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The role of mushroom polyphenols on enzyme inhibition

A Dissertation

submitted in partial fulfilment

of the requirements for the Degree of

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by

YU-YI, CHU

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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.

The role of mushroom polyphenols on enzyme inhibition

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Shiitake mushroom (*Lentinula edodes*), black ear mushroom (*Auricularia auricula*) and silver ear mushroom (*Tremella fuciformis*) are a common type of mushrooms. Recent year, bioactivity phenolic compound isolated from them has been attended and investigated by researchers and further focusing on their inhibition of starch digestion as well as the improvement of quality of starch in the food industry. Therefore, we hypothesize that the pasting properties and hydrolysis of red sorghum flour may affect by bioactivity phenolic compounds inhibiting the digested enzyme, such as α-amylase and intestinal α-glucosidase, derived from the mushroom. The objective of this study was to investigate the extract bioactive polyphenolic compounds from three different species of edible mushrooms, namely shiitake mushroom, black ear mushroom and silver ear mushroom as ingredients. The results of this study suggest that water extraction at 50℃ for 3 hours in neutral pH could obtain optimal extraction condition for a great TPC and antioxidant activity. Shiitake mushroom water extract was the highest TPC and antioxidant activity among all mushroom extract, followed by shiitake mushroom 50% ethanol extracts. Besides, addition with each mushroom extracts all reduced the pasting properties of red sorghum paste, in particular, peak viscosity and final viscosity. Shiitake water extract has a significant ($p<0.05$) high comparing with others. Moreover, the TPC, antioxidant activity and protein digestibility were observed an increasing trend during in vitro digestion of all red sorghum paste incorporated in each mushroom extracts, while the reducing sugar and AUC were significant ($p<0.05$) decrease. Shiitake mushroom water extracts remain to present the greatest TPC and antioxidant activity during in vitro digestion of red sorghum paste, followed by shiitake ethanol mushroom extracts. Our preliminary result supported that mushroom extract, ie. shiitake mushroom, black ear mushroom and silver ear mushroom could be used as a functional ingredient of red sorghum food products development, which provides bioactivity phenolic compounds and enhances antioxidant activity. What is more, it may display as a modification agent by decrease the hydrolysis of starch during digestion for further reducing sugar release as well as raising protein digestibility of red sorghum flour products.

Keywords: Shiitake, Black ear mushroom, Silver ear mushroom, Red Sorghum, Enzyme inhibition

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Chapter 1

Introduction and Thesis Outline

Mushrooms have been consumed in the human diet in many countries for a few centuries. There are many different species of mushrooms has been reported their potential for medical properties, such as anti-cancer (Alonso et al., 2017; Hara et al., 2003; J. Li et al., 2014), anti-oxidant (Heleno, Barros, Sousa, Martins, & Ferreira, 2010) and anti-infection (Béni et al., 2018; Masterson et al., 2020). Shiitake mushroom, Black ear and Silver ear mushrooms are a common type of mushrooms. Recent year, bioactivity phenolic compound isolated from them has been attended and investigated by researchers and further focusing on their inhibition of starch digestion as well as the improvement of quality of starch in the food industry.

Starch was formed by complex carbohydrates. Rapid degradation of carbohydrates may be resulting in the postprandial hyperglycemia elevation. Pancreatic α-amylase and intestinal α-glucosidase well know the two major starch digestive enzymes, which involved in starch hydrolysis and degradation into glucose. Glucose is the smallest molecular weight sugar, which is also the can be rapidly absorbed in the blood-stream (Adefegha, 2018). The previous study has demonstrated that foods riching in polyphenols, such as flavonoids, phenolic acids, stilbenes, lignans and polymeric lignans could reduce glycemic index and alleviating hyperglycemia (Bahadoran, Mirmiran, & Azizi, 2013; Coe, Clegg, Armengol, & Ryan, 2013).

Phenolics compounds have been used as one of the modification methods on the quality of starch in recent years (An, Bae, Han, Lee, & Lee, 2016). Mushroom powder and its extract also have been increasingly incorporated in the formula as functional ingredients because their bioactivity phenolic compounds (Lu et al., 2018). Additional, their unique polyhydric structures also have been demonstrated could interact with the protein, starch and non-starch polysaccharides, which benefits improving food products (Y. Wu, Niu, & Xu, 2020). Therefore, we hypothesize that the pasting properties of red sorghum flour may affect by bioactivity phenolic compounds derived from the mushroom. However, to our best knowledge, there have been very few works of literature focused on the effects of bioactivity phenolic compounds derived from Shiitake, Black ear mushroom and Silver ear mushroom on the pasting properties and *in vitro* digestion of red sorghum flour. Therefore, the objective of this study was to investigate the extract bioactive polyphenolic compounds from three different species of edible mushrooms, namely shiitake mushroom, black ear mushroom and silver ear mushroom as ingredients. Furthermore, we also try to clarify how bioactive polyphenolic compounds altering the starch digestion of starch-based system. We wish that this study would enhance our understanding of inhibition of the digested enzyme in bioactivity phenolic compounds extracted from

Shiitake, Black ear and silver ear mushrooms and increase the worth and for further quality improvements of red sorghum flour products.

1.1 Aims

This research aimed to extract bioactive polyphenolic compounds from three different species of edible mushrooms, namely shiitake mushroom, black ear mushroom and silver ear mushroom as ingredients. Three mushroom extracts were each incorporated into red sorghum paste and made them a portion of food that potential benefits for human health.

First of all, the results of the extraction of bioactive polyphenolic compounds from three mushrooms using water and Ethanol were presented and discussed. The effects of the different extraction condition, such as solvent ratio, pH and temperature on the extraction yields and their antioxidant activity were analysed for each mushroom.

Additionally, the results regarding the effect of bioactive polyphenolic compounds in mushrooms on the starch degradation and release of reducing sugars during in vitro starch digestion were presented. To summarize, the aims of the study include three key points below.

1. To screen appropriate extraction methods for improving the extractability of bioactive polyphenolic compounds from three species of mushrooms.

2. To compare the extraction yields and to analyze the antioxidant activity of bioactive polyphenolic compounds between three different mushrooms.

3. To clarify how bioactive polyphenolic compounds affecting the starch digestion, using red sorghum paste as the starch-based system.

1.2 Hypothesis

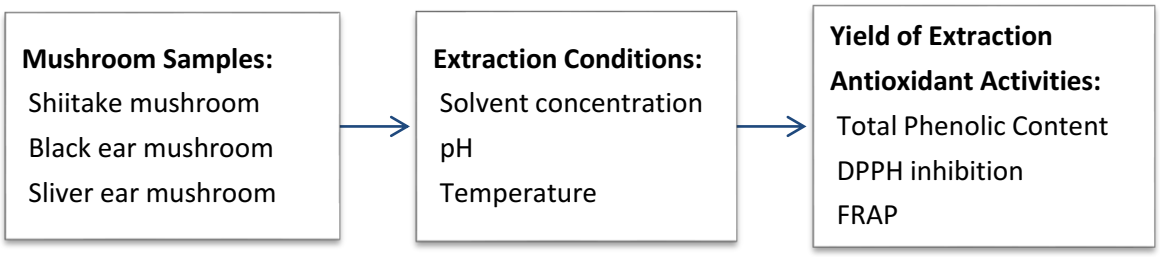
1. Different extraction conditions such as solvent concentrations, pH and temperature will affect the content of polyphenolic compounds and antioxidant capacities between each mushroom.

2.Addition of mushroom extracts will enhance the total phenolic compounds content and antioxidant activity as well as will have beneficial impacts on the antioxidant capacities of red sorghum paste.

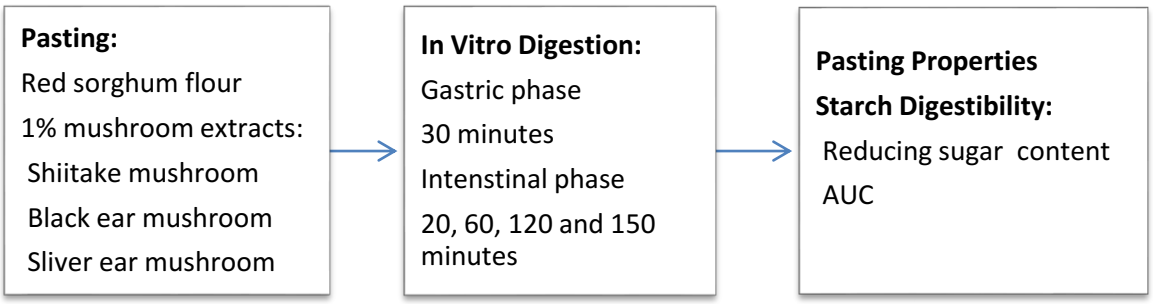
3. Addition of mushroom extracts will decrease the starch degradation and release of reducing sugars during *in vitro* starch digestion of red sorghum paste.

1.3 Thesis outline

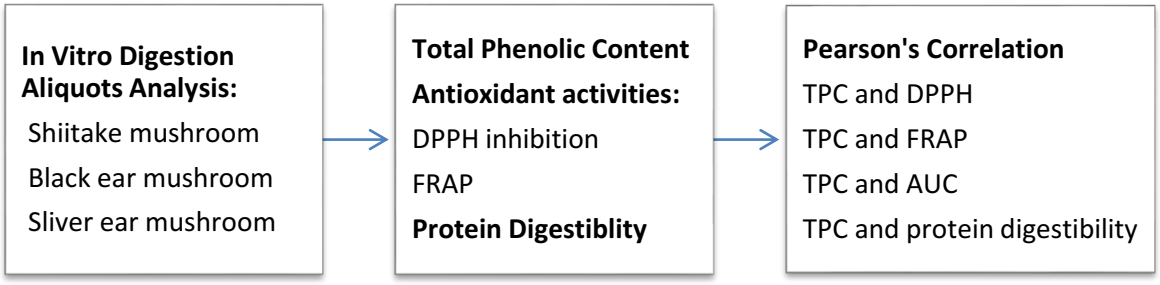
- **Project Title:** The role of mushroom polyphenols on enzyme inhibition
- **Chapter 1:** Introduction and thesis outline
- **Chapter 2:** Literature review
- **Chapter 3:** Material and method
- **Chapter 4:** Extraction of polyphenol compounds from mushroom samples



- **Chapter 5:** In vitro digestion of red sorghum paste with addition of mushroom extracts



- **Chapter 6:** Phenolic compounds release and protein digestibility during *in vitro* digestion of red sorghum paste with mushroom extracts



- **Reference**

Chapter 2

Literature review

2.1 Mushrooms

Mushrooms have many different species and have been consumed as a common vegetable all over the world. In fact, mushrooms cannot be classified as a true vegetable because they lack the part of plants, such as leaves and no chlorophyll, roots and seeds. Furthermore, they can grow well in a dark environment without any light. To further explain, mushrooms are fungus and its growing through by releasing spores Tiram (2013). The fruiting body of mushrooms, such as carpophore and mycocarp, present above ground, and its lifetime only about 10–14 days. Some of them have mycelia, which live in symbiosis with roots of the tree.

Mushrooms have been used in the human diet in many countries, such as Greeks, Roman and Asia since a few centuries (Valverde, Hernández-Pérez, & Paredes-López, 2015). Nowadays, mushrooms still were popular foods worldwide. According to FAOSTAT data (2020), the most production quantity of mushrooms (including truffles) is China in 2019, followed by Japan as well as the United States of America, which are approximately 8.9, 0.47 and 0.38 million tonnes per year, respectively. This means that mushrooms play an important role in economic value.

Mushrooms are not only the unique aroma and texture but also have a great nutritional value. They are rich in dietary fibre, protein and essential amino acids, minerals as well as vitamins. Also, mushrooms are low-fat content, which made them well suited as a low-calorie diet (Kozarski et al., 2015). Nevertheless, they also contain various important fatty acids, in particular a high proportion of polyunsaturated fatty acids (PUFA) (Kalač, 2013). Furthermore, mushrooms have been reported that bioactive polyphenolic compounds isolated from mushroom could be used as an excellent antioxidant (X. Li, Zhang, & Xu, 2009). In the past few decades, the increasing of considerable interest of mushrooms in the researchers was due to their medicinal properties.

In this section will focus on chemical composition, nutritional values and medicinal properties of mushrooms as well as introduce the characters of three mushrooms used in this study, namely Shiitake mushroom (*Lentinula edodes*), black ear mushroom (*Auricularia auricula*) and silver ear mushroom (*Tremella fuciformis*).

2.1.1 Chemical Composition and nutritional values

Mushrooms have a great nutritional value, which rich in macronutrient, such as protein and essential amino acids and dietary fibre but low lipids. Additionally, edible mushrooms also contain various micronutrient, minerals as well as vitamins.

Normally, mushrooms contain moisture in the range of 87 to 95% wet basis (Rhim & Lee, 2011). Their dry matter around 8-14g per 100g fresh matter. Total contents of crude protein, crude ash of

mushrooms are approximately 20-25 and 5-12g per 100g dry matter, respectively, while crude fat content about 2-3g per 100g dry matter. The rests completion of the dry matter of mushrooms is carbohydrates and dietary fibre. This means that mushrooms are high protein, high fibre, low-fat and low calories foods (Kalac, 2016). Valverde et al. (2015), also presented that percentage of protein, fat and carbohydrate of some edible mushrooms are around 4.5-37.4, 1.0-4.3 and 55.3-87.1g per kg dry weight, respectively. Moreover, the ash and total energy of them are 4.7-9.3 % and 325-772 kcal (dry basis), respectively. The difference in composition between mushrooms depends on species, growth characteristics, stage and postharvest.

Mushrooms are reported to be a good source of protein, which contains 20–25 g per 100g of dry matter (Mattila et al., 2001). The ranges of protein digestibility of mushroom are between 60 and 70% and characterised by a great thermal and pH stability (Masterson et al., 2020). The amino acid composition has a different level among mushroom species. The change of the proportion of essential amino acids in total amino acid content was approximately 40% among wild species, while the cultivated mushrooms were from 30% to 35% (Kalac, 2016). The most common type of amino acid in mushrooms contain Leucine, valine, glutamine, glutamic and aspartic acids are the most abundant amino acids in mushrooms (Reis, Barros, Martins, & Ferreira, 2012; Valverde et al., 2015). In addition, mushrooms also contain a less amount of free amino acids, such as alanine, glycine, serine, and threonine, which form and enhance the sweet taste of mushrooms (Kalac, 2016).

Furthermore, due to the various advantages of plant protein-based meat analog, exploring new plant protein to replace animal protein has become a more popular trend in the last decades. Although the nutritional value of mushroom proteins is less than animal protein, such as hen egg whites, milk, or meat, it is greater than most plant protein. Thus, the mushroom protein has been successful used to develop a mushroom-soy protein fibrous-structured meat analog via the single-screw extrusion technique (Mohamad Mazlan et al., 2020).

On the other hand, bioactivity proteins isolated from mushrooms, such as water-soluble polysaccharide–protein, lectins, ribosome-inactivating proteins (RIP), fungal immunomodulatory proteins (FIP), ribonucleases, laccases, have been reported the potential in medicine and biotechnology field, which include antitumor, antiviral, antimicrobial, antioxidative, and immunomodulatory agents (Liu, Chen, Chen, Huang, & Cheung, 2016; Xu, Yan, Chen, & Zhang, 2011).

Lipids in the mushroom are formed by neutral and polar lipids. The former consist of fats, esters of trifunctional alcohol glycerol, and fatty acids with fully esterified glycerol and waxes as well as esters of monofunctional higher alcohol, and a fatty acid. The later one is Phospholipids. Fat in mushrooms is low, which approximately 2-3 g per 100g of dry matter or around 0.2-0.3 g 100 g−1 fresh matter. Thus, the advantage of low-fat content can be used in low-calorie diets. Nevertheless, mushrooms still contain various fatty acid, within some of them are benefiting human health. There are greater than 40 fatty acids

has been identified from different species of mushroom. The important fatty acid includes Palmitic (C16:0), Stearic (C18:0), Oleic (C18:1), Linoleic (C18:2) and Linolenic (C18:3). Aliphatic and monocarboxylic are the two main components that existed in mushroom, while the minor one is fatty acids with an odd number of carbons with branched-chain or hydroxy fatty acids. In another word, the fatty acid of mushroom is Unsaturated fatty acids, the main components include alpha-linoleic acid from the ω-6 family and by oleic acid, while the amounts of the ω-3 family are low. Overall, the proportion of fat in mushrooms is low and the ratio of saturated and ω-3 fatty acids are also low (Valverde et al., 2015).

Carbohydrates form the main proportion of mushroom, which comprise approximately one-half of mushroom dry matter. It includes sugars, such as monosaccharides and their derivatives, oligosaccharides contain from two to ten monosaccharides, as well as various polysaccharides formed by tens to thousands of linked monosaccharides linked by glycosidic bonds (Kalac, 2016; Kalač, 2013).

The previous studies have indicated that various carbohydrates have been isolated from different mushrooms. Major monosaccharides include glucose, fructose and arabinose, while oligosaccharides are melezitose and trehalose. The median value of mannitol and trehalose are 28.9 and 39.2 g per kg dry matter, respectively (Kalač, 2013). The contents occur significantly (p<0.05) change between various species and within each species. Mannitol involves in volume growing and firmness of mushrooms fruiting bodies. Although it couldn't be digested completely by humans, its high content is beneficial for diabetics (Kozarski et al., 2015).

Polysaccharides are a specific group among carbohydrates, which were reported that they can antitumor and immunomodulatory. The reserve polysaccharide of mushrooms was present as glycogen. Its content was from 50 to100 g per kg of dry matter. Chitin is a structural N-containing polysaccharide, which characterized by a water-insoluble. It is a β-(1→4)-branched N-acetylglucosamine units. Chitin comprises approximately 80–90% dry matter in mushroom cell walls. Although human can't digest chitin, it is an essential structural polysaccharide in mushroom. Noticeable, most of the time the glycogen and chitin were found in the animal source. However, mushrooms also can isolate them, this fact is different from plant finding the starch and cellulose (Kalac, 2016; Valverde et al., 2015).

Dietary fibre means a sum of the indigestible carbohydrates in the human gut environment. It formed by soluble and insoluble fibre. For soluble fibre, enhancing the viscosity of foods and chyme is due to its structure forming by the gel with water. Otherwise, they can reduce the postprandial blood glucose, insulin-releasing and cholesterol level. The insoluble one can increase stool bulk and reduce the stay time of stool in intensity, which is benefits colon health. The Contents of soluble and insoluble fibre are approximately from 40 to 90 and from 220 to 300 g per kg dry matter, respectively. Wang et al. (2014) reported that total dietary fibre is a range of 5.1% and 40.0% in the dry matter among 12 wild-growing mushroom species.

Vitamins collected from mushrooms include water-soluble vitamins: thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B6), folates (vitamin B9), cobalamin (vitamin B12), vitamin C as well as fat-soluble vitamins: D2 and E (Mattar et al., 2018; Valverde et al., 2015). Vitamin D2 produced from mushrooms was recommended as the best sources of natural vitamin D to vegetarians (Kalac, 2016).

Mineral in mushrooms are included various macro-and micronutrients mineral constituents, which includes essential elements: K, P, Mg, Ca, Na, Zn, Cu, Mn, Ni, and Co, and those ones that have no known biological function. Metals, such as K, P, Mg, Ca, Na, Cu, and Zn involve in body liquids and tissues. Meanwhile, they also are playing the role of co-factors of enzymes. K, P can be found in the flesh of mushroom, which also rich in both Cu and Zn. K often occurred in caps of B. edulis. Its concentration is a range of 250 to 293g per kg dry matter. P also is an abundant source in wild-grown edible mushrooms, in particular in the caps. The total range of P is approximately from 101 to 152kg per kg dry matter. The concentration of Mg occurring in wild-grown mushrooms is similar to cultivated ones, which from 590 to 1300 mg per kg dry matter on average. All mushroom species can isolate Selenium, but its content has a wildly change between different one. Similarly, this situation also can be found in Sodium elements. The total content of Sodium is around from 17 to 1200mg per kg dry matter. The mean value of Ca is between 38 and 1100mg per kg dry matter among several mushrooms species (Falandysz & Borovička, 2013). Overall, mushrooms have a very low content of sodium, which benefits hypertensive controlling, while its high concentrations of K and P is an important role in orthomolecular aspect (Kozarski et al., 2015).

2.1.2 Antioxidants of Edible Mushrooms

Mushrooms have been recognized as an excellent resource of bioactive compounds, such as phenolic compounds. Meanwhile, its antioxidant capacity also has begun to receive high attention in researchers. Phenolic compounds, which are families of aromatic hydroxylated compounds. They usually possess a high antioxidant capacity because of their hydroxyl groups. Phenolic compounds have many subclasses, which includes flavonoids, phenolic acids stilbenes, lignans, tannins, and oxidised polyphenols. Additionally, these subclasses display a diversity of structures (Çayan, Deveci, Tel-Çayan, & Duru, 2020; Kozarski et al., 2015).

Various chemicals have been isolated from mushrooms and demonstrated possess antioxidant activity. Zhang et al. (2018) has collected thirteen boletus mushrooms representing five different species collected in Southwest China and found that water-soluble polysaccharides may play an important role in antioxidant of mushroom. Furthermore, the latest study shows that a new galactoglucan from Pleurotus djamor, which also has been illustrated presenting antioxidant activities (Maity et al., 2021). The relationship of Total Phenolic Contents and Antioxidant Activities of mushroom also received high attention. The flavonoid, phenolic compounds, tannin, saponin, alkaloids, and steroids were isolated from Pleurotus Ostreatus ethanol extracts, which may make the mushroom a great antioxidant activity (Rahimah, Djunaedi, Soeroto, & Bisri, 2019). Two mechanisms of antioxidant activity of flavonoids have been demonstrated. Firstly, it is

through by direct scavenging or quenching of oxygen free radicals excited oxygen species, while another one is through by inhibiting oxidative enzymes (Zhang (N. Zhang et al., 2015).

2.1.3 Medicinal properties

Mushrooms not only a vegetable in human meal, but some of them also have been used as a traditional medicinal use for a long time in many Asian countries, such as Chinese and Japanese. The most common one is the Basidiomycetes family. The most popular uses are their anti-oxidation properties (Heleno et al., 2020). Besides this, mushrooms have received considerable attention is due to their biological activities, such as antitumor, antiviral, anticomplementary, anticoagulant, antidiabetic, hypolipidemic, hepatoprotective, immunostimulant and immunological activities, which made them well suited for use in wild industry, such as food development, cosmetics as well as biomedicine (Hetland, Johnson, Bernardshaw, & Grinde, 2021; Kozarski et al., 2015).

Currently, many kinds of bioactivity compounds from mushrooms that have therapeutic effects have been isolated, including two main groups (De Silva, Rapior, Fons, Bahkali, & Hyde, 2012), namely high molecular weight metabolites, such as polysaccharides (X. Li et al., 2009), proteins and lipids, and low molecular weight metabolites, such as lectins, lactones, terpenoids (Qiao et al., 2014), alkaloids, sterols and phenolic compounds (Çayan et al., 2020; Shomali et al., 2019). This section will focus on the importance and relationship between mushroom bioactivity compounds and medicinal properties.

Mushroom and cancer

The research of anti-cancer action of Mushroom for a few decades, with an advantage of a minimum of side effects. The previous study has been reported that the increase of mushroom intake can reduce the risk of cancer. A Hospital-based case-control study in Japan has found that mushrooms higher mushroom intake is associated with a reduction of risk of stomach cancer (Hara et al., 2003). According to Li et al. (2014), conducted a meta-analysis of observational studies and report that the relative risk of the breast cancer is 0.97 (95% CI: 0.96–0.98) for per 1 g per day increment in mushroom consumption. This means that the rising of mushroom intake and reduction of risk of breast cancer show a linear dose-response association.

Currently, various potential anti-cancer compounds from mushrooms have been identified from more than 20,000 species of mushrooms. These bioactivity substances include p-hydroxybenzoic acid, protocatechuic acid, sinapic acid, cinnamic acid and ferulic acid in P. ostreatus mushrooms (Patel & Goyal, 2012). Additionally, trans-cinnamic acid, p-hydroxy-benzoic acid, protocatechuic acid and caffeic acid were isolated in A. bisporus (Muszyńska, Kała, Rojowski, Grzywacz, & Opoka, 2017). The

chemical structure of these compounds was presented as Figure 2.1 (Neergheen, Kam, Pem, Ramsaha, & Bahorun, 2020).

On the other hand, polysaccharides and glucan isolated from mushroom have been reported that both are the primary bioactive compounds of antitumor activity, including heterosaccharide chains of xylose, mannose, galactose, or uronic acid, or β-D-glucan-protein complexes (Sullivan, Smith, & Rowan, 2006). Polysaccharides receiving higher attention is due to its complex structure and different bioactivities, which were formed by the β-d-glucan group and monosaccharides units, linked with the glycosidic bond. Its bioactivity of anti-cancer has supported by a large number of results of *in vitro* and in vivo experiments. For example, polysaccharides (glucans) from fruiting body extract or mycelia extract of the Pleurotus pulmonarius has been demonstrated its inhibition of colitis-associated colon carcinogenesis in a mice model. Alonso et al. (2017) also demonstrate that proteoglucan from mushroom can decreases tumour burden and lung metastases in a murine model of breast cancer.

Mushroom and infection

Mushrooms can produce various secondary metabolites, which have been reported that they can be applied as a novel antimicrobial compound, including terpenes, steroids, benzoic acid derivatives, anthraquinones, and quinolones. Béni et al. (2018) found that secondary metabolites isolated from Tapinella atrotomentosa extracts possess a significant antibacterial activity, in particular multiresistant Acinetobacter baumannii and extended-spectrum β-lactamase (ESBL)-producing Escherichia coli. Mushroom rich in lentinan, which also has been shown benefits to the innate immune system. The Ireland researcher team (Masterson et al., 2020) reported that β-glucans from the Shiitake mushroom can reduce cell counts of Klebsiella pneumonia, as well as infiltration of white cell in a rodent pneumonia model. The mechanism of lentinan in reducing the sepsis-induced lung injury might due to its immunomodulatory manner.

Figure 2.1 Chemical structures of common phenolic acids, flavonoids and ergothioneine present in mushrooms, (Neergheen et al., 2020).

2.2 Mushroom Samples

2.2.1 Shiitake Mushroom (*Lentinula edodes*)

Shiitake mushrooms (Lentinus edodes), which is one of the world's famous edible mushroom. It was the second most cultivated edible mushroom around the world, which second only to Agaricus bisporus (Kalač, 2013; Valverde et al., 2015). Normally, mushrooms contain more than 57 % of carbohydrates, 25 % of protein, 5.7 % of fat, and 12.5 % of ash (Mattar et al., 2018). Various important compounds have been isolated from their fruiting body, mycelia, and culture medium, which include dietary fibre, bioactive polysaccharides (lentinan), ergosterol, vitamin B (B1 and B2) and C as well as various minerals.

Shiitake mushroom is well known for its unique aroma, as well as a high concentration of umami compounds and could be used as a natural seasoning in the meal. Additionally, its aqueous extracts were often used to prepare dashi. The volatile compounds that isolated from mushroom can be classified as two types, namely volatile and nonvolatile compounds. Free amino acids, 5-nucleotides and soluble carbohydrates are water-soluble compounds and playing the main role of providing taste in mushrooms (Her, Kim, Kim, & Lee, 2015). Volatile compounds are primary contributors of flavour in mushroom, which were comprised by a series of aliphatic eight carbon (C8) components, which includes 1-octen-3-ol, 3-octanol, 1-octanol and 3-octanone. What is more, sulphur compounds also provide shiitake mushroom with a unique flavour, which includes dimethyl trisulfide (DMTS), dimethyl disulfide (DMDS) and cyclic sulphur compounds. Mattar et al. (2018) using shiitake mushroom extract as a natural taste-enhancer to reduce the NaCl content in low-sodium beef burgers development. The results indicate that adding 20% of mushroom homogenate can offer a higher salinity perception in a burger that NaCl reduced to 50%, which also improve its acceptance of colour, aroma, flavour, texture, and overall perception. This can help a new product development achieving the goal of clean-label products. The reason for Shiitake mushrooms incorporating into cuisines in many countries is not only due to its strong aroma and umami taste but also due to its nutritional as well as medicinal properties.

Due to mushrooms have been recognized as a good source of bioactive compounds. Shiitake mushroom has been reported that its bioactive compounds could against cardiovascular diseases, in particular Water-soluble fractions, which presents high antioxidant activities (Morales, Piris, Ruiz-Rodriguez, Prodanov, & Soler-Rivas, 2018). Furthermore, its antioxidant capacity has begun to arouse high interest among researchers and added in many processing foods. Some recent studies have reported that Shiitake mushrooms have been used to produce a functional food, such as semolina pasta in New Zealand (Lu et al., 2018) and functional rice muffins in Korea (Olawuyi & Lee, 2019), both aim to increase bioactive composition and antioxidant activity in the end products.

2.2.2 Black Ear Mushroom (*Auricularia auricula*)

Black ear mushroom (*Auricularia auricula*), which is one of edible mushroom for a few centuries in the world, in particular in Chinese and European. Numerous evidence has demonstrated that Black ear mushroom contains bioactivity and benefit human health. Black ear mushroom is rich in dietary fibre, higher amounts of crude proteins, but it contains lower amounts of carbohydrates and soluble sugars. This advantage benefits hypoglycaemic effects *in vivo* (Bandara et al., 2017). In addition, a large amount of evidence of the potential use of black ear mushroom bioactive compounds have been reported, such as reducing cholesterol and weight manipulating (Chen et al., 2008; T. Zhang, Zhao, Xie, & Liu, 2020). Recently, a systematic review and network meta-analysis were evaluated by Ma et al. (2018). They suggested that the black ear was an effective and safe drug for anti-cancer, especially in new and adjuvant treatments of gastrointestinal cancers. Various bioactivity compounds have been purified from black ear mushroom. Polysaccharide extract from black ear mushroom has demonstrated could exhibit free radical scavenging activity (Qian et al., 2020). Black ear mushroom also has been incorporated into new food products as a functional ingredient due to its hypoglycaemic and antioxidant activity, such as extrudates foods (Vallée et al., 2017) and bread (Yuan, Zhao, Yang, McClements, & Hu, 2017).

2.2.3 Siliver Ear Mushroom (*Tremella fuciformis*)

Silver ear mushroom (*Tremella fuciformis*) belongs to the order Tremellaces and the family Tremellacea, which also known as edible white jelly mushroom (H. Li, Lee, Kim, Moon, & Lee, 2014). It has been used as a medicinal ingredient of traditional medicine in many East Asian countries (Y.-j. Wu et al., 2019). Due to its various bioactivities, Silver ear mushroom has attracted increasing attention in several decades. It is rich in polysaccharides. The polysaccharides isolated from Silver ear mushroom consisted mainly of mannose, glucuronic acid, glucose, galactose, xylose, and rhamnose (Ge et al., 2020). Its main structure is (1→3)-α-mannan backbone. Additional, the amount of xylose and glucuronic acid from Silver ear mushroom were both lower than other mushroom polysaccharides (Hu et al., 2019). Various phenolic acids have been identified in Silver ear mushroom and possessing antioxidant activity as well as *in vitro* anti-inflammatory activity (H. Li et al., 2014). Silver ear mushroom water extract has been reported could improve memory in a rat model (Kim et al., 2007). Polysaccharides from silver ear mushroom contain uronic acid, which makes it can be used as a portion of potential functional food for skin protective effect (Wen et al., 2016). A recent study has used silver ear mushroom mixed with Flos Sophorae Extract to modify the quality of Low-Fat Yogurts for improving physicochemical, textural, rheological, and antioxidant properties (Tang et al., 2020).

2.3 Red Sorghum Flour

Starch is as well known the cheapest and the most important carbohydrate resource in human life. It is formed by two polymers: branched amylopectin and linear amylose. The former has a linear structure of D-glucan units bonded together by α-(1,4) bonds, while the latter is composed of linear chains with α-(1–4) bonds and α-(1–6) bonds (Khlestkin, Peltek, & Kolchanov, 2018).

Sorghum is an important crop, which originated in sub-Saharan Africa (Irondi et al., 2019). In the beginning, it was produced to feed livestock and industrial usage. Nowadays, sorghum received attention by more and more people is due to its beneficial health-promoting properties. For instance, it rich in slowly digestible starch (SDS) and bioactive flavonoids (Teferra & Awika, 2019), which would benefit consumer health. Therefore, sorghum has a potential used in the food industry for a novel starch resource. The Nutrition analysis of red sorghum flour we used is listed below:

Figure 2.2 Nutrition analysis of red sorghum flour (per 100g quantity).

Energy (kJ)	Protein (g)	Fat (g)	Carbohydrate (g)	Sugars (g)	Dietary Fibre (g)	Ash (g)	Moisture (g)	Particle Size <200 micron
1559	12.3	4.1	67.4	1.6	6.6	1.2	8.4	>75%

Chapter 3

Material and methods

3.1 Materials

3.1.1 Mushroom Powder

Dried shiitake mushroom, dried black ear mushroom and dried sliver ear mushroom were purchased from the local Asian Supermarket (Sunson Asian Supermarket, Christchurch, New Zealand).

Three mushroom samples were dry-milled by a mill (model: BCG200; Coffee Grinder, Breville, Sydney, Australia). Each sample mill for four to five minutes until as powder and to minimise possible differences among mushroom samples. Then the powder was put into a few sealed container and stored at room temperature in the dark.

3.1.2 Red Sorghum Flour

Red sorghum flour was purchased from the local Food Ingredients Company (Davis Food Ingredients, Pty Ltd, Auckland, New Zealand). It made in Australia from locally grown sorghum. This flour contains 67.4g, 12.3g and 6.6g of carbohydrate, protein and dietary fibre, respectively.

3.2 Sample preparation

3.2.1 Mushroom Extracts

To assess the effect of extraction condition on polyphenol, weigh out 0.5 g of each mushroom powder into 50 mL plastic bottle and add 20 mL of RO water, 50 and 80% ethanol as extraction solvent. Using a pH meter to adjust the pH of the solvent to pH2 and pH7. Stir 3 hours on the multi-stirrer at 25, 50 and 80 °C. Centrifuge each extract solutions at 4500 rpm for 15 minutes at 20°C. Collect the supernatant into plastic tubes and store at -20°C to minimize degradation.

To prepare mushroom extracts used in all experiments, weigh out 5 g of each mushroom powder into 250 mL Schott bottle and add 200 mL of RO water or ethanol, pH7. Stir 3 hours on the multi-stirrer at 50°C. Centrifuge each extract solutions at 4500 rpm for 15 minutes at 20°C. Collect each supernatant and using a rotary vacuum evaporator (Buchi Rotavapor-R Rotary Evaporator, USA) at 55 °C for

removing RO water or ethanol and concentrated. The concentrated solution stored at 4 °C for measurement of the extraction yield and preparing as 1% (W/V) extract solutions.

3.2.2 Measurement of the Extraction Yield

5ml of concentrated extract solutions were placed into a container and put in the oven at 95 °C overnight. The weight of the dried extract of each mushroom samples was measured triplicate. The calculation of yield percentage was using the following formula:

$$\text{Percentage of Extraction Yield (\%)} = \frac{\text{Weight of dried extract}}{\text{Weight of dried mushrooms samples}} \times 100\%$$

3.2.3 Red Soughum Paste and Measurement of Pasting Profile

Red sorghum paste was prepared by RVA. The pasting properties of starch and starch blends were measured by a Rapid Visco Analyser (RVA4500) (Perten Instruments, Hägersten, Sweden). Red sorghum flour was weighed in an aluminium container, then 25ml of 1% concentrated each mushroom extract solution was added to each sample. The mixture was stirred using a plastic paddle for at least 1 min. The heating and cooling program was set following the procedure of He et al (2018), and some modification. A programmed heating and cooling cycle was used, in which the samples were held at 50 °C for 1 min, heated to 95°C over 7.5 minutes, and held at 95 °C for 5 min before cooling to 50 °C over 7.5 minutes and then being held at 50 °C for 2 minutes. Six parameters were measured on the visco-amylogram: PT: pasting temperature (°C); Pt: peak time (min); PV: peak viscosity (cP); TV: trough viscosity (cP); BV: breakdown viscosity (cP); SV: setback viscosity (cP); FV: final viscosity (cP). The analyses were performed in triplicate and mean values were calculated.

3.3 Determination of Total Phenolic Compounds (TPC)

The total phenolic content (TPC) of all mushrooms extracts was determined by 0.2N Folin–Ciocalteu reagent (Sigma, St Louis, USA), following the method of Singleton and Rossi (1965) and with slight modification. Mushrooms extract (0.5 mL) were mixed with 2.5 mL of 0.2N Folin-Ciocalteu reagent and 2 mL of 7.5 % sodium carbonate. The sample was incubated at room temperature for 2 hours in a dark covered with aluminum foil. Then absorbance was measured at 760 nm (V-1200 spectrophotometer, Global Science). The TPC of mushrooms extracts was calculated from the calibration curve of standard gallic acid (25-250 μg/L) and expressed as mg gallic acid (Sigma, St Louis, USA) equivalent (GAE) per

gram dry weight, with each sample being analysed in triplicate. Additional dilution was needed if the TPC value determined was over the linear range of the standard curve.

3.4 Antioxidant analysis

3.4.1 Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH)(TCI America, Tokyo, Japan) radical scavenging activity of all mushrooms extracts was measured by the method adopted by Floegel et al (2011) with slight modifications. 0.1mM of DPPH stock solution was prepared with 100 % methanol and stirred for 30 minutes. Then, 0.5 mL of standard or sample were mixed with 1mL of DPPH solution and 1.5 mL of 100 % methanol and incubated in the dark for 30 minutes covered with aluminium foil. The absorbance was measured at 517 nm (V-1200 spectrophotometer, Global Science). The DPPH radical scavenging activity of mushrooms extracts was calculated from the calibration curve of standard Trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, USA), 0–1000 µmol. All results were expressed as micromoles of Trolox per gram dry weight, with each sample is analysed in triplicate. Additional dilution was needed if the DPPH value determined was over the linear range of the standard curve.

3.4.2 Determination of Ferric-ion Reducing Antioxidant Power (FRAP)

The Ferric-ion reducing antioxidant power (FRAP) of all mushrooms extracts was measured by the method adopted by Wootton-Beard, Moran and Ryan, (2011) with slight modifications. Prepare a fresh working solution of FRAP reagent on the day of the analysis by mixing together 300 µM Acetate buffer pH 3.6, 10 mM TPTZ (Sigma, St Louis, USA) in 40 mM HCl and 20 mM Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) (Sigma, St Louis, USA) at a ratio of 10:1:1 (v/v/v). Iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Sigma, St Louis, USA) was prepared as a standard solution. Then, 250 µL of each standard or sample extract and 2.5 mL of the FRAP reagent were mixed well and incubated in the dark for 2 hours covered with aluminium foil. The absorbance was measured at 593 nm (V-1200 spectrophotometer, Global Science). The FRAP of mushrooms extracts was calculated from the calibration curve of standard $FeSO_4$ (50–1000 µM) and expressed the results as µM Fe^{2+} per gram dry weight, with each sample is analysed in triplicate. Additional dilution was needed if the FRAP value determined was over the linear range of the standard curve.

3.5 In vitro gastrointestinal starch digestibility analysis

3.5.1 Glycemic glucose equivalent (GGE) ASSAY

The *in vitro* digestion method developed by Foschia et al. (2015) and improved by Hossain et al. (2017) was used to evaluate carbohydrate digestibility of red sorghum paste incorporated with mushroom extracts.

This method used to evaluate the amount of free glucose in incubation during enzymatic hydrolysis with time. Weigh 8.25g red sorghum paste addition 1% mushroom extracts solution 25ml held at 37 °C for 10 min with constant stirring. Adding 0.8 mL of 1 M HCl to each sample container made low pH. Then, 1 mL of 10% pepsin solution (Thermo Fisher Scientific, San Jose, CA, USA) (1 g pepsin in 10 mL 0.05 M HCl) was added to mimic gastric digestion and incubated for 30 min. 1 mL aliquots were taken (time 0) and added to 1 mL absolute ethanol to stop the further reaction. Add 2 mL 1 M $NaHCO_3$ and 5 mL 0.1 M sodium maleate buffer pH 6 to each sample container. 0.1mL of Amyloglucosidase was added to prevent end-product inhibition of pancreatic α-amylase. 5mL of 2.5% Pancreatin solution (Sigma Aldrich, St Louis, USA) (2g Pancreatin powder in 80mL 0.1 M sodium maleate buffer, pH 6) was added to represent ileal digestion and aliquots were taken after 20, 60 and 120 minutes and treated as before. The samples were stored at 4 °C for the subsequent analysis of reducing sugar, TPC, antioxidant activity and protein digestibility analysis.

3.5.2 Determination of Reducing Sugar Content (RSC)

Reducing Sugars release during *in vitro* digestion was measured by 3.5-dinitrosalicylic acid (DNS). All aliquots were centrifuged at 1000 rpm for 5 minutes. 50µL of standard or sample placed to test tube. Add 0.25 mL of enzyme solution A (1% invertase and 1 % amyloglucosidase in acetate buffer pH 5.2) and leave to digest for a minimum of 20 minutes at room temperature. Add 0.75 mL of DNS mixture (ratio of 1 : 1 : 5 of 0.5 mg/mL glucose : 4 M NaOH : DNS reagent) to each test tube. Cover test tubes in metal racks with tinfoil and incubated in boiling water bath at 95 – 100 °C for 15 minutes. Place test tube racks in a cold water bath and add 4 mL of RO water. The absorbance was measured at 530 nm (V-1200 spectrophotometer, Global Science). The results of glucose released were calculated from the calibration curve of standard glucose (0–10mg/mL) and expressed the results as mg glucose per gram sample and plotted versus time area under the curve (AUC) was calculated by dividing the graph into trapezoids., with each sample being analysed in triplicate. Additional dilution was needed if the glucose value determined was over the linear range of the standard curve.

3.6 Bicinchoninic Acid (BCA) Assay

The soluble protein of 1% concentrate extract solution and all digested samples during *in vitro* digestion were measured by the method adopted by Bainor et al. (2011) with slight modifications. Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, USA) was used to determine soluble protein content. Add 10 µL of standard or sample to each tube containing reagent and mix well. Incubate at 37°C for 2 hours in the dark and covered with aluminium foil. The absorbance was measured by a microplate reader (FLUOstar Omega, BMG Labtech GmbH, Ortenberg, Germany) at 562 nm. The soluble protein of mushrooms extracts was calculated from the calibration curve of standard bovine serum albumin (0-2000 ug/ml) and expressed the results as g protein per gram dry weight, with each sample being analysed in triplicate. Additional dilution was needed if the protein value determined was over the linear range of the standard curve.

3.7 Statistical Analysis

All experiments were performed in triplicate. The results were presented as means ± standard deviation (SD) and analyzed using Minitab statistical software Version 19 (Minitab Pty Ltd; Sydney, NSW, Australia) for windows 10. Statistical differences were determined by one-way analysis of variance (ANOVA) and Tukey's comparison test, $p < 0.05$ was considered as the significant level.

Chapter 4

Extraction of polyphenol compounds from mushroom samples

4.1 Effect of different extraction conditions on TPC and Antioxidant activity of mushroom extracts

Normally, the extraction yield and efficiency are depending on various conditions, which include extraction methods, extraction solvent, solvent pH, temperature and time. Screening appropriate extraction conditions are the most important step in the research. It can build up a stable techniques system of extraction, which also can enable the maximum amount of bioactive compounds releasing from materials. It has been found that polyphenolic compounds and flavonoids are the major contributors to antioxidant activities among plant and mushroom (Choi, Lee, Chun, Lee & Lee, 2006; Zhang et al., 2015; Liu, Xiao, Wang, Chen & Hu, 2017). Thus, the purpose of this work was to evaluate the effect of different extraction condition on the changes in the TPC (Table 4.1) and antioxidant activities of extracts of Shiitake mushroom, black ear mushroom and silver ear mushroom. TPC of all mushrooms extracts was determined by 0.2N Folin–Ciocalteu reagent, while antioxidant activities were measured by diphenylpicryl-hydrazyl (DPPH) radical scavenging activity (Table 4.2) and ferric reducing antioxidant power (FRAP) (Table 4.3).

4.1.1 Extraction method

Different extraction methods can affect the recovery of polyphenols from mushroom, which usually can be separate as conventional and non-conventional. Using a water bath as a method to extraction polyphenol compounds from plant issue is a conventional extraction method used for decades. The best advantage of the water bath is that indirect and slowly heating method can enable a large amount of polyphenol compounds extraction from plant tissue. Thus, this is the reason we used the water bath as an extraction method in this study (Rajbhar, Dawda & Mukundan, 2015).

4.1.2 Extraction solvent

The solubility of bioactive phenolic compounds in various solvents is depending on their specific structure. In the other words, their extraction yield was affected by the extraction solvent and the isolation procedures. Thus, the selection of an extraction solvent is the first step (Złotek, Mikulska, Nagajek & Świeca, 2016).

With the consideration of the objective of the present study is that mushroom extracts incorporated into red sorghum paste and made them a portion of food that potential benefits for human health. Thus, we choose the RO water as the first extraction solvent, which avoids the undesirable residue. Additionally, recent studies have shown that ethanol is also a better solvent of bioactive phenolic compounds extraction and presenting a higher antioxidant activity (Librán Cuervas-Mons, Mayor López, García Castelló & Vidal Brotons, 2013; Khatri & Chhetri, 2020). Therefore, ethanol is the secondary solvent, which has different concentrations, namely 50% and 80% mixed with RO water. The results presented that the value of TPC, Trolox concentration and FRAP in RO water extract were all greater than 50% and 80% ethanol extracts. Meanwhile, they decrease depending on the increase of ethanol concentration. According to Rajbhar et al. (2015), this is due to the strengthening of the hydrogen bonds between polyphenols and protein. Likewise, decreasing the strengthening of hydrogen bonds can through by adding a higher percentage of water into absolute ethanol, so that improve the solubility of the polyphenols. As a result, the RO water could be identified as the most efficient solvent system for extracting polyphenolic compounds from all mushrooms powder in for polyphenol extracting in this study. In contrast, 80% of ethanol was shown as the most inefficient solvent in extracting phenolic compounds from all mushrooms.

4.1.3 Extraction pH of the solvent

The effect on different pH on phenolic compounds from Shiitake, black ear and silver ear mushroom has not been extensively studied before this study. Overall, the results revealed that extraction at pH7 was significantly greater than pH2 (p<0.05) in the TPC, Trolox concentrations and FRAP. TPC value of Shiitake RO water extract in pH7 (7.39±0.05) was significantly (p<0.05) higher than in pH2 (6.41±0.24). The Trolox concentration of Shiitake RO water extract in pH7 (24.09±0.85) presents greater value than in pH2 (5.76±0.92). Similarly, TPC and Trolox concentration of BE RO water extract in pH7 (2.57±0.01 and 2.76±0.12) both show a significant difference comparing with pH2 (1.99±0.03 and 1.63±0.05). For SE RO water extract, antioxidant activity in Trolox concentration and FRAP value in pH7 were both significant differ to pH2. Librán Cuervas-Mons et al. (2013), assayed the influence on different pH in polyphenol from grape waste and found that the extraction yield has increased in basic pH, while it decreases in acid pH. Our results appeared in line with the results of theirs. Therefore, the solvent of pH 7 has been selected for further extraction condition.

4.1.4 Extraction temperature

The usual extraction temperatures of a water bath for extracting bioactivity phenolic compounds are ranged from 20 to 50°C (Rajbhar et al., 2015). In this work, 25, 50 and 80 °C were used in the experiment to discuss the effect on the temperature of TPC and antioxidant activity. From 30 extracts of 3 mushrooms, RO water extracts at 50°C for 3 hours of black ear mushroom and silver ear mushrooms exhibited the highest value of TPC, which are 2.74±0.06 and 3.21±0.08 mg GAE /g DW, respectively. The results of Trolox concentration and FRAP also present a similar trendy. The highest TPC of Shiitake mushroom extract was RO water extraction at 25°C (7.39±0.05 mg GAE /g DW) and followed by RO water extraction at 50°C (6.15±0.03 mg GAE /g DW). However, it falls once the extraction temperature rasing at 80°C.

The previous study has reported that heat treatment can significantly (p<0.05) enhance the antioxidant activities of Shiitake mushroom (Choi et al., 2006). However, it is well known that most of the bioactivity compounds could be damaged during cooking and thermal processing, which is due to their characters of unstable to heat. According to Rajbhar et al. (2015), the phenolic compounds might be degradation sharply once the temperatures higher than 70°C. This is due to heat enhancing the cell walls permeable and increasing solubility and diffusion coefficients. Meanwhile, the viscosity of the solvent decreasing also beneficial the solid substrate releasing. Nevertheless, phenolic compounds such as might be degradation once the temperature above 70°C. This results also were demonstrated in our data. Consequently, the 50°C is the appropriate temperature for extracting among the three mushrooms, which is the temperature we selected to prepare the mushroom extract in all experiment.

In addition, the positive correlation between the TPC and antioxidant activity, Trolox concentration (r =0.843, p<0.001) and FRAP (r =0.949, p<0.001) of the studied extracts has been found (Table 4.4), while concentration Trolox also present a positively correlated with FRAP (r =0.863, p<0.001). Based on the above comparison of the various extraction methods used for TPC, Trolox and FRAP, the best process conditions of phenolic compounds from 3 mushrooms were water bath at 50°C for 3 hours of extraction in RO water at pH7.

Table 4.1 Effect of different extraction conditions on TPC of three mushrooms.

Extraction condition			TPC (mg GAE /g DW)		
pH	Temperature (°C)	Solvent	Shiitake	BE	SE
2	25	RO Water	6.41±0.24^{bc}	1.99± 0.03^{d}	1.72±0.02^{e}
		50% EtOH	4.87±0.29^{e}	1.00± 0.01^{f}	1.23± 0.16^{f}
		80% EtOH	3.26±0.27^{f}	0.88± 0.01^{g}	0.36± 0.02^{f}
7	25	RO Water	7.39±0.05^{a}	2.57± 0.01^{b}	1.35±0.02^{d}
		50% EtOH	5.08±0.15^{de}	1.03± 0.01^{f}	0.68±0.02^{e}
		80% EtOH	3.37±0.14^{f}	0.79± 0.02^{h}	0.46± 0.01^{f}
7	50	RO Water	6.15±0.03^{bc}	2.74± 0.06^{a}	3.21±0.08^{a}
		50% EtOH	6.49±0.03^{b}	1.31± 0.01^{e}	2.15±0.04^{c}
7	80	RO Water	5.50±0.12^{d}	2.42± 0.06^{c}	2.66±0.06^{b}
		50% EtOH	6.01±0.03^{c}	1.29± 0.03^{e}	2.28±0.33^{c}

Data was expressed as Mean ± SD (n = 3).
$^{a-f}$ Mean values with different lowercase superscript letters in separate sample are significantly different ($P < 0.05$).

Table 4.2 Effect of different extraction conditions on DPPH concentration of three mushrooms.

Extraction condition			Trolox concentration (μM/g DW)		
pH	Temperature (°C)	Solvent	Shiitake	BE	SE
2	25	RO Water	5.76±0.92^{d}	1.63± 0.05^{b}	0.59±0.06^{de}
		50% EtOH	5.92±0.01^{d}	2.68± 0.12^{a}	0.39±0.12^{e}
		80% EtOH	5.33±0.53^{d}	2.91± 0.07^{a}	1.24±0.10^{bc}
7	25	RO Water	24.09±0.85^{a}	2.76± 0.12^{a}	0.93±0.03^{cd}
		50% EtOH	17.49±0.30^{b}	1.66± 0.01^{b}	1.22±0.02^{bc}
		80% EtOH	14.21±0.61^{c}	1.75± 0.08^{b}	1.08±0.04^{bc}
7	50	RO Water	24.55±0.46^{a}	3.02± 0.71^{a}	1.84±0.12^{a}
		50% EtOH	25.28±0.33^{a}	1.84± 0.01^{b}	1.74±0.09^{a}
7	80	RO Water	13.76±1.10^{c}	2.78± 0.11^{a}	0.78±0.42^{de}
		50% EtOH	24.55±0.43^{a}	1.97± 0.21^{b}	1.47±0.24^{ab}

Data was expressed as Mean ± SD (n = 3).
$^{a-e}$ Mean values with different lowercase superscript letters in separate sample are significantly different ($P < 0.05$).

Table 4.3 Effect of different extraction conditions on FRAP of three mushrooms.

Extraction condition			FRAP (μM Fe^{2+}/g DW)		
pH	Temperature (°C)	Solvent	Shiitake	BE	SE
2	25	RO Water	144.81 ± 4.06^{b}	23.60 ± 0.25^{b}	13.83 ± 0.27^{bc}
		50% EtOH	108.29 ± 3.10^{e}	16.52 ± 0.25^{de}	11.49 ± 0.09^{cd}
		80% EtOH	083.86 ± 0.31^{f}	18.57 ± 0.15^{d}	11.21 ± 0.19^{d}
7	25	RO Water	130.79 ± 1.71^{a}	32.60 ± 0.37^{c}	16.44 ± 0.30^{cd}
		50% EtOH	100.52 ± 1.92^{de}	18.10 ± 0.16^{e}	13.72 ± 1.78^{d}
		80% EtOH	081.65 ± 3.15^{f}	19.39 ± 0.18^{de}	11.16 ± 0.24^{d}
7	50	RO Water	113.84 ± 3.41^{cd}	39.61 ± 2.48^{a}	20.40 ± 1.73^{a}
		50% EtOH	119.76 ± 5.55^{c}	25.43 ± 0.52^{c}	17.02 ± 0.35^{b}
7	80	RO Water	112.47 ± 2.80^{cd}	33.93 ± 1.21^{b}	18.23 ± 0.30^{ab}
		50% EtOH	121.29 ± 0.80^{c}	25.33 ± 0.51^{c}	17.14 ± 1.70^{b}

Data was expressed as Mean ± SD (n = 3).
$^{a-f}$ Mean values with different lowercase superscript letters in separate sample are significantly different ($P < 0.05$).

Table 4.4 Pairwise Pearson Correlations between TPC and antioxidant activity.

		Correlation (r)	95% CI for ρ	P-Value
TPC	Trolox	0.843	(0.771, 0.894)	0.000
TPC	FRAP	0.949	(0.923, 0.966)	0.000
Trolox	FRAP	0.863	(0.798, 0.908)	0.000

Unit: TPC: GAE mg/g DW; Trolox: μM /g DW; FRAP:μM/g DW.

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4.2 Comparison of yield between RO water and 50% ethanol extracts

The yield of RO water and 50% ethanol extracts of three mushrooms were shown in Table 4.5. The results show that the yield of RO water extracts and 50% ethanol extracts derived from Shiitake mushroom was 33.48 ± 1.92 and 36.29 ± 1.88 %, respectively. There were no significant differences between the two extracts. For BE, the yield of RO water extracts was shown significantly (p<0.05) higher than 50% ethanol extracts, which were 10.81 ± 0.90 and 6.00 ± 0.34 %. Similarly, there was a significant difference between RO water extracts and 50% ethanol extracted from SE. The yield was 16.00 ± 0.00 and 12.32 ± 0.63 %, respectively. BE had the minimum ethanol extract yields among the three mushroom species and followed by its water extract. There were significant differences (P < 0.05) in the extraction yields between the extracts of these three mushroom species. Overall, the results demonstrate that RO water extracts generated from three mushrooms were all reach a higher yield in this study.

Zhang et al. (2015) also compared the yield between water and 80% ethanol extracts among five mushrooms. The results show the yield of 80% ethanol extract and antioxidant activity were greater than water extracts in Lentinus edodes, Auricularia polytricha and *Tremella fuciformis*. They prepared the water extract with 200 mL of water three times at 100 °C for 2 hours, which differ to ours that 50 °C for 2 hours. The might be the possible cause of the difference.

Table 4.5 The yield of RO water and 50% ethanol extracts of three mushrooms.

	Yield of extracts (%)(W/W)		
Extraction Solvent	Shiitake	BE	SE
RO Water	33.48 ± 1.92[a]	10.81 ± 0.90 [a]	16.00 ± 0.00 [a]
50% EtOH	36.29 ± 1.88 [a]	06.00 ± 0.34[b]	12.32 ± 0.63[b]

Data were expressed as Mean ± SD (n = 3).
[a- b] Mean values with different lowercase superscript letters in the separate sample are significantly different (P < 0.05).

4.3 Comparison of antioxidant analysis of 1% concentrated extracts solution

Contents of TPC, antioxidant activity and soluble protein were determined in the 1% (W/V) concentrated extract solution from the three mushrooms (Table 4.7). The 1% (W/V) concentrated extracts solution was used to prepare red sorghum paste. The final extraction condition was selected from 4.1 effects of different extraction conditions on TPC and Antioxidant activity of mushroom extracts. Weigh out 5 g of each mushroom powder into 250 mL Schott bottle and add 200 mL of RO water or ethanol, pH7. Stir 3 hours on the multi-stirrer at 50°C. Centrifuge each extract solutions at 4500 rpm for 15 minutes at 20°C. Collect each supernatant and using a rotary vacuum evaporator (Buchi Rotavapor-R Rotary Evaporator, USA) at 55 °C for removing RO water or ethanol and concentrated to 1% (W/V).

For TPC, the water extracts derived from three mushrooms were significantly (p<0.05) higher than ethanol extracts. The shiitake water extracts (24.57±0.42 mg GAE /g DW) were the maximum value among the three mushrooms, followed by its 50% ethanol extracts (17.79±0.21 mg GAE /g DW) and water extracts derived from BE (18.89±0.66 mg GAE /g DW) and SE (18.30±1.7 mg GAE /g DW).

For antioxidant activity measurement, shiitake mushroom water extracts were found to have the highest concentration in Trolox (44.50±0.07 µM/g DW) and FRAP (222.06±16.46 µM Fe^{2+}/g DW). Its 50% of ethanol extracts were reached at 39.98±0.12 µM/g DW in Trolox and 106.53±11.83 µM Fe^{2+}/g DW in FRAP, which makes it the second high among all extracts. However, the rest of the mushrooms extracts had a significantly (p<0.05) lower concentration in Trolox and FRAP and were in the descending order of BE and SE. It was obvious that the antioxidant activities of water extracts in the DPPH and FRAP method were excellent for Shiitake mushrooms, and low for the two ear mushrooms.

Soluble protein content was determined by the BCA assay. The results show there was no significant difference between water extracts and 50% ethanol extract isolated from Shiitake mushroom, which were 11.61 ± 0.46 and 11.11 ± 0.43 mg/g DW, respectively. Likewise, SE water and ethanol extract presenting a similar trend, which was 7.44 ± 0.17 and 7.33 ± 0.18 mg/g DW. In contrast, the least protein content was detected in BE ethanol extract (3.10 ± 0.15 mg/g DW) among all extracts, which was significantly (p<0.05) lower than water extracts (4.96 ± 0.45 mg/g DW).

Table 4.6 The results of TPC, antioxidant activity and soluble protein content of 1% (W/V) concentrated extracts solution of RO water and 50% ethanol in three mushrooms.

Samples	Solvent	TPC (mg GAE /g DW)	Trolox (μM/g DW)	FRAP (μM Fe^{2+}/g DW)	Protein (mg/g DW)
Shiitake	RO Water	24.57 ± 0.42[a]	44.50 ± 0.07[a]	222.06 ± 16.46[a]	11.61 ± 0.46[a]
	50% EtOH	17.79 ± 0.21[b]	39.98 ± 0.12[b]	106.53 ± 11.83[b]	11.11 ± 0.43[a]
BE	RO Water	18.89 ± 0.66[b]	12.11 ± 0.67[d]	71.04 ± 5.25[c]	4.96 ± 0.45[c]
	50% EtOH	12.00 ± 0.23[d]	16.43 ± 0.36[c]	39.43 ± 4.36[d]	3.10 ± 0.15[d]
SE	RO Water	18.30 ± 1.7[b]	6.36 ± 0.46[e]	19.72 ± 5.92[de]	7.44 ± 0.17[b]
	50% EtOH	15.50 ± 0.3[c]	7.10 ± 0.54[e]	4.76 ± 3.08[e]	7.33 ± 0.18[b]

Data were expressed as Mean ± SD (n = 3).

[a-f] Mean values with different lowercase superscript letters in columns are significantly different ($P < 0.05$).

Chapter 5

Pasting properties and *in vitro* glycaemic index of red sorghum paste with addition of mushroom extracts

This study hypothesises that the pasting properties of red sorghum flour may affect by bioactivity phenolic compounds derived from the mushroom. Therefore, the objectives of this study were to investigate the effects of phenolic compounds derived from mushroom water and ethanol extract on the gelatinisation and *in vitro* starch digestibility in a red sorghum starch system and to assess how these compounds affect starch-digestive enzyme activity. 1% concentrated mushroom extract solutions were separately incorporated in red sorghum flour for pasting preparation and compared with control. The pasting properties were measured by Rapid-Visco-Analyser (RVA). RVA is a rotational viscometer, which could continuously record the viscosity and many parameters of starch samples under conditions wanted, such as temperature and shear. It helps the samples measured to suspend in the solvent and keeping them in suspension during testing, which then offers the appropriate heating, cooling and shear. Eventually, the viscosity changes and a characteristic pasting curve of starch paste were recorded. Pasting properties were the most critical aspects of the research field of starch and its derivative products. It could be obtained by studying a pasting profile of a starch-water suspension, as a function of the time and temperature. Structure and morphology of the starch granules will occur a massive change during the processing of starch cooking with water. Then, the exudated products release from the starch granules forms a network, which further enhances the viscosity in starch-water suspensions (Rincón-Londoño et al., 2016).

5.1 Pasting properties that extracts affect the gelatinisation of red sorghum starch

The RVA pasting properties of red sorghum paste with the addition of 1% shiitake, black ear and silver ear mushroom extracts were presented in Table 5.1. In this study, there six parameters were measured: pasting temperature (PT); peak time (Pt); peak viscosity (PV); trough viscosity (TV); breakdown viscosity (BV); setback viscosity (SV); final viscosity (FV). The meaning of pasting temperature (PT) is the temperature of starch paste at which viscosity started to rise. A low pasting temperature illustrates a lower resistance to swelling and rupture of starch granules (Harasym, Satta & Kaim, 2020). After temperature reaching this point, starch granules becoming expand and the viscosity raise to the peak viscosity (PV), which means that the maximum swelling degree of starch granules during heating. Then, paste continued to heat and resulting in the starch granules rupture occurring. Then, the viscosity falls

to the through viscosity (TV). Lastly, the starch granules, namely amylose and amylopectin rearrange, which viscosity reached the final viscosity (FV) during the cooling step (Rincón-Londoño et al., 2016; Xiao, Xu, Lu & Liu, 2020). Breakdown viscosity refers to the level of starch granule disruption once it reaching at the maximum viscosity (Harasym et al, 2020).

Overall, a significant decrease in the final viscosity of red sorghum pastes with all mushroom extract during RVA analysis was observed in the order of shiitake mushroom ethanol extract, shiitake mushroom water extract, SE water extract, SE ethanol extract, BE ethanol extract and BE water extract. Among all mushroom extract, shiitake mushroom water and ethanol extract had the lowest peak viscosity (PV), trough viscosity (TV), breakdown viscosity (BV), setback viscosity (SV) and final viscosity (FV), while black ear mushroom water extract exhibited the highest values of PV (404.1±2.6 cP), TV (386.8±1.1 cP) and BV (17.3±1.5 cP), which all significant strong than control. The pasting temperature of BE and SE mushroom extracts were lower than control, while shiitake mushroom was rather similar in the control. The peak time of shiitake water and ethanol extract both shorter than control, while there was no significant difference in BE and SE compared with control. The results indicated that the pasting properties of red sorghum paste contain 1% mushroom extracts solution differed depending on species of mushroom and types of extraction solvent.

Harasym et al. (2020) reported Buckwheat Grains rich in polyphenol. They evaluated the effects of ultrasound treatment of buckwheat grains on pasting properties of resulting flour and observed a reduction of FV. According to Han et al. (2020), report that three different polyphenols they used significantly decreased the setback values of rice starch. This result is similar to ours, in particular shiitake mushroom water extract. He mentioned that reduced setback refers to polyphenols a short-term retrogradation and reassociation during cooling of RS was altered. Wu et al. (2020) investigated the effects of green tea polyphenols on the pasting properties of rice flour. The results show green tea polyphenols could significantly decrease all pasting attributes of tested rice flour, which is due to a cross-linking were built between starch granule and green tea polyphenols. The above evidence may help us to clarify our result presented.

The pasting curve was shown in Figure 5.1. Figure 5.1 shows the viscosity of the suspension during the heating and cooling of the red sorghum flour. Similarly, the shape of the pasting curve differed depending on species of mushroom extract and types of extraction solvent. All RVA curves of red sorghum addition 1% mushroom extract shifted down, which indicate that the final viscosity of red sorghum flour with mushroom extract was significantly lower than control ($p < 0.05$). Final viscosity indicates the ability of the material to form a viscous paste or gel after cooking and cooling. It was evident that mushroom extracts change the characterise of red sorghum paste and its capability of forming a viscous paste or gel.

Based on the primary result, all mushroom extract could effectively decrease the PV and FV of red sorghum paste, which means that starch granules expanding was decreased. Meanwhile, the ability of the red sorghum flour to form a viscous paste after heating and cooling was decreased. Furthermore, a reduction of starch granules expanding also decrease starch digestibility, which can be demonstrated by the fall of reducing sugar concentration and AUC. This result was further discussed in section 5.2.

Table 5.1 Average RVA starch pasting properties of red sorghum paste that contain 1% different mushroom extracts.

Samples	PT (°C)	Pt (min)	PV (cP)	TV (cP)	BV (cP)	SV (cP)	FV (cP)
Control	95.2±0.1^{a}	6.7±0.1^{ab}	287.3±9.6^{b}	283.3±9.0^{b}	4.0± 1.0^{c}	606.0±9.0^{a}	889.3±9.5^{a}
Shiitake							
Water	95.1±0.0^{ab}	6.6±0.2^{b}	163.3±2.5^{de}	160.7±2.5^{d}	2.7± 0.6^{c}	316.7±8.5^{d}	477.3±11.0^{e}
50% EtOH	95.2±0.1^{a}	6.2±0.2^{c}	150.0±1.0^{e}	148.7±1.2^{d}	1.3± 0.6^{c}	278.7±1.2^{d}	427.3±1.2^{f}
Black Ear							
Water	94.6±0.0^{c}	6.9±0.1^{ab}	404.1±2.6^{a}	386.8±1.1^{a}	17.3± 1.5^{a}	381.7±12.1^{c}	768.4±11.0^{b}
50% EtOH	94.8±0.3^{abc}	7.0±0.0^{a}	264.0±32.5^{bc}	249.0±28.3^{c}	15.0± 4.2^{a}	444.0±72.1^{b}	693.0±43.8^{c}
Sliver Ear							
Water	94.7±0.2^{bc}	7.0±0.0^{ab}	251.0±6.1^{c}	235.0±8.9^{c}	16.0± 3.0^{a}	423.0±7.0^{b}	658.0±15.9^{cd}
50% EtOH	94.9±0.3^{abc}	7.0±0.0^{a}	179.7±1.5^{d}	170.0±1.0^{d}	9.7± 0.6^{b}	475.7±8.4^{b}	645.7±9.3^{d}

PT: pasting temperature; Pt: peak time; PV: peak viscosity; TV: trough viscosity ; BV: breakdown viscosity; SV: setback viscosity; FV: final viscosity. Data was expressed as Mean ± SD (n = 3).
$^{a-f}$ Mean values with different lowercase superscript letters in columns are significantly different (P < 0.05).

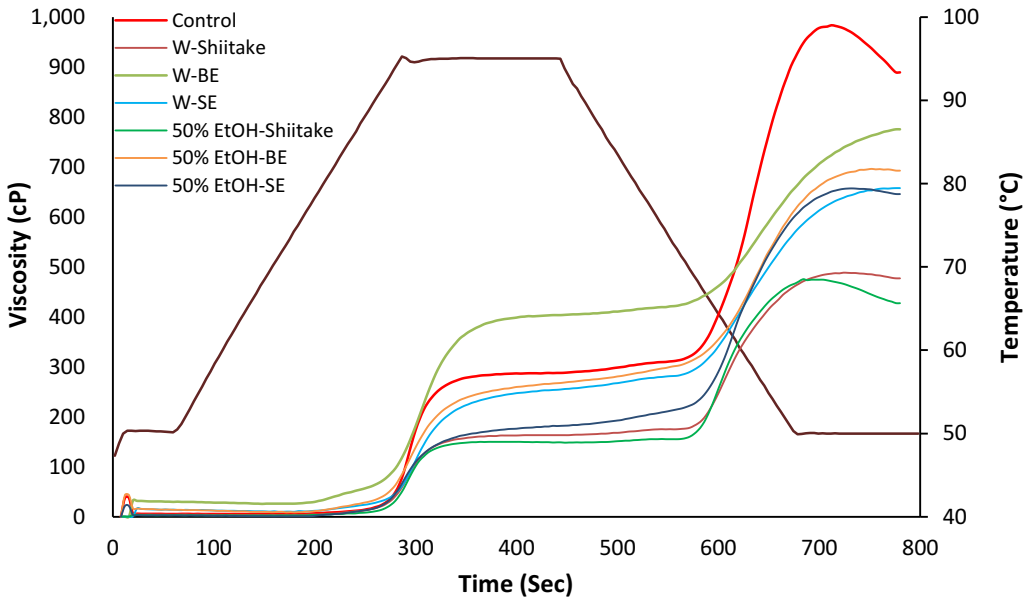

Figure 5.1 Pasting properties of red sorghum flour that contain 1% different mushroom extracts determined by rapid visco analyzer.

5.2 *In vitro* starch digestion and reducing sugars released during gastric and intestinal digestion.

Reducing sugars released sampling was performed at 0, 20, 60 and 120 minutes during *in vitro* intestinal digestion. The effects of mushroom extract derived from shiitake, BE and SE on *in vitro* starch digestion in red sorghum paste were investigated by measuring the released glucose contents during starch hydrolysis. The reducing sugars content degraded from starch were presented as glucose release curves (Figure 5.2.). Figure 5.2 shows the starch hydrolysis curves of shiitake, BE and SE-incorporated red sorghum paste comparing with the control. Overall, starch hydrolysis of red sorghum addition three mushroom extracts all presents a sharp rise between 0 to 20 minutes during *in vitro* starch digestion, and then it presented a slowly increasing trend from 20 to the last minutes. This trend could be attributed to that most of Rapidly digested starch (RDS) was digested at around 20 minutes of digestion (Englyst, Kingman & Cummings, 1992; Lu et al., 2018). Moreover, a significant decrease of reducing sugar content in the *in vitro* intestinal digestion was observed in the order of shiitake ethanol extract, shiitake water extract, BE water extract, SE water extract, BE ethanol extract and SE ethanol extract. It was obvious that the shiitake mushroom was excellent presenting for inhibiting reducing sugar release, in particular ethanol extract. In section 5.1, we have demonstrated all mushroom extract both could effectively decrease the PV and FV, which means that the degree of starch granules expanding was inhibited.

The area under the curve (AUC) was calculated from the values for the amount of reducing sugar released in the in vitro starch digestion. The comparison of the AUC between mushroom extracts and control was presented in Figure 5.3. The Figure illustrates a similar trend with the reducing sugar content. As expected, the maximum AUC value was recorded in the control paste (508.21±8.52mg/g). The AUC of all paste addition with mushroom extracts were significantly lower than control. Comparison of the effect of reducing AUC value between water and ethanol extract found that BE (432.51±8mg/g) and SE (431.53±9.13mg/g) ethanol extract lower than their water extract, which was 444.77±5.43 and 438.03±10.42 mg/g, respectively. However, these differences did not reach significance statistically. Among all mushroom, Shiitake mushroom water extract (403.80±21.49 mg/g) was the lowest one, while the highest one was BE water extract (444.77±5.43mg/g).

Bae et al. (2016) evaluate the influence on the release of reducing glucose of buckwheat noodles containing flavonoid extract and rutin-enriched flavonoid extract. The result demonstrated that the amount of released glucose was significantly reduced, which clarified that polyphenol plays a critical role in affecting sugar release and through by depressing starch hydrolysis. Also, Lu et al. (2018) has developed a functional cereal food product that incorporation of shiitake mushroom powder. His results indicated that the mushroom extract reducing the degree of gelatinisation is due to its hydrophilic components could retard the starch granules' swelling. Zhu et al. (2009) mentioned that

the ability of swelling of starches may be altered through by polyphenols with hydroxyl groups occurring a competition with them for hydration. Li et al. (2020), further clarify how phenolic complexation affecting starch digestibility. The result suggests that phenolics may complex with starch through non-covalent CH-π bonds along α-(1 → 4) glycosidic chains. Meanwhile, after starch gelatinization, the complexation between starch and phenolics has remained, which could alter both pasting properties and digestibility of starch. The other report indicated that inhibition of α-Amylase is due to polyphenol-enzyme binding interactions, whereas this inhibition depending on its binding affinity to the α-Amylase (Narita & Inouye, 2011). Vallée et al. (2017) found that addition with BE powder into extruded products could significantly decrease glycaemic AUC. This mechanism is due to its 61.4% insoluble dietary fibre in 71% of the total dietary fibre, which could reduce glycemic response. However, mushroom extracts extracted by water and 50% ethanol were used as a tested sample rather than mushroom powder in the present study. The reduction in sugar release and AUC after in vitro digestion of red sorghum paste addition with mushroom extract in the current study may, therefore, be largely due to the polyphenols soluble phenolic compounds or soluble dietary fibre in the extract, such as β-glucan. Soluble dietary fibre has been reported to decrease the glycemic response (Chillo, Ranawana, Pratt & Henry, 2011).

Based on the obtained results, it can be concluded that that the addition of shiitake, BE and mushroom extract could inhibit the *in vitro* digestion and hydrolysis of starch to a certain extent, thereby reducing the starch hydrolysis rate and AUC value of red sorghum paste.

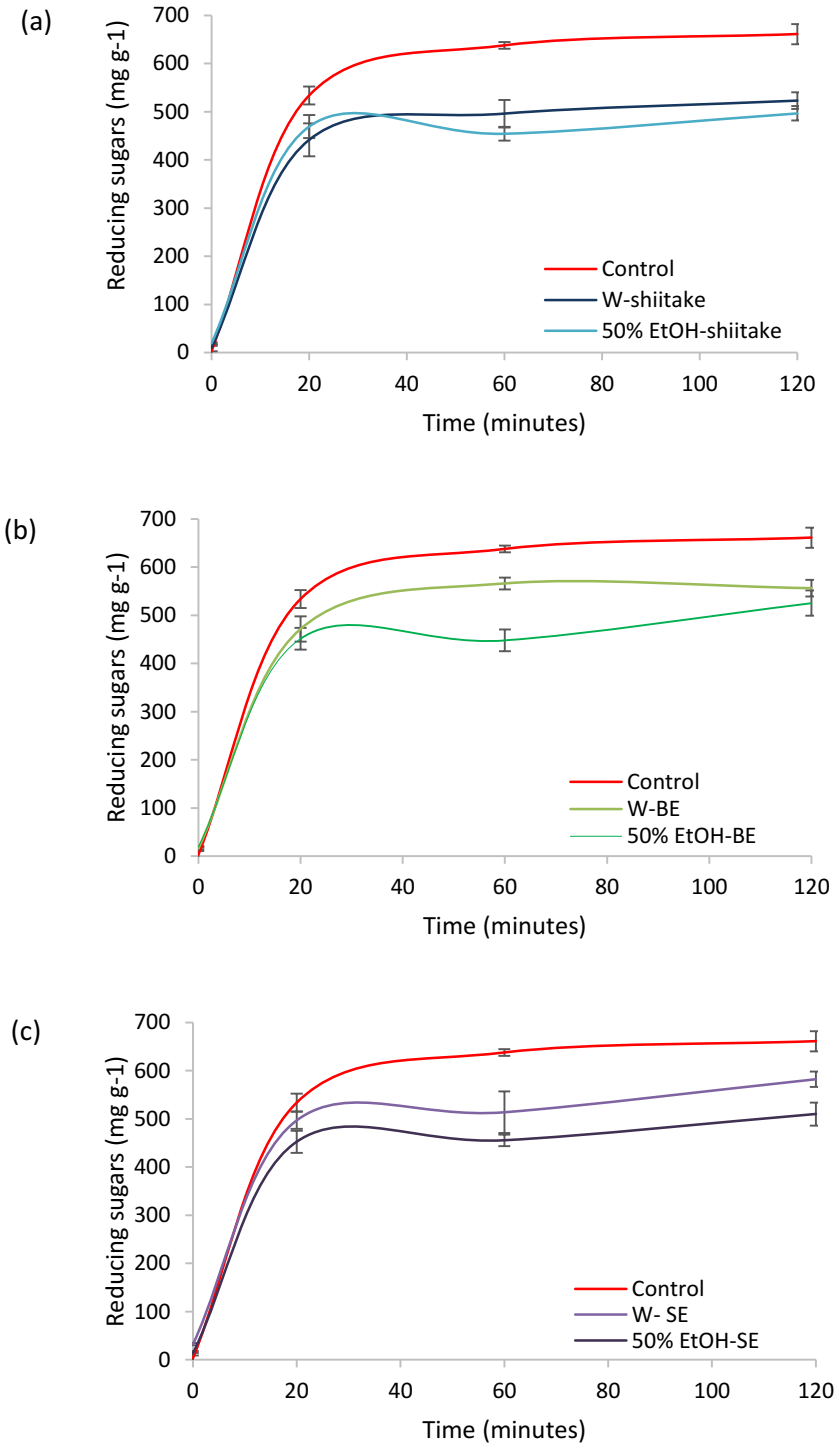

Figure 5.2 Amount of reducing sugars released during *in vitro* digestion comparing control to mushrooms extracts.

RO water and 50% EtOH shiitake extracts (a); RO water and 50% EtOH BE extracts (b); RO water and 50% EtOH SE extracts (c).

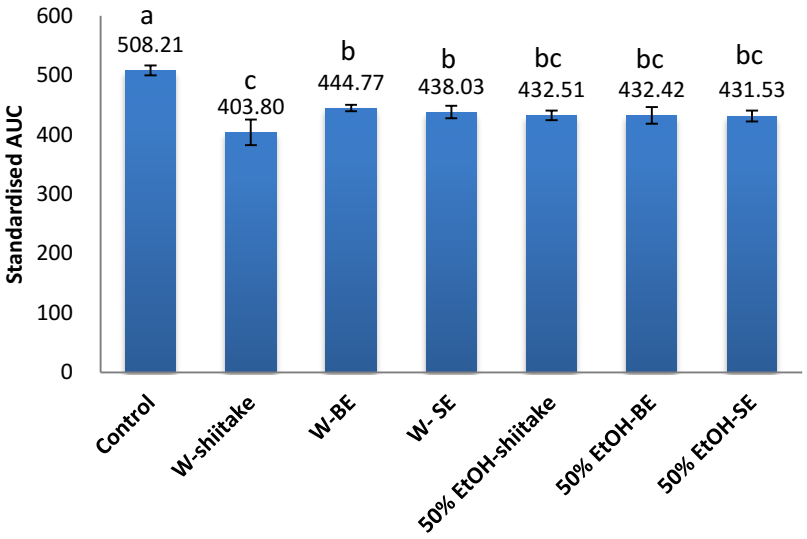

Figure 5.3 Comparison of area under the curve (AUC) between mushroom extract and control.

Error bars represent the standard deviation of replicates. The same letter is not significantly different from each other ($p < 0.05$). AUC represents the reducing sugar content after digestion. Error bars represent the standard deviation of replicates. The same letter is not significantly different from each other ($p < 0.05$).

5.3 Pearson's Correlation between pasting properties and AUC

Pearson correlation among pasting properties parameters and AUC was shown in Table 5.2. The results illustrated that FV (0.717; ***) and SV (0.783; ***) are positively correlated with AUC, while Pt (-0.06) and BV (-0.242) are negatively correlated with AUC.

Table 5.2 The Pearson's correlation coefficient (r) of pasting properties parameters and AUC.

	PT	Pt	PV	TV	FV	BV	SV
Pt	-0.594**						
PV	-0.619***	0.452*					
TV	-0.583**	0.413	0.998***				
FV	-0.279	0.532**	0.760***	0.766***			
BV	-0.834***	0.767***	0.665***	0.618**	0.416		
SV	0.042	0.458*	0.339	0.346	0.868***	0.131	
AUC	0.333	-0.06	0.304	0.340	0.717***	-0.242	0.783***

PT: pasting temperature (°C); Pt: peak time (min); PV: peak viscosity (cP); TV: trough viscosity (cP); BV: breakdown viscosity (cP); SV: setback viscosity (cP); FV: final viscosity (cP). *, significant at $p \leq 0.05$. **, significant at $p \leq 0.01$. ***, significant at $p \leq 0.001$.

Chapter 6

Release of the phenolic compounds, antioxidant activity and protein digestibility during *in vitro* starch digestion of red sorghum paste incorporated with mushroom extracts

6.1 Release of the phenolic compounds during *in vitro* starch digestion of red sorghum paste incorporated with mushroom extracts.

The changes of TPC during *in vitro* starch digestion of red sorghum paste incorporated with mushroom extracts was presented in Table 6.1. Overall, Table 6.1 shows that TPC of red sorghum paste incorporated with mushroom extract with digested enzyme treatment was all significantly greater than without enzyme treatment both in gastric and intestinal phase based on Tukey's test, $P < 0.05$, $n = 3$. Comparison of TPC between gastric and intestinal phase found that the amount of TPC in the intestinal phase presented an excellent performance than in the gastric phase. Otherwise, each water extract derived from three mushrooms was all higher than their ethanol extract.

Comparison of TPC value between enzyme and without enzyme treatment found that shiitake water extract enzyme-treated presenting the highest among all mushrooms, which increases from 2.71±0.02 to 4.99±0.29 mg/g at the intestinal phase of *in vitro* digestion. It was no difference comparing with shiitake ethanol extract. Similarly, at 120 minutes of *in vitro* digestion, the TPC of water extract from BE (4.85±0.02mg/g) and SE (4.90±0.17mg/g) that enzyme-treated were both shows significantly high than their ethanol extract (4.12±0.08 and 4.40±0.09mg/g, respectively).

The amount of TPC all presents a raising trend during *in vitro* digestion. In addition, comparison of TPC value between the gastric and intestinal phase of *in vitro* digestion indicates that the percentage of rising in the intestinal phase was all higher than in the gastric. The increasing percentage of TPC in all samples ranged from 115% to 160 % in the gastric phase, while its ranged from 184% to 213% in the intestinal phase. Shiitake water and ethanol extract were raise from 3.12±0.13 and 3.10±0.16 in gastric phase to 4.99±0.29 and 4.58±0.21 in the intestinal phase, respectively. Likewise, water and ethanol extract of BE also increased separately from 2.81±0.01 and 2.69±0.10 to 4.85±0.02 and 4.12±0.08. For SE water and ethanol extract, a significant increase also was recorded, which up from 2.61±0.07 and 2.25±0.08 to 4.90±0.17 and 4.40±0.09, respectively.

Table 6.1 Changes in TPC during *in vitro* starch digestion of red sorghum paste incorporated with three mushroom extracts.

	TPC (mg GAE g DW^{-1})					
	Shiitake		BE		SE	
	NC	Enzyme	NC	Enzyme	NC	Enzyme
Gastric phase						
RO Water	2.71±0.02^{b}	3.12±0.13^{a*}	2.37±0.03^{b}	2.81±0.01^{a*}	2.06±0.03^{c}	2.61±0.07^{a*}
50% EtOH	2.23±0.05^{c}	3.10±0.16^{a*}	1.83±0.04^{c}	2.69±0.10^{a*}	1.40±0.06^{d}	2.25±0.08^{b*}
Intestinal phase						
RO Water	2.71±0.02^{b}	4.99±0.29^{a*}	2.47±0.06^{c}	4.85±0.02^{a*}	2.30±0.04^{c}	4.90±0.17^{a*}
50% EtOH	2.29±0.01^{b}	4.58±0.21^{a*}	1.96±0.05^{d}	4.12±0.08^{b*}	2.16±0.10^{c}	4.40±0.09^{b*}

Data was expressed as Mean ± SD (n = 3).
$^{a-b}$ Mean values with different lowercase superscript letters in columns are significantly different ($P < 0.05$). Upper star (*) represent the significance between enzyme and NC. NC, negative control, without digestive enzymes treatment.

6.2 Antioxidant analysis during *in vitro* starch digestion of red sorghum paste incorporated with mushroom extracts.

Antioxidant activity of red sorghum paste incorporated with mushroom extracts after 120 minutes of *in vitro* digestion was also investigated, which measuring by DPPH and FRAP and comparing with control paste. The changes in DPPH and FRAP were separately shown in Figure 6.1 and Figure 6.2. Figure 6.1 shows that a significant increasing trend of Trolox concentration of red sorghum paste incorporating with each mushroom extracts was observed in the digested enzyme-treatment group during 120minutes of *in vitro* digestion. Also, Trolox value of addition with both water and ethanol mushroom extract was significantly higher than control paste. Shiitake ethanol extract has shown the highest Trolox concentration and significantly greater than its water extract (Figure 6.1a), while there is no difference between water and ethanol extract in BE (Figure 6.1b) and SE (Figure 6.1c).

Figure 6.2. presents the changes in FRAP during 120 minutes of *in vitro* starch digestion of red sorghum paste incorporated with three mushroom extracts. Similarly, the FRAP value recorded showed a similar trend to Trolox value, which was a significant raising in all mushroom extract with enzyme treatment comparing with no enzyme addition during *in vitro* digestion. Comparison of varies between water and ethanol extract found that shiitake ethanol extracts were significantly lower than water extract (Figure 6.2a), but didn't observe a difference between two extracts in BE (Figure 6.2b) and SE (Figure 6.2c).

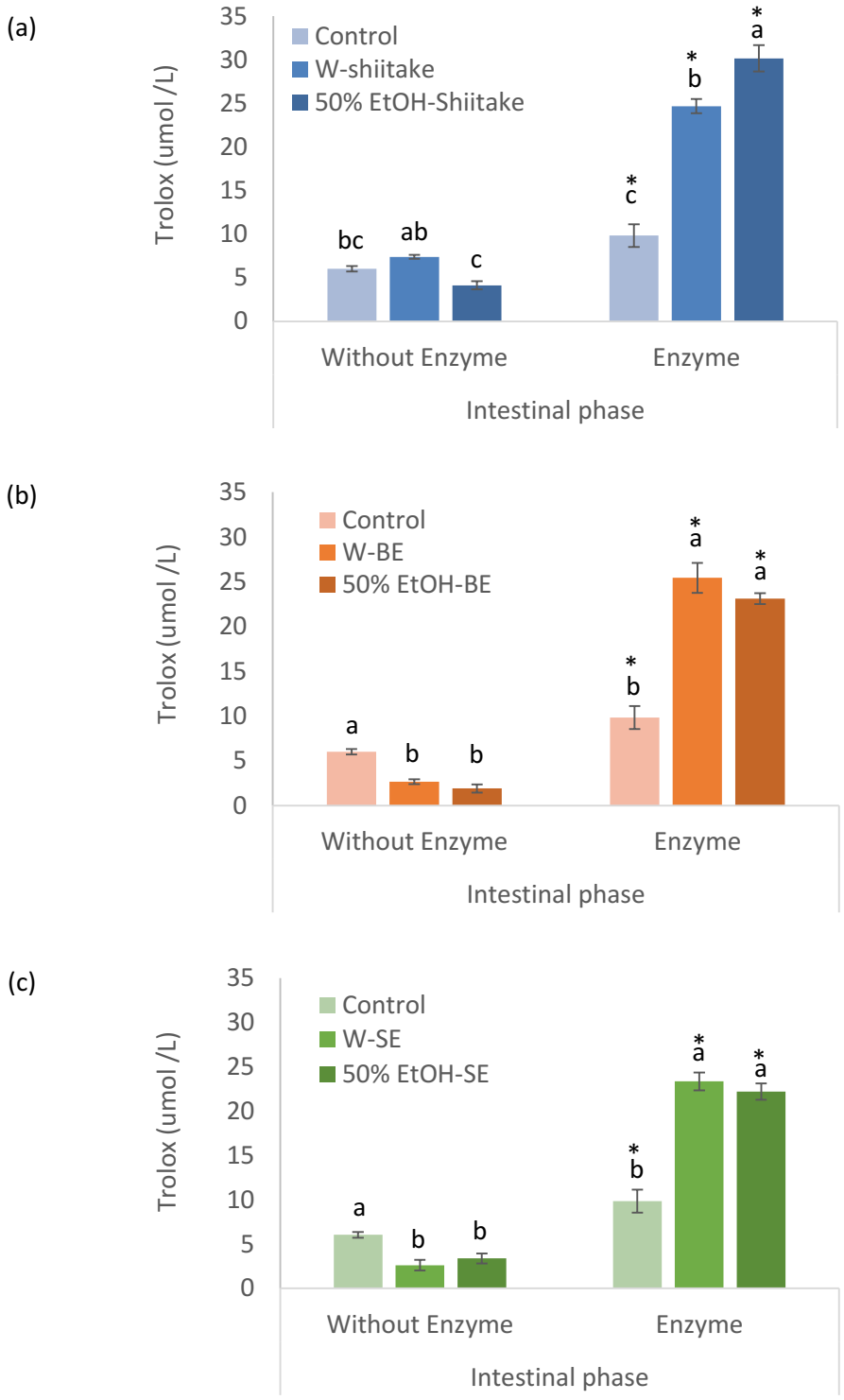

Figure 6.1 Changes in Trolox concentration during 120 minutes of *in vitro* starch digestion of red sorghum paste incorporated with three mushroom extracts comparing with control.

RO water: RO water extracts; 50% EtOH: 50% Ethanol extracts. Mean values with different lowercase superscript letters in separate phase are significantly different (P < 0.05). Upper star (*) represent the significance between enzyme and without digestive enzymes treatment. (a) Shiitake; (b) Black ear; (c) Sliver Ear

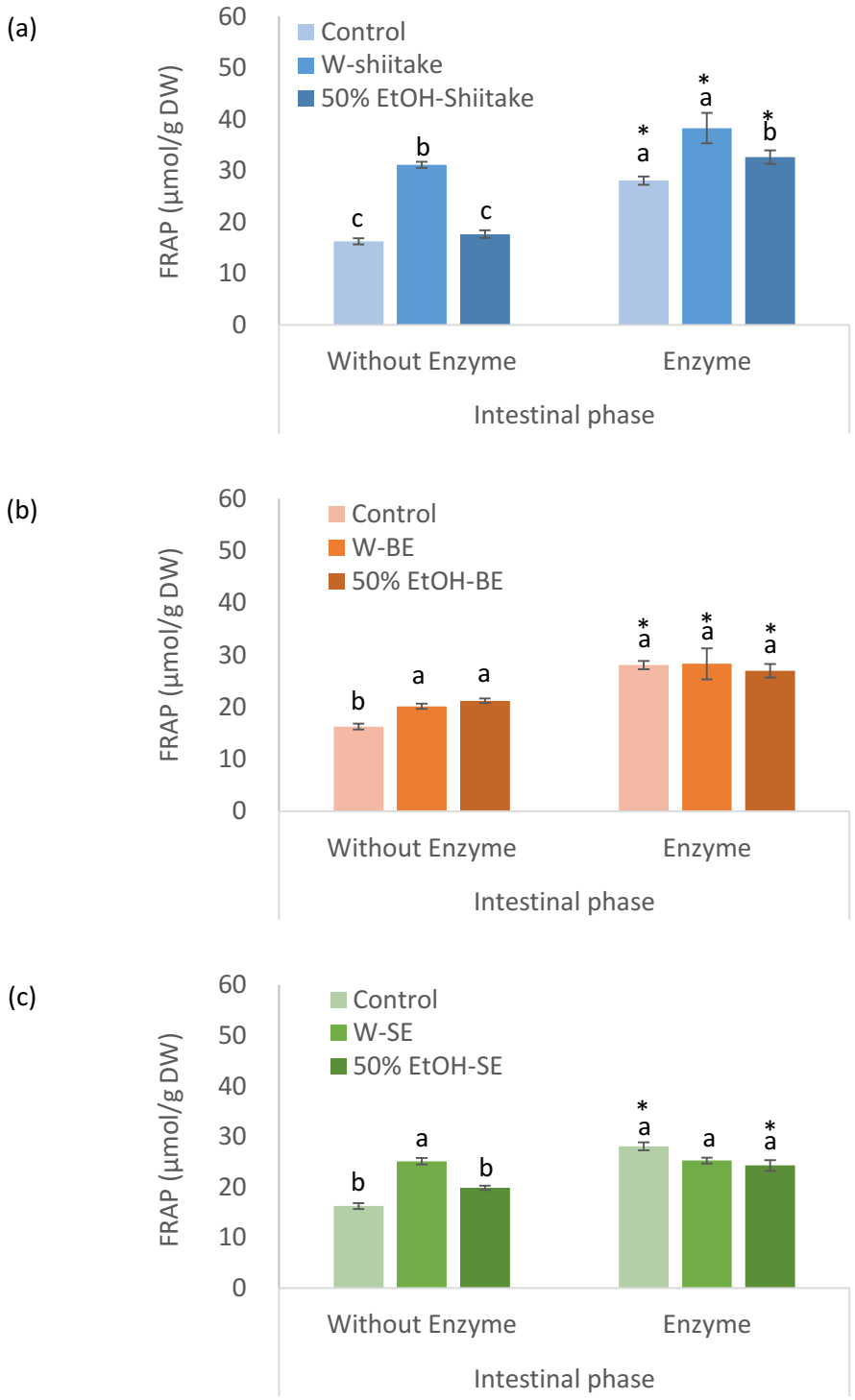

Figure 6.2 Changes in FRAP during 120 minutes of *in vitro* starch digestion of red sorghum paste incorporated with three mushroom extracts comparing with control.

RO water: RO water extracts; 50% EtOH: 50% Ethanol extracts. Mean values with different lowercase superscript letters in separate phase are significantly different ($P < 0.05$). Upper star (*) represent the significance between enzyme and without digestive enzymes treatment. (a) Shiitake; (b) Black ear; (c) Sliver Ear

As the best tool for evaluating the digestive stability and release of bioactivity compounds, in vitro digestion offers various advantages, such as simple, cheap and reproducible. Meanwhile, it also could address the disadvantages of in vivo trials, such as expensive and time-consuming (He, et al. 2017). Therefore, we used simulated digestion to assess the release of the phenolic compounds and antioxidant activities of each mushroom extracts in this study.

According to Tenore et al. (2015), the best form of polyphenols should be bioavailable, which means that it can be released from the food matrix during the gastrointestinal digestion and possess a specific function as well as achieving a specific effect. However, gastrointestinal digestion may also lead to structural changes of polyphenol and further to affect their stability, bioavailability and bioactivity. Thus, some studies have observed a decrease in both polyphenols content and antioxidants after in vitro digestion. This was due to the changes in the pH values of simulated gastrointestinal digestion, i.e. the acidic pH of gastric digestion and the alkaline pH of intestinal digestion resulting in degradation of polyphenols (Thomas-Valdés, Theoduloz, Jiménez-Aspee & Schmeda-Hirschmann, 2019).

Nevertheless, our results presented a significant increase of TPC and antioxidant activities during in vitro digestion of red sorghum paste addition with all mushroom extracts. This appeared in line with the results of the previous study. An in vitro simulated digestion of 22 fruit juices was investigated by He et al. (2017) and found that the polyphenol content and FRAP value of tested fruit juices presented a significant increase during digestion. This trend could be attributed to the release and the interactions of polyphenol compounds in fruit juices, which improve the solubility and bioavailability of these compounds. Likewise, Coe et al. (2013), have been observed an increasing trend of total phenol content in polyphenol-rich baobab fruit during an *in vivo* digestion, which indicated the TPC release and its bioaccessibility was raised in digesting phase. Likewise, Coe et al. (2013), have been observed an increasing trend of total phenol content in polyphenol-rich baobab fruit during an in vivo digestion, which indicated the TPC release and its bioaccessibility was raised in digesting phase. Others, Rodríguez & Tironi (2020) found that some phenolics were released from gastrointestinal digestion and improving the antioxidant activity, i.e. ORAC and ABTS of tested flour extracts. Glycosylated derivatives, which are the most common type of polyphenols that occur in nature (Cirkovic Velickovic & Stanic-Vucinic, 2018). Rodríguez & Tironi (2020) indicated that polyphenol could interact with protein and form an insoluble form in flour, which can be released through acid treatment. This might help to illustrate the increase of TPC and antioxidant activities during in vitro digestion.

On the other hand, He et al. (2017) also observed the increased content of polysaccharides of juice samples. A stabled structure of polysaccharide could prevent the degradation of bioactivity phenolic compounds. A previous study has reported the antioxidant activity of mushroom extracts was associated with the various compounds, such as organic acids, phenolics, polysaccharides and other

small weight molecular, but polyphenols were considered the most associated with antioxidant capacity (Wang, Hu, Nie, Yu & Xie, 2016).

Although our data support that all mushroom extracts used in this study were shown to be good sources of polyphenols and can enhance the bioactivity, such as antioxidant activities, during in vitro digestion, they are the crude extracts that contain various compositions. Mushrooms are widely acknowledged that rich in the polysaccharides (Kalaˇ, 2016; Wu et al., 2020), thus, we were not able to directly demonstrate that the only contributor to antioxidant activities is polyphenols rather than other compounds in our samples. To clarify more truth, more determinations, such as polysaccharides content and HPLC analysis of polyphenols, were suggested in further study.

6.3 *In vitro* Protein Digestibility and soluble protein content during gastric and intestinal digestion.

In this work, in vitro protein digestibility of red sorghum paste addition with different mushroom extract were examined, which including the pepsin and pancreatin digestion. The changes of protein digestibility (%) during in vitro starch digestion of red sorghum paste incorporating with three mushroom extracts were presented in Table 6.2 and Figure 6.3. Table 6.2 shows that the protein digestibility of adding each mushroom extracts all significantly higher than control in both gastric and intestinal phase. Shiitake mushroom water and ethanol extract both presenting a significant-high protein digestibility at 30minutes of gastric digestion. Shiitake ethanol extract was the highest one at 47.75±2.40%, followed by water extract at 45.81±2.62 %, which increase 205% and 196% of control, respectively. Additionally, there is no statistical difference was observed between water and ethanol extract. Although BE and SE extract were also higher than control, the results show a significant difference between their water and ethanol extracts. For intestinal phase, the protein digestibility of red sorghum addition with shiitake mushroom extracts was all recorded greater than control. It was apparent that the protein digestibility of mushroom extracts in the water extraction was excellent high during each stage which was 57.84±2.43%, 63.13±1.75% and 71.62±1.03%, respectively. It raises from 57.84±2.43% in 50 minutes of digestion to 71.62±1.03% in the 150minutes. They both higher than control in the end of digestion (Figure 6.3a). Surprisingly, it was followed by SE water extract, which increased from 50.22±0.57% in the first stage of intestinal to 70.36±2.87% in the end-stage. However, the BE and SE using ethanol as extraction solvent didn't obtain high protein digestion (Figure 6.3b and 6.3c).

The soluble protein content of red sorghum incorporated in three mushrooms extracts during *in vitro* simulated gastrointestinal digestion was also measured and presented in Table 6.3. Overall, shiitake

water and ethanol extract both significant increases during in gastric and intestinal phase of *in vitro* digestion comparing with all mushroom extract and control. It remains the highest one until the end-stage of digestion. BE and SE extracts also present a statistical difference compared with control during a digested period, but they were observed that each water extract greater than ethanol extract. This increasing trend is similar to protein digestibility of red sorghum incorporated in three mushrooms extracts during *in vitro* simulated gastrointestinal digestion. The results suggested that mushroom extract could enhance the soluble protein content during *in vitro* digestion.

Although Phenolic compounds possess specific bioactivity effect of human health, such as reduce the glycemic response, they also bring negative influences on digestion. For instance, the inhibition of protein digestibility is unacceptable, which leads to the amounts of valuable amino acids decrease (Cirkovic Velickovic & Stanic-Vucinic, 2018). Thus, it is necessary to clarify the impacting of phenolic compounds on protein digestibility. Normally, polyphenols lead to protein digestibility reduce during in vitro digestion, which is due to covalent and noncovalent between polyphenols and proteins. However, our experiments revealed that all mushroom extract incorporating in red sorghum paste enhances both protein digestibility and soluble protein content during in vitro digestion. Glycosylated derivatives, which are the most common type of polyphenols that occur in nature (Cirkovic Velickovic & Stanic-Vucinic, 2018). As the previous present, polyphenol could interact with protein and form an insoluble form in flour, which can be released through acid treatment (Rodríguez & Tironi, 2020). This not only increases polyphenol release but also enhance the soluble protein release, which further to raise protein digestibility.

Table 6.2 The changes of protein digestibility (%) of red sorghum incorporated in three mushrooms extracts during *in vitro* simulated gastrointestinal digestion.

		Protein Digestibility (%)			
Samples	Solvent	Gastric digestion	Intestinal digestion		
		30 Minutes	50 Minutes	90 Minutes	150 Minutes
Control		23.28 ± 0.21^{d}	41.97 ± 2.69^{c}	49.71 ± 2.28^{de}	58.20 ± 1.78^{cd}
Shiitake	RO Water	45.81 ± 2.62^{a}	57.84 ± 2.43^{a}	63.13 ± 1.75^{a}	71.62 ± 1.03^{a}
	50%	47.75 ± 2.40^{a}	53.31 ± 2.85^{ab}	56.23 ± 0.90^{bc}	66.23 ± 3.14^{ab}
BE	RO Water	34.79 ± 0.73^{bc}	48.75 ± 2.77^{bc}	52.65 ± 0.61^{cd}	61.60 ± 1.18^{bc}
	50%	30.51 ± 0.19^{c}	41.63 ± 3.55^{c}	42.45 ± 1.86^{f}	52.49 ± 2.23^{de}
SE	RO Water	37.56 ± 0.49^{b}	50.22 ± 0.57^{b}	60.04 ± 2.92^{ab}	70.36 ± 2.87^{a}
	50%	30.51 ± 1.87^{c}	41.77 ± 2.63^{c}	45.92 ± 2.90^{ef}	50.74 ± 3.99^{e}

Data was expressed as Mean ± SD (n = 3).
Mean values with different lowercase superscript letters in columns are significantly different ($P < 0.05$).

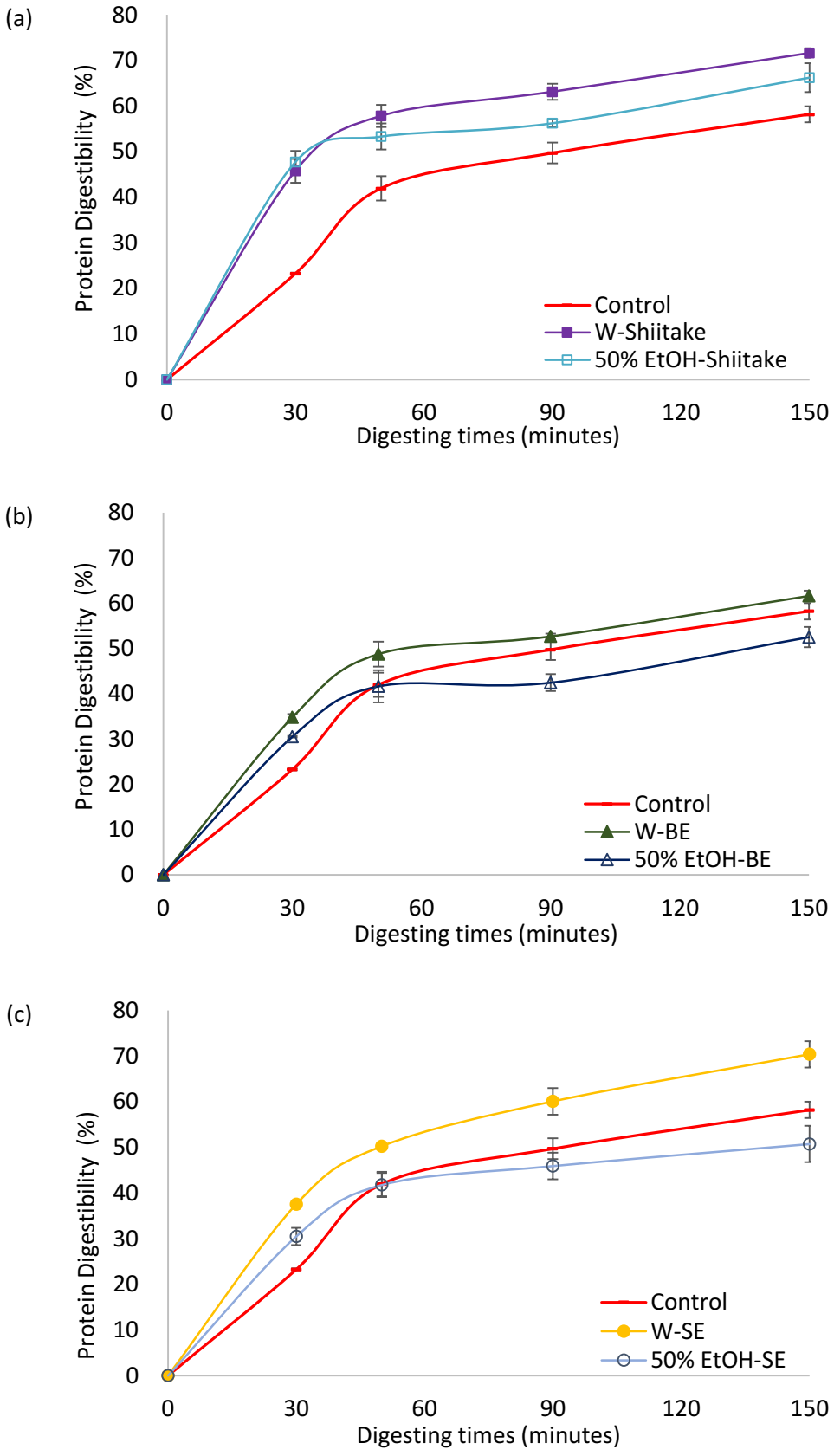

Figure 6.3 The changes of protein digestibility (%) of red sorghum incorporated in three mushrooms extracts during *in vitro* simulated gastrointestinal digestion.

(a) Shiitake RO water and 50% EtOH extracts; (b) Blcak Ear RO water and 50% EtOH extracts; (c)Sliver Ear RO water and 50% EtOH extracts.

Table 6.3 The changes of soluble protein content of red sorghum incorporated in three mushrooms extracts during *in vitro* simulated gastrointestinal digestion.

		Soluble Protein Content (mg/g DW)			
Samples	Solvent	Gastric digestion	Intestinal digestion		
		30 Minutes	50 Minutes	90 Minutes	150 Minutes
Control		0.00±0.00^{e}	0.00±0.00^{f}	0.00±0.00^{e}	0.00±0.00^{d}
Shiitake	RO Water	537.2±30.70^{a}	678.20±28.60^{a}	740.30±20.50^{a}	839.86±12.04^{a}
	50%	557.6±28.10^{a}	622.50±33.30^{ab}	656.57±10.53^{b}	773.30±36.60^{a}
BE	RO Water	384.9±8.03^{bc}	539.30±30.60^{cd}	582.44±6.70^{c}	681.37±13.05^{b}
	50%	331.9±2.03^{d}	452.80±38.60^{e}	461.60±20.20^{d}	570.90±24.30^{c}
SE	RO Water	424.8±5.48^{b}	568.02±6.42^{bc}	679.00±33.00^{b}	795.80±32.50^{a}
	50%	344.7±21.20^{cd}	471.90±29.70^{de}	518.80±32.80^{d}	573.30±45.10^{c}

Data was expressed as Mean ± SD (n = 3).
$^{a-e}$ Mean values with different lowercase superscript letters in columns are significantly different ($P < 0.05$).

6.4 Pearson's Correlation Analysis

6.4.1 Pearson's Correlation between TPC and Antioxidant Activity after *in vitro* digestion

Pearson's Correlation of TPC, antioxidant capacities, AUC, protein digestibility after in vitro digestion of red sorghum paste incorporated in mushroom extracts were shown in Table 6.4. The correlation between TPC and Antioxidant activity, ie. Trolox and FRAP were shown in Figure 6.4a and 6.4b, while the Graphical representation of the correlation between total phenolic content and antioxidant activity were shown in Figure 6.4. The statistical analysis showed a positive correlation (r=0.351) between TPC and Trolox (Figure 6.4a), while it was also presented a positive (r=0.474) and significant (P<0.05) correlation between TPC and FRAP (Figure 6.4b). Additionally, a positive (r=0.543) and significant (p<0.05) correlation was found between Trolox and FRAP (Figure 6.4c). As a result, the total phenol content was presented a positive correlation between both antioxidant activities. Our results appeared in line with the results of Garcia et al. (2020), they found that water extract of shiitake mushroom presents a high antioxidant activity. Additional, a positive correlated between phenolic compounds content and antioxidant activity was also observed.

6.4.2 Pearson's Correlation Analysis between TPC and AUC after *in vitro* digestion

Pearson's Correlation between TPC and AUC after *in vitro* digestion of red sorghum paste incorporated in mushroom extracts was also examined. The correlation value (r) as shown in Table 6.4, while the Graphical representation of the correlation was presented in Figure 6.5. The statistical analysis obtained indicated that there is a negative correlation (r=-2.89) between TPC and AUC. On the other hand, AUC also shows negatively and significantly correlated with both Trolox and FRAP, which were r=-0.494 ($p<0.05$) and r=-0.458 ($p<0.05$), respectively. Consequently, the results suggested that the decreasing of AUC depending on the increase of total phenol content from mushroom extracts and presenting a negatively correlated with their antioxidant activities.

6.4.3 Correlation Analysis between TPC and Protein Digestibility after *in vitro* digestion

The correlation coefficient (r) between TPC and digestibility after *in vitro* digestion of red sorghum paste incorporated in mushroom extracts was shown in Table 6.4, while the Graphical representation of the correlation was assumed in Figure 6.6. The result of the statistical analysis shows a strong positive (0.755) and significant ($p<0.001$) correlation between TPC and protein digestibility. Furthermore, protein digestibility was also observed positively and significantly correlated with antioxidant activity from mushroom extract, which was reached at 0.577 ($p<0.01$) between Trolox and 0.563 ($p<0.01$) between FRAP. In conclusion, our results supported that protein digestibility was enhanced during *in vitro* digestion of red sorghum paste addition with 1% mushroom extract and it increases with total phenol content and the antioxidant activities.

Table 6.4 Pearson's Correlation coefficient (r) of TPC, antioxidant capacities, AUC, protein digestibility after *in vitro* digestion of red sorghum paste incorporated in mushroom extracts.

	TPC	Trolox	FRAP	AUC
Trolox	0.351			
FRAP	0.474*	0.543*		
AUC	-0.289	-0.494*	-0.458*	
Protein digestibility	0.755***	0.577**	0.563**	-0.62**

Unit: TPC (mg GAE g/g DW), Trolox (µmol /L), FRAP (µmol/g DW), Protein digestibility (%).
*, significant at $p \leq 0.05$. **, significant at $p \leq 0.01$. ***, significant at $p \leq 0.001$.

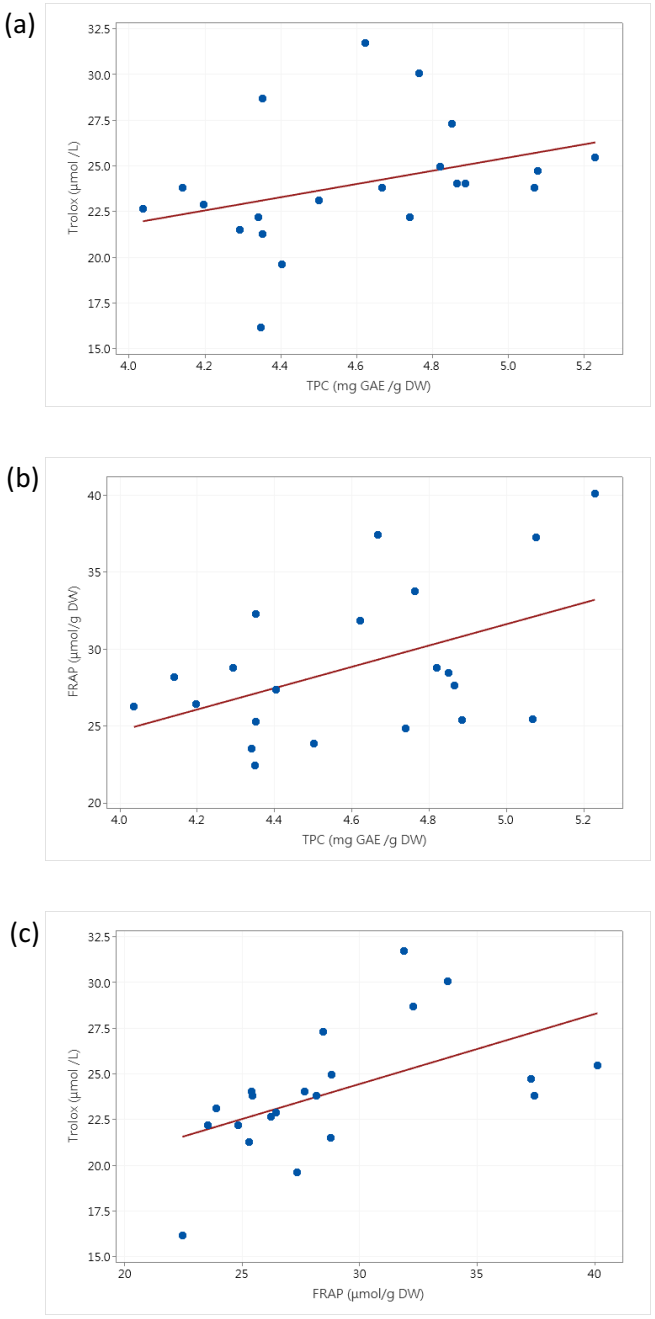

Figure 6.4 Graphical representation of the correlation between total phenolic content and antioxidant activity (a) TPC and Trolox, (b) TPC and FRAP, (c) Trolox and FRAP after *in vitro* digestion.

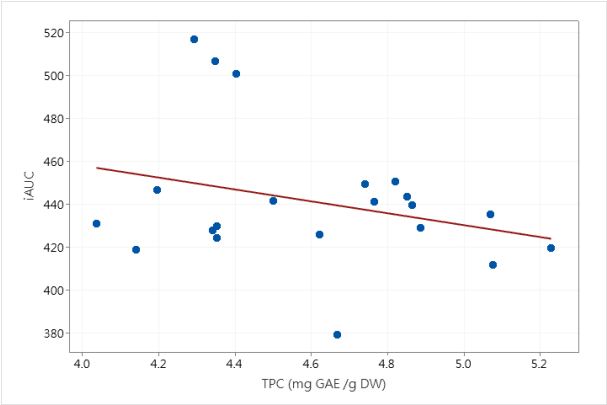

Figure 6.5 Graphical representation of the correlation between TPC and AUC after *in vitro* digestion.

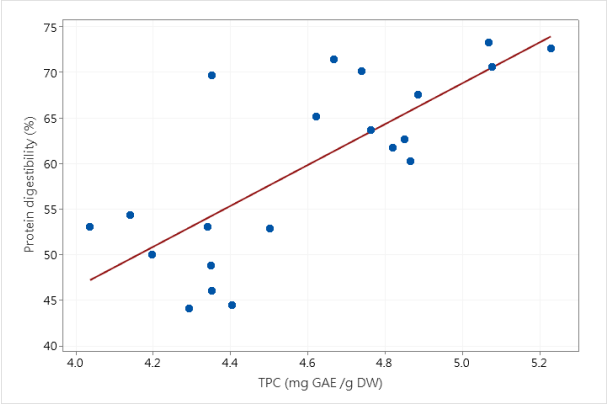

Figure 6.6 Graphical representation of the correlation between TPC and protein digestibility after *in vitro* digestion.

Conclusion

The results of this study suggest that water extraction at 50℃ for 3 hours in neutral pH could obtain optimal extraction condition for a great TPC and antioxidant activity. Shiitake mushroom water extract was the highest TPC and antioxidant activity among all mushroom extract, followed by shiitake mushroom 50% ethanol extracts. In addition, addition with each mushroom extracts all reduced the pasting properties of red sorghum paste, in particular, peak viscosity (PV) and final viscosity (FV). Shiitake water extract has a significant ($p<0.05$) high comparing with others. Moreover, the TPC, antioxidant activity and protein digestibility were observed an increasing trend during in vitro digestion of all red sorghum paste incorporated in each mushroom extracts, while the reducing sugar and AUC were significant ($p<0.05$) decrease. Shiitake mushroom water extracts remain to present the greatest TPC and antioxidant activity during in vitro digestion of red sorghum paste, followed by shiitake ethanol mushroom extracts. Our preliminary result supported that mushroom extract, ie. shiitake mushroom, black ear mushroom and silver ear mushroom could be used as a functional ingredient of red sorghum food products development, which provides bioactivity phenolic compounds and enhances antioxidant activity. What is more, it may display as a modification agent by decrease the hydrolysis of starch during digestion for further reducing sugar release as well as raising protein digestibility of red sorghum flour products.

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