	23.79 ± 1.04^{d}	13.49 ± 0.47^{c}	15.71 ± 0.63^{d}	13.66 ± 0.80^{d}

Abbreviations: NE (No Enzyme), E (with enzyme). Data was expressed as Mean ± Sd (n=3). Mean

values sharing same letters are not significantly different (P>0.05)

6.3.2. Effect of addition of mushroom extracts on the protein digestibility white sorghum flour during digestion

The level of protein digestibility after gastric and intestinal digestion is summarized in Table 8. The protein digestibility of control samples after gastric digestion was 15.7% and it increased to 40.2% after intestinal digestion. In case of flour enriched with water extracts of *L. edodes,* the protein digestibility was 38.12% after gastric digestion and increased to 60.2% after intestinal digestion. The results showed that the protein digestibility of samples enriched with mushroom extracts was higher than the control. In case of samples with 50% mushroom extracts, the protein digestibility was lower than that of samples with water mushroom extracts. The flour with *L. edodes* extracts showed highest protein digestibility followed by *T. fuciformis* and *A. auricula.* The addition of mushroom extracts into the flour significantly ($p < 0.05$) increased the protein digestibility after gastric and intestinal digestion. Protein digestibility after enzymatic gastric and intestinal digestion was negatively correlated with total phenolic contents released enzymatic gastric and intestinal digestion (Table 9). However, protein digestibility was significantly positively correlated with the ferric reducing antioxidant power after enzymatic and non-enzymatic gastric and intestinal digestion (Table 9).

Table 8 Level of protein digestibility (% age) in digested white sorghum flour with mushroom extracts.

		Protein Digestibility (%age)			
Samples	**Solvent**	**Gastric Digestion**	**Intestinal Digestion**		
		30 min	50min	90min	150min
Control		15.73 ± 2.04^{d}	32.77 ± 1.43^{d}	39.42 ± 1.8^{c}	40.21 ± 2.95^{e}
L. edodes	RO Water	38.12 ± 1.80^{ab}	48.45 ± 2.13^{a}	55.64 ± 2.11^{a}	60.26 ± 0.74^{a}
	50%MetOH	37.33 ± 2.03^{a}	44.45 ± 1.94^{abc}	50.25 ± 2.18^{b}	53.97 ± 3.23^{abc}
A. auricula	RO Water	27.33 ± 1.11^{c}	40.47 ± 0.78^{bc}	41.63 ± 2.16^{c}	51.35 ± 2.93^{bcd}
	50%MetOH	26.98 ± 2.02^{c}	33.29 ± 3.25^{d}	39.13 ± 1.40^{c}	43.80 ± 3.21^{de}
T. fuciformis	RO Water	32.00 ± 2.87^{bc}	45.10 ± 1.16^{ab}	49.14 ± 1.04^{b}	56.91 ± 2.68^{ab}
	50%MetOH	28.76 ± 2.53^{c}	39.38 ± 0.48^{c}	47.45 ± 1.87^{b}	46.56 ± 2.57^{cde}

Data was expressed as Mean ± Sd (n=3). Mean values sharing same letters are not significantly different (P > 0.05).

Table 9 Pearson's correlations between the total phenolic contents (mg GAE/g dry weight), ferric reducing antioxidant power (µmol Fe2+ E/g), and protein digestibility (% age) of digested white sorghum flour with three mushroom samples.

	TPC GE	TPC G NE	TPC I E	TPC I NE	FRAP GE	FRAP G NE	FRAP I E	FRAP I NE	Protein Digestibility (30 min)	Protein Digestibility (50 min)	Protein Digestibility (90 min)
TPC G NE	-0.525*										
TPC I E	1	-0.525*									
TPC I NE	-0.666***	0.911***	-0.666***								
FRAP GE	-0.326	0.817***	-0.326	0.722***							
FRAP G NE	-0.35	0.815***	-0.35	0.724***	0.972***						
FRAP I E	-0.228	0.8***	-0.228	0.708***	0.959***	0.945***					
FRAP I NE	-0.321	0.805***	-0.321	0.705***	0.98***	0.979***	0.961***				
Protein Digestibility (30 min)	-0.539*	0.705***	-0.539*	0.775***	0.639**	0.729***	0.621**	0.665***			
Protein Digestibility (50 min)	-0.396	0.678***	-0.396	0.797***	0.56**	0.656***	0.632**	0.582**	0.82***		
Protein Digestibility (90 min)	-0.333	0.499*	-0.333	0.645**	0.591**	0.668***	0.656***	0.599**	0.765***	0.881***	
Protein Digestibility (150 min)	-0.398	0.749***	-0.398	0.826***	0.611**	0.659***	0.664***	0.595**	0.72***	0.89***	0.794***

Abbreviations: TPC, Total phenolic contents; FRAP, Ferric reducing antioxidant power; GE Gastric fractions with enzyme; G NE, Gastric fractions No enzyme; TPC IE, Intestinal fractions with enzyme; TPC I NE, Intestinal fractions no enzyme; The values denote the coefficient of Pearson correlation coefficient. Significance: ***Significant at $p < 0.001$; **Significant at $p < 0.01$; *Significance at $p < 0.05$.

6.4. Conclusion

It has been shown that the total phenolic contents and antioxidant activity increased after enzymatic and non-enzymatic gastric and intestinal digestion. The higher total phenolic contents and antioxidant power was observed in samples enriched with water mushroom extracts than 50% methanolic mushroom extracts. Moreover, the sample enriched with *L. edodes* extracts showed the higher total phenolic contents and antioxidant power after digestion than *T. fuciformis* and *A. auricula*. In addition to it, after enzymatic gastric and intestinal digestion higher phenolics and antioxidant activity was observed than non-enzymatic gastric and intestinal digestion. The protein digestibility increased on addition of mushroom extracts after gastric and intestinal digestion. Therefore, mushrooms enhanced the phenolic contents and antioxidant activity of the white sorghum flour.

Chapter 7

General discussions and conclusions

Three different species of edible mushroom powder (*Lentinula edodes, Auricularia auricula,* and *Tremella fuciformis*) were used for the extraction of polyphenols at different conditions (methanol concentration, temperature, and pH). The effect of different conditions on the polyphenol content was determined. In order to determine the effect of mushroom aqueous extracts on the white sorghum flour, a series of parameters were analysed, including total phenolic contents, DPPH radical scavenging activity, ferric reducing antioxidant power, pasting properties of sorghum flour, *in vitro* starch digestion with and without enzyme, protein content, and protein digestibility after digestion. The antioxidant activity of the water extracts was higher than the methanolic extracts. Moreover, the antioxidant activity decreases on decreasing the pH during extraction. Mushroom polyphenols inhibited the starch degrading enzymes, as a result, reduced their glycaemic index. On addition of mushroom extracts to the white sorghum flour, the polyphenol content and protein digestibility increased after *in vitro* digestion. The results support that mushroom extracts cause a significant impact in reducing the starch gelatinisation and stimulating the total phenolic contents, antioxidant activity and protein digestibility of the white sorghum flour. Incorporation of mushroom extracts could not only add the nutritional benefits into the white sorghum flour but also acts as altering agent by decreasing the hydrolysis of starch throughout the digestion and elevating the antioxidant capacities of white sorghum flour. This research presents mushroom extracts inclusion as a feasible way of inhibiting starch from gelatinisation.

The extraction conditions affected differently the mushroom polyphenols. The total phenolic contents, DPPH radical scavenging activity, and ferric reducing power of three mushrooms extracted at different pH and different methanol concentration is shown in Table 2. The results revealed that the RO water extracts retained higher polyphenols than methanol extracts. The concentration of the solvent has a great impact on the extraction proficiency hence the polarity of a solvent contributes to increase the yield of bioactive compounds (Pham, Vuong, Bowyer, & Scarlett, 2017). Current study revealed that with the increase in the solvent concentration the amounts of polyphenols decreased. This could be due to the presence of phenolic acids, phenylpropanoids, flavonoids, and quinones in mushrooms which are more soluble in water (Haminiuk, Maciel, Plata-Oviedo, & Peralta, 2012). The polyphenolic content decreased on decreasing pH (Table 2). The study conducted on sweet potato leaf revealed that the extraction performed at pH 5-7 solutions showed higher recovery rate of polyphenolic contents (Sun, Mu, & Xi, 2017). Moreover, the different temperature conditions also showed significant effect on the recovery of polyphenolic contents of three different mushrooms

(Figure 2). Current study revealed that, the recovery of polyphenolic contents was different in the water and methanol extracts of three different mushrooms in consideration with different temperature conditions. *L. edodes* higher recovery of polyphenol contents than *A. auricula* and *T. fuciformis.* In case total phenolic contents, *L. edodes* showed higher recovery rate at 25^{o} C than 50^{o} C and 80^{o} C. However, *A. auricula* and *T. fuciformis* showed higher recovery rate at 50^{o} C. The different recovery rate of polyphenols at different temperatures might be because of the solubility of type of polyphenols of different species at different temperatures. For instance, higher temperature results in higher solubility of some substances and on the contrary, less solubility of other substances due to modification if their structure (Casazza, Pettinato, & Perego, 2020). Meanwhile, a study showed that, at constant pH, the solubility of proteins reduced with temperature because of the thermal denaturation of proteins (Casazza, Pettinato, & Perego, 2020).

Mushrooms are good sources of antioxidants and possess several health benefits. Many different are being conducted that are showing the effect of mushroom polyphenols on the properties of cereals and mushroom powders are incorporated into the cereal products to enhance the nutritional value of the product (Lu et al., 2018; Tu, Brennan, & Brennan, 2020; and Wang et al., 2020). Current study focused on the addition of 1% concentrated water and methanol extracts of three different mushrooms into white sorghum flour to investigate the effect on pasting properties and starch digestibility of white sorghum flour as compared to control. The results (Table 4, Fig. 5.1, Fig 5.2, and Table 5) showed that the peak viscosity, trough viscosity, breakdown viscosity, final viscosity, and setback viscosity of control was higher than the samples enriched with mushroom extracts. This showed that mushroom extracts negatively significantly affected the pasting properties of white sorghum flour. Moreover, the samples enriched with mushroom extracts also showed decreased level of reducing sugars compared to control. These results support the idea that the presence of the mushroom extracts restricts the digestion of carbohydrate in white sorghum flour. A research conducted on wheat flour noodles investigated that addition of shiitake powder interfered with the digestion of starch in wheat flour noodles (Wang et al.., 2020). It has also been investigated that mushroom phenolic contents were positively correlated to reduction in glycaemic index of extruded products (semolina snacks) (Lu et al., 2020).

Furthermore, inclusion of mushroom extracts in the white sorghum flour also affected significantly the bioactive compounds and protein digestibility after enzymatic and non-enzymatic digestion (Table 6, Table 7, Table 8, and Table 9). The results showed that, an increase in total phenolic contents, ferric reducing antioxidant power, and protein digestibility was observed after gastric and intestinal digestion in samples enriched with mushroom extracts than control group. These results are consistent with the studies conducted on hot extruded products which also showed the improved antioxidant activity of semolina snack samples (Lu et al., 2020). Another research

conducted on the wheat flour noodles with shiitake powder in it, showed increase in phenolic contents and protein content than control after digestion (Wang et al., 2020). This study revealed that mushroom extracts possess several benefits in terms of increasing the nutritional value of the cereals. The mushroom extract enriched white sorghum flour had significant reduction in glucose release throughout the *in vitro* digestion and increase in the antioxidant capabilities as compared to control, which explains that there is a considerable ability of utilizing edible mushroom materials in modifying the postprandial glucose response of cereal goods. Moreover, further research is needed to determine the role of mushrooms polyphenols in quality of cereal products.

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