COMPARISON OF NUTRITIONAL COMPOSITION, ANTI-INFLAMMATORY AND ANTIOXIDATIVE ACTIVITIES BETWEEN THE RAW AND BOILED *Raphanus sativus* subsp. *longipinnatus* ROOTS

By

ONG YIN JIE

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ABSTRACT

COMPARISON OF NUTRITIONAL COMPOSITION, ANTI-INFLAMMATORY AND ANTIOXIDATIVE ACTIVITIES BETWEEN THE RAW AND BOILED *Raphanus sativus* subsp. *longipinnatus* ROOTS

Ong Yin Jie

Raphanus sativus subsp. *longipinnatus* (daikon radish) is widely consumed by Asians and claimed to have anti-inflammatory and antioxidant activities. However, this statement was not proven scientifically. Many studies mainly focused on red radishes (*Raphanus sativus* L.), with limited research done on daikon radish. This study analysed the nutritional composition, anti-inflammatory, and antioxidant activities of the raw and boiled daikon radish roots. The nutritional composition including moisture, ash, crude protein, crude fibre, and crude fat were analysed according to the AOAC guidelines, while the mineral content was determined using Flame Atomic Absorption Spectrometry (FAAS). In this research, 80% methanol was used to obtain sample extracts for bioactivities analysis. The protein denaturation assay (PDA) was used to examine the anti-inflammatory capabilities; while DPPH radical scavenging assay, total phenolic content (TPC) and ferric-reducing antioxidant power (FRAP) assays were utilised to analyse the antioxidant activities. Student's T-test was used to statistically compare the raw and boiled results. The moisture (96.64 ± 0.05% FW), ash (25.10 ± 1.10% DW) and crude fibre (16.41 ± 1.60%

DW) contents were found higher in the boiled daikon radish roots. The raw daikon radish roots have a higher crude protein content at 12.69 ± 0.44% DW. No significant differences in crude fat were found between both samples. Calcium and magnesium remained to be two of the most abundant minerals in the raw and boiled samples. Besides, the boiled daikon radish root extracts exhibited lower half-maximal inhibitory concentration (IC_{50}) values in PDA (664.79 ± 27.25 μg/mL) and DPPH (1.59 ± 0.09 mg/mL) assays, giving a higher anti-inflammatory and antioxidant activity. Although the raw daikon radish had higher TPC (6.36 ± 1.52 μg GAE/g sample), no significant differences were found between the raw and boiled sample extracts in the FRAP. Therefore, the boiled daikon radish roots were noted to give higher nutritional value.

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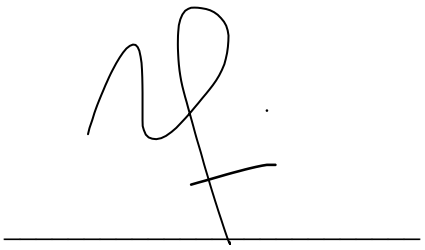
DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.



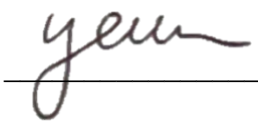
Ong Yin Jie

APPROVAL SHEET

This final year project report entitled "**COMPARISON OF NUTRITIONAL COMPOSITION, ANTI-INFLAMMATORY AND ANTIOXIDATIVE ACTIVITIES BETWEEN THE RAW AND BOILED *Raphanus sativus* subsp. *longipinnatus* ROOTS**" was prepared by ONG YIN JIE and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

(Dr. Sit Nam Weng) Date: 31 May 2022
Supervisor
Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman



(Dr. Tan Yen Nee) Date: 31 May 2022
Co-Supervisor
Department of Agricultural and Food Science
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: 31 May 2022

PERMISSION SHEET

It is hereby certified that **ONG YIN JIE** (ID No: **18ADB04169**) has completed this final year project report entitled "COMPARISON OF NUTRITIONAL COMPOSITION, ANTI-INFLAMMATORY AND ANTIOXIDATIVE ACTIVITIES BETWEEN THE RAW AND BOILED *Raphanus sativus* subsp. *longipinnatus* ROOTS" under the supervision of Dr. Sit Nam Weng (Supervisor) from the Department of Allied Health Sciences, Faculty of Science, and Dr. Tan Yen Nee (Co-Supervisor) from the Department of Agricultural and Food Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project report in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

(ONG YIN JIE)

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LIST OF ABBREVIATIONS

DPPH	2, 2-diphenyl-1-picrylhydrazyl
TPTZ	2, 4, 6-Tris(2-pyridyl)-s-triazine
Al	Aluminium
NH_3	Ammonia
NH_4^+	Ammonium
AOAC	Association Of Official Analytical Chemists
BSA	Bovine Serum Albumin
Cd	Cadmium
Ca	Calcium
CNS	Central Nervous System
Cl	Chloride
Cr	Chromium
Co	Cobalt
Cu	Copper
DAMP	Damage-Associated Molecular Patterns
DRI	Dietary Recommendation Index
DW	Dry Weight
Fe^{2+} E	Fe^{2+} Equivalent
FRAP	Ferric-Reducing Antioxidant Power
$FeSO_4$	Ferrous Sulphate
FW	Fresh Weight
GAE	Gallic Acid Equivalent

IC_{50}	Half-Maximal Inhibitory Concentration
HCl	Hydrochloric Acid
Fe	Iron
$FeCl_3$	Iron (III) Chloride
Pb	Lead
LOQ	Limit Of Quantification
Mg	Magnesium
Mn	Manganese
NHS	National Health Service
Ni	Nickel
HNO_3	Nitric Acid
PBS	Phosphate-Buffered Saline
PDA	Protein Denaturation Assay
RDA	Recommended Dietary Allowance
ROS	Reactive Oxygen Species
R^2	Regression Coefficient
NaOH	Sodium Hydroxide
H_2SO_4	Sulphuric Acid
TPC	Total Phenolic Content
Zn	Zinc

CHAPTER 1

INTRODUCTION

Raphanus sativus subsp. *longipinnatus*, generally referred to as daikon radish or 'lobak', belongs to the Brassicaceae family (Schippers, 2004). The whole plant of the daikon radish is edible, but the root is the most commonly consumed part, which is normally harvested before the plant flowers (Mohammed and Hameed, 2018). The daikon radish root (Figure 1.1) has a white skin with white flesh that imparts a sweet, pungent flavour. It is popular in Asia particularly Japan, where it is served raw in a salad or grated, boiled, dried, or pickled (Gordenker, 2015).

Figure 1.1: *Raphanus sativus* subsp. *longipinnatus* roots.

Asians believed the daikon radish possesses anti-inflammatory properties to prevent pulmonary disease, which draws much attention during this Covid-19 pandemic. However, this statement is yet to be proven. Since ancient times, the *Raphanus sativus* family has been used as traditional folks medicine to treat

diseases such as stomach disorders, urinary infections and more (Manivannan et al., 2019). Hence, to allow a better understanding of the health benefits of daikon radish, a systematic assessment should be carried out. This research provides an insight into the health benefits of the daikon radish roots including its nutritional composition, antioxidant activities and anti-inflammatory properties.

It is important to fully understand food's nutritional composition in order to obtain adequate information on the amount of nutrients present to build the recommended nutrient intake for the food-based dietary guidelines (Elmadfa and Meyer, 2010). On the other hand, understanding the bioactivities of food is vital in nutraceutical fields (Zaky et al., 2021). These include anti-inflammatory, antioxidative, anticancer and more, in which the bioactivities are attributed to a small amount of bioactive compound in food. According to the National Center for Complementary and Integrative Health (2013), antioxidants are proven effective in preventing or delaying cell damage. In addition, dietary anti-inflammatory substances reduce the release of inflammatory markers (Casas et al., 2014), which can reduce and prevent chronic inflammation in the body and improve overall health.

The nutritional composition and the biological activity of the daikon radish may vary according to the cooking method. Since other common cooking methods such as pickling is heavily subjective to different recipes, therefore, in this research, raw and boiled methods were selected to evaluate the natural properties of the daikon radish. Boiling has proven to have various effects in altering the nutritional composition, biological activities and physicochemical properties of

plants (Arias-Rico et al., 2020). Therefore, as one of the most commonly consumed methods, the effects of boiling daikon radish roots were determined to compare the nutritional and nutraceutical benefits.

The objectives of the study are: (1) to analyse and compare the nutritional composition between raw and boiled daikon radish roots; (2) to investigate and compare the anti-inflammatory activities of the raw and boiled daikon radish roots; and (3) to examine and compare the antioxidant properties of the raw and boiled daikon radish roots.

CHAPTER 2

LITERATURE REVIEW

2.1 Nutritional Composition

The moisture, ash, crude protein, crude fibre and crude fat content were classified under commonly analysed nutritional composition of food. These are the components of interest especially in the food industry (Thangaraj, 2016).

2.1.1 Moisture Content

The moisture content represents the amount of water present in the food, which indirectly influences the shelf life, taste and appearance of the food. According to Moore (2020), a high moisture content in food encourage microbial growth, which decreases the shelf life. This complicates the transportation of the food, as spoilage often occur before being transported to the destination. The moisture content varies widely in different types of food. According to Pomeranz and Meloan (1994), the radishes family has the highest moisture content among the root vegetables.

Pomeranz and Meloan (1994) further mentioned that water molecules in food can exist in free or bound form. Isengard (2001) stated that bonding of water molecules in food influence their detectability. Therefore, various methods have been developed to accurately analyse moisture contents. The most commonly used method is oven drying, classified under the direct method based on the

physical separation of water. It utilises heat to produce mass loss which dries out the water and other volatile substances in food, until it reaches constant weight. The water content can then be calculated by the gravimetric differences before and after the drying.

2.1.2 Ash and Mineral Content

The ash analysis is conducted by burning out the organic compounds present in food, leaving behind the inorganic minerals. This is positively associated with the amount of minerals present in food (Hoenig, 2005; Harris and Marshall, 2017; Ismail, 2017). Examples of minerals in foods are calcium (Ca), iron (Fe) and potassium (K).

The minerals can be categorised into macrominerals and trace minerals according to the body's requirements (National Library of Medicine, 2019). Macrominerals are the minerals that are required by the body in a larger quantity, such as calcium (Ca), chloride (Cl), magnesium (Mg) and more. These are vital for the normal functions of organs including bone, muscle, heart and brain (Johnson, 2021). For example, magnesium is essential in more than 300 enzymatic metabolism pathways (Schwalfenberg and Genuis, 2017). On the other hand, trace minerals such as chromium (Cr), copper (Cu) etc. are required in a relatively lower amount. Although they are essential for better health, it causes nutritional disorder when consumed too little and toxic effect may be produced in excessive consumption (Johnson, 2021).

Nonetheless, minerals in food may also cause adverse effects on human health. These include aluminium, cadmium and lead, which are considered as food contamination. Aluminium has been reported to interfere with protein and nucleic acid function, increase reactive oxygen species (ROS) production and disturb enzymatic activities. This is known as aluminium poisoning, when the accumulation of aluminium in blood is higher than 100 ug/L (Rahimzadeh et al., 2022). Besides, according to Genchi et al. (2020), cadmium is associated with an increased risk of cancer and osteoporosis. As the liver and kidneys are able to synthesise cadmium-inducible proteins to protect the cells from damage, they are highly prone to be affected by cadmium toxicity. Moreover, high exposure to lead is proven to cause damage to the central nervous system (CNS), and is life-threatening in severe toxicity (World Health Organization, 2019). Therefore, it is important to examine the heavy metal contamination in food besides analysis of health-benefit minerals.

2.1.3 Crude Protein Content

Protein is categorised as one of the macronutrients which plays an important role in normal metabolic function (Brazier, 2020). Proteins are made up of amino acids with peptide bonds, that fold into primary, secondary, tertiary or quaternary structures for their functions (Watford and Wu, 2018). Among the amino acids, there are 9 essential amino acids that the human body is unable to synthesise. Therefore, the source of these amino acids heavily depends on dietary intake. The dietary recommendation index (DRI) for adults is 0.8 g protein per kg bodyweight daily (Trumbo et al., 2002). Extra proteins are needed for pregnant and lactating women. Insufficient dietary protein may lead to

protein deficiency, including life-threatening diseases in children such as kwashiorkor and marasmus (Brazier, 2020). Hence, it is important to obtain a sufficient amount of protein through a balanced diet.

Crude protein analysis examines the amount of nitrogen, which is required to build amino acids (Hay, 2021). The Kjeldahl method, introduced by Johan Kjeldahl in 1883 is commonly used to estimate the crude protein level by determining the total nitrogen content (Varelis, 2016). According to Jiang et al. (2014), the Kjeldahl method consisted of three procedures, that is digestion, distillation and titration. Digestion boils the food sample with concentrated sulfuric acid to convert the nitrogen into ammonium sulfate, which can then be distilled into the receiving solution. This is achieved by boiling with base and subsequently condensing the ammonium (NH_4^+) to ammonia (NH_3). As the ammonia ions will be collected in the receiving solution, the standard acid such as sulphuric acid (H_2SO_4) or hydrochloric acid (HCl) can be used in titration to calculate the amount of ammonia ions present (AOAC, 1999).

2.1.4 Crude Fat Content

According to the National Health Service (NHS) (2022), dietary fat in an appropriate amount is vital for a healthy diet. As the human body is unable to synthesise essential fatty acids, it is important to acquire them through diet for better body health. Besides, dietary fat can also help in the absorption of fat-soluble vitamins, including vitamin A, D, E and K (Bockisch, 1998). Since the endogenous-synthesised vitamins are insufficient, dietary vitamins are an

excellent source for physiological processes, in which the fat-soluble vitamins played important roles in vision, immunity, blood coagulation and more (Reddy and Jialal, 2021). Therefore, intake of dietary fat is crucial in assisting the absorption of fat-soluble vitamins.

However, not all fat in food is needed in the same proportion. Multiple researches have associated atherosclerosis and coronary heart disease with the consumption of dietary saturated fat, in which the high saturated fat intake increases the risk of cardiovascular disease (Liu et al., 2017). On the other hand, trans fats are found to elevate the level of blood cholesterol (NHS, 2022). Therefore, analysis of fat in food is vital in providing guidelines for the recommended consumption.

Hewavitharana et al. (2020) mentioned that analysis of lipid in foods is traditionally done by the Soxhlet method. The common solvent used is petroleum ether, which can be connected to the Gerhardt analytical systems for rapid determination (Gerhardt GmbH & Co., 2022). As the petroleum ether has a low boiling point, the extraction time can be shortened for a faster result (Hawach Scientific, 2018). To obtain a reliable result, the sample must be dried before analysis, as the principle of the Soxhlet method relies on the weight differences between the residues and sample (Strugnell, 1989).

2.1.5 Crude Fibre Content

Dhingra et al. (2011) define dietary fibre as a part of plants that is resistant to digestion such as cellulose and noncellulosic polysaccharides. It consists of components that are resistant to human digestion and can be categorised into the soluble and insoluble fibre. Barber et al. (2020) stated that fruits and vegetables are the main sources of soluble fibre, whereas cereals and whole-grain products are rich in insoluble fibre. Nonetheless, soluble and insoluble fibre are simultaneously present in high-fibre foods.

Dietary fibre is associated with enhancing gut motility to prevent constipation (Woo et al., 2015) and improving overall health in various metabolic pathways. These include regulation of glucose uptake and insulin sensitivity (Honsek et al., 2018; Kabisch et al., 2019) and reducing risks of colorectal carcinomas (Song et al., 2015), cardiovascular diseases (Threapleton et al., 2013) and chronic inflammation (Knudsen et al., 2018). Crude fibre analysis provides insight into the indigestible fibre by using chemical digestion based on the AOAC method. The acid extracts starch and sugar while the base removes protein, hemicellulose and lignin. The sequential chemical digestions remove the undesirable compounds, leaving crude fibre to be quantified gravimetrically (AOAC, 1999).

2.2 Anti-inflammatory Properties

Inflammation is a type of immune response of the body to irritants (Institute for Quality and Efficiency in Health Care, 2018). It occurs upon encountering pathogens, injuries or chemicals (Okoli et al., 2008). In the process of inflammation, inflammatory mediators including bradykinin and histamine will be released for vasodilation, which helps in the extravasation of inflammatory cells such as neutrophils and monocytes (Zuchtriegel et al., 2016). The secretion of inflammatory mediators is responsible for the cardinal signs of inflammation including rubor (redness), tumour (swelling), calor (heat), dolor (pain) and functio laesa (loss of function) (Punchard et al., 2004).

Inflammation is important to eradicate external irritants. However, in some cases, the inflammation may persist to cause chronic diseases, such as rheumatoid arthritis, psoriasis, and inflammatory bowel diseases (Institute for Quality and Efficiency in Health Care, 2018). Kalavani et al. (2016) stated that the occurrence of certain arthritic diseases may be attributed to the denatured protein accumulated in body cells. Hence, it is crucial for the pro-inflammatory and anti-inflammatory factors to be tightly regulated. Examples of the endogenous anti-inflammatory agents include regulatory T cells, anti-inflammatory neuropeptides and cytokines such as IL-4, 6, 10 and 11 (Anderson and Delgado, 2008). Besides, dietary anti-inflammatory substances are also important to help prevent inflammatory-related diseases (Casas et al., 2014).

To assess the anti-inflammatory properties *in vitro*, a membrane lysis assay can be used to examine the ability of a substance in stabilising the cell membrane to prevent further damage caused by inflammation (Gunathilake et al., 2018a). Okoli et al. (2008) mentioned that the lysosomal contents of leucocytes such as bactericidal enzymes and proteases will be released during inflammation and cause damage to the surrounding cells. Membrane stabilisation in the leucocytes limits the release of lysosomal content and prevents exacerbation of inflammatory response (Okoli et al., 2008).

One of the simple and cost-effective membrane lysis assays is the protein denaturation assay (PDA). In tissue necrosis, protein degradation occurs, causing the release of damage-associated molecular patterns (DAMP) to promote inflammation (Opie, 1962). Therefore, protein denaturation is associated with inflammation and may lead to inflammatory diseases (Osman et al., 2016). Hence, the ability of the compound to inhibit protein denaturation reflects the anti-inflammatory capacity.

2.3 Antioxidant Activity

According to the National Center for Complementary and Integrative Health (2013), antioxidants are defined as substances that are able to delay cell damage. Free radicals can be endogenous or exogenous, which can cause oxidative stress to the cells and cause damage. Endogenous free radicals are generated during the metabolic processes or during inflammation such as in aerobic respiration (Das and Roychoudhury, 2014) or the release of reactive oxygen species (ROS)

by polymorphonuclear neutrophils (PMN) (Bassal et al., 2021). External ROS in environmental stressors can be produced by cigarette smoke or environmental stressor. These damage body cells and further worsen inflammation to release more ROS. Examples of ROS include free radicals and non-radicals to induce oxidative damage (Das and Roychoudhury, 2014). Excessive ROS has been associated with various diseases such as cancer, neurodegenerative diseases and more. Hence, an antioxidant is important in scavenging the free radicals to prevent cell damage.

The antioxidative mechanism can be generated endogenously (Liu et al., 2018). The endogenous antioxidant can be protein or nonprotein, which have different mechanisms (Aguilar et al., 2016). One of the most well studied nonprotein antioxidants is glutathione, which in its reduced form can scavenge the radicals. The antioxidants are responsible for maintaining redox balance to regulate ROS. Dietary antioxidants play an important role in the endogenous mechanisms (Jacob, 1995). Therefore, consumption of dietary antioxidants helps in the antioxidant interactions and is proven to improve various diseases, particularly age-related degenerative diseases (Salminen et al., 2014).

On the other hand, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay, total phenolic content (TPC) and ferric-reducing antioxidant power (FRAP) can be used to determine antioxidant activity of extracts. DPPH is a stable free radical molecule solubilised in methanol (Njoya, 2021) that has an absorption band of 515 nm in its radical form (Brand-Williams et al., 1995). Upon reduction by an antioxidant, the absorption band disappears. The antioxidants are able to

reduce the DPPH molecule by contributing a hydrogen atom or an electron (Njoya, 2021). This assay is widely used to analyse the antioxidant properties of the crude extract because of its simple and easy method.

In addition, phenolic compounds are found in plants as secondary metabolites. These include simple phenols, phenolic acids, flavonoids and more, and have a protective effect against UV light and may also act as antioxidants (Blainski et al., 2013). Besides participating in the reaction, some of the phenolic compounds may induce endogenous antioxidant synthesis and further aid in oxidative disease prophylaxis (Aryal et al., 2019). The phenolic content can be examined by the Folin-Ciocalteu reagent. In the presence of phenolic compounds, the Folin-Ciocalteu reagent will be reduced to produce a colour compound with an absorbance of 760 nm (Malta and Liu, 2014). As the concentration of the phenolic increases, the intensity of the colour increases to allow quantification of the compound.

Ferric-reducing antioxidant power (FRAP) has been widely used to examine the antioxidant content of extracts. It measures the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in acidic pH (Shahidi and Zhong, 2015). The acidic pH is important to drive the electron transfer while maintaining iron solubility (Shahidi and Zhong, 2015). As the Fe^{2+}gives blue colour, the antioxidant activity can be measured as the colour intensity which can be quantified spectrophotometrically at 593 nm. A blue compound will be formed by either reduction of Fe^{3+} to Fe^{2+} or binding of the antioxidant to the free Fe^{3+} (Shahidi

and Zhong, 2015). The FRAP assay is commonly used in the determination of antioxidants as it is time-saving and simple to perform (Rubio et al., 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

The chemicals and reagents used throughout the study are listed in Table 3.1.

Table 3.1: List of chemicals and reagents used with respect to their brand and manufacturer.

Chemicals / Reagents	Manufacturer, Country
2, 4, 6-Tris(2-pyridyl)-s-triazine (TPTZ)	Merck KGaA, Germany
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Alfa Aesar, United States
Acetylsalicylic acid, 99%	Acros Organics, Pittsburgh
Aluminium standard solution, 1000 ppm	Merck KGaA, Germany
Boric acid	Bendosen, Norway
Bovine serum albumin	SRL Chemical, India
Cadmium standard solution, 1000 ppm	Merck KGaA, Germany
Calcium standard solution, 1000 ppm	Merck KGaA, Germany
Chromium standard solution, 1000 ppm	Merck KGaA, Germany
Cobalt standard solution, 1000 ppm	VWR International Ltd, England
Copper (II) sulfate-5-hydrate	Bendosen, Norway
Copper standard solution, 1000 ppm	Fischer Scientific, UK
Ferrous sulphate heptahydrate 98.5%	Loba Chemie, India
Folin-Ciocalteu's reagent	Merck KGaA, Germany
Glacial acetic acid	Merck KGaA, Germany
Hydrochloric acid, 37%	R&M Chemicals, UK
Iron (III) chloride hexahydrate	Merck KGaA, Germany
Iron standard solution, 1000 ppm	Merck KGaA, Germany
L-Ascorbic acid	Systerm, Malaysia
Lead standard solution, 1000 ppm	Merck KGaA, Germany
Magnesium standard solution, 1000 ppm	Merck KGaA, Germany
Manganese standard solution, 1000 ppm	Merck KGaA, Germany

Table 3.1 continued: List of chemicals and reagents used with respect to their brand and manufacturer.

Chemicals / Reagents	Manufacturer, Country
Methanol, analytical grade	Merck KGaA, Germany
Nickel standard solution 1000 ppm	Merck KGaA, Germany
Nitric acid, 70%	Labscan Ltd, Thailand
Petroleum ether, 40°C - 60°C	Bendosen, Norway
Potassium chloride	Systerm, Malaysia
Potassium dihydrogen phosphate	Merck KGaA, Germany
Potassium sodium tartrate	R&M Chemicals, UK
Potassium sulphate	SRL Chemical, India
Sodium acetate 3-hydrate	Bendosen, Norway
Sodium carbonate	Systerm, Malaysia
Sodium chloride	Merck KGaA, Germany
Sodium hydrogen phosphate	Merck KGaA, Germany
Sodium hydroxide	Chemiz sdn bhd, Malaysia
Sulfuric acid, 98%	Labscan Ltd, Thailand
Zinc standard solution, 1000 ppm	Merck KGaA, Germany

3.2 Experimental Design

The research was conducted according to the flowchart in Figure 3.1.

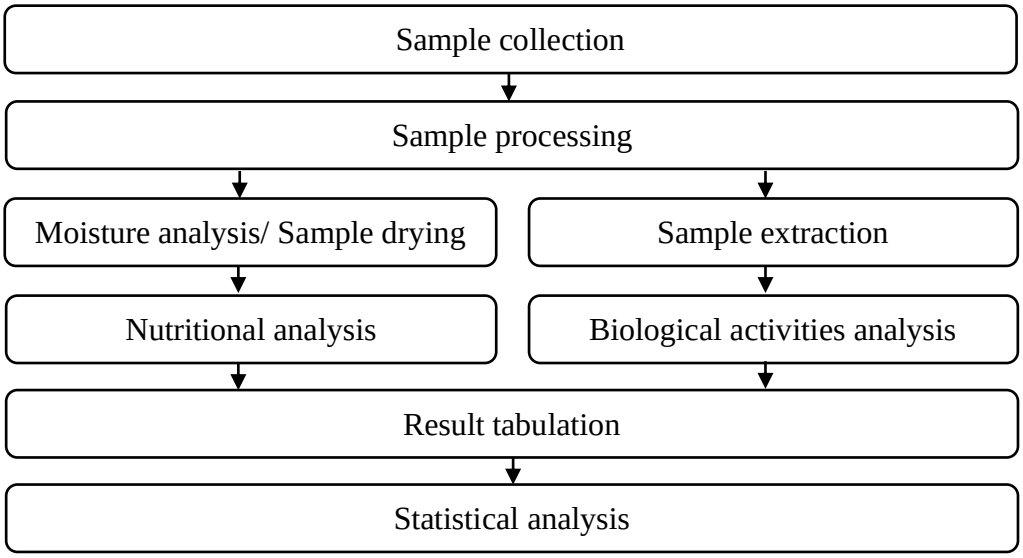

Figure 3.1: An overview of the experimental design.

3.3 Sample Collection

A total of 4 kg of the fresh *Raphanus sativus* subsp. *longipinnatus* roots were collected from Cameron Highlands, Pahang on 25th October 2021. The radish species was confirmed by Dr. Suguraman Manickam, a botanist affiliated with the Faculty of Science, University of Malaya, Malaysia.

3.4 Sample Processing

Raw and boiled *R. sativus* subsp. *longipinnatus* were prepared in UTAR Faculty of Science, Kampar, Perak. The daikon radish roots were cleaned under running tap water and the skin of the daikon radish roots was removed using a peeler. For the raw sample, 2 kg of the daikon radish roots were cut into cubes (~1 cm on each side) using a knife (Figure 3.2). Another 2 kg of the daikon radish roots were cut into cubes and boiled in deionised water at 100°C for 10 minutes to obtain the boiled sample. For nutritional analysis, 1.65 kg each of the raw and boiled samples cubes were dried using a drying oven (Binder GmbH, Tuttlingen, Germany) and crushed using a grinder (Pensonic PB-4004V, Malaysia) into powders.

Figure 3.2: Daikon radish roots cubes.

3.5 Sample Extraction

The remaining radish cubes (350 g each for raw and boiled) were soaked in 80% methanol-water for optimum extraction (Ishida et al., 2011) to obtain the sample extract for biological activities analysis (Figure 3.3). The radish cubes were fully submerged in 500 mL of solvent in the conical flask at room temperature for each raw and boiled sample. The flasks were shaken orbitally using the shaker incubator (Yihder LM-450D, Taiwan) overnight. A total of three rounds of extraction were done by changing the solvents for each cycle. Extraction of the sample was done by rotary evaporation (Büchi Labortechnik, Switzerland) to evaporate the methanol at 180 mbar, 40°C. After most of the solvents were evaporated, the sample extract was then freeze-dried using a freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) for 12 hours to remove the remaining water. The sample extract was weighed until constant value was obtained to ensure the elimination of solvents.

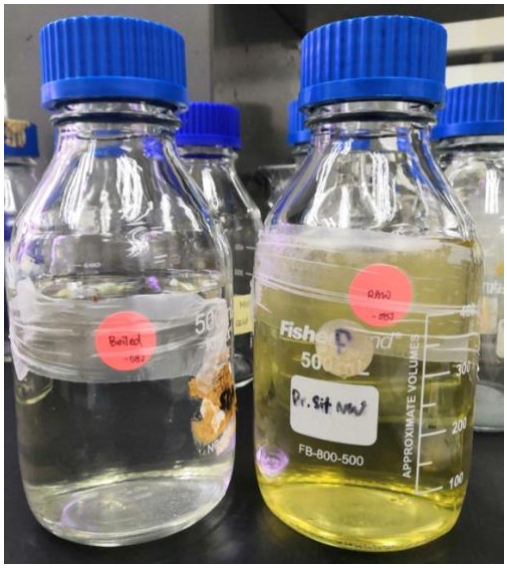

Figure 3.3: 80% methanol filtrate of the raw and boiled daikon radish roots after soaking.

3.6 Nutritional Composition Analysis

The raw and boiled roots were subjected to moisture, ash, crude protein, crude fibre, and crude fat analysis according to the Association of Official Analytical Chemists (AOAC) methods. All the analyses were performed thrice to obtain average data.

3.6.1 Moisture Analysis

The moisture analysis was done according to the AOAC method 934.06 (AOAC, 1999). Briefly, 10 g of the fresh raw and boiled samples were weighed and dried in the glass petri dish for 24 hours in a drying oven (Binder GmbH, Tuttlingen, Germany) at 105°C. The weight of the sample after drying was recorded and the moisture content was calculated according to the following formula: Moisture content (%) = $\frac{(W1-W2)}{W1} \times 100$; where: W1 is the weight of sample before drying (g) while W2 is the weight of sample after drying (g).

3.6.2 Ash Analysis

Two grams each of the dried raw and boiled samples was placed in crucibles and burnt in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 600°C for 12 hours (AOAC method 942.05). The formula used to calculate the ash content of the sample was: % Ash = $\frac{W3-W1}{W2} \times 100$; where W1 = weight of crucible (g), W2 = weight of sample (g) and W3 = weight of crucible and ash (g) after burning.

3.6.3 Crude Protein Analysis

The crude protein of the samples was analysed using the Kjeldahl method according to the AOAC method 920.152. Two grams each of dried raw and boiled samples were added with 20 mL of 98% sulfuric acid, 0.8 g of copper (II) sulphate and 7 g of potassium sulphate. A blank reagent was prepared without the addition of sample and served as a negative control for calibration. The process was done using the Kjeldahl system provided by BÜCHI Labortechnik AG, Flawil, Switzerland. The mixture was then digested using Kjeldahl Speed Digester for 1.5 hours (Figure 3.4). The distillation was done using Kjeldahl Distillation Unit K-355 after adding distilled water and 32% NaOH at the ratio of sample to reagents of 1:2 v/v and 1:3 v/v respectively (Figure 3.5). Lastly, the ammonium collected in 4% boric acid as the receiving solution was titrated using 1 M HCl. The volume of titrant used for each sample was recorded and the crude protein content were calculated using a conversion factor of 6.25. The total protein (%) can be calculated using the equation below:

$$\%P = \frac{[V(1)-V(B1)]\cdot c\cdot M(N)}{m\cdot 1000} \cdot 100 \cdot 6.25$$

Where:

V(1): consumption of titrant, sample (mL)

V(B1): average consumption of titrant, blank (mL)

c: concentration of titrant (mol/L)

M(N): molecular weight of N (14.007 [g/mol])

m: sample weight (g)

1000: conversion factor (mL in L)

%P: % of weight of protein

Figure 3.4: Kjeldahl Speed Digester.

Figure 3.5: Kjeldahl Distillation Unit K-355.

3.6.4 Crude Fibre Analysis

The crude fibre analysis was done using the Fibertherm® FibreBag analysis system (C. Gerhardt GmbH & Co., Königswinter, Germany) according to the AOAC method 962.09. One gram each of the raw and boiled samples was put in the fibrebag and digested using 360 mL of 0.13 M H_2SO_4 and 360 mL of 0.23 M NaOH by alternating between boiling and simmering processes thrice (Figure 3.6). The filtered residues were then rinsed with deionised water until the solution was clear. The samples in the fibrebag were then dried in the drying oven at 105ºC for 24 hours. This was followed by incineration at 600ºC for 12 hours in the muffle furnace to get the weight of the ash. The weights of the sample before digestion, after digestion and after incineration were recorded to calculate the fibre content using the following formula: $\% \, Crude \, Fibre = \frac{[(M3-M1-M4)-(B3-B1-B4)]}{M2} \times 100$;

where:

M1= weight of fibrebag (g)

M2= sample weight (g)

M3 = pre-ashed crucible and dried fibrebag after digestion (g)

M4 = pre-ashed crucible and ash (g)

B1 = weight of blank fibrebag (g)

B3 = pre-ashed crucible and dried blank fibrebag after digestion (g)

B4 = pre-ashed crucible and blank ash (g).

Figure 3.6: Digestion of samples by boiling and simmering in acid and base.

3.6.5 Crude Fat Analysis

The crude fat content was analysed using Soxtherm® rapid extraction system developed by C. Gerhardt GmbH & Co according to the AOAC method 963.15 (Figure 3.7). Petroleum ether (40ºC - 60ºC) was used as the solvent for the extraction. Five grams each of the raw and boiled samples were weighed and wrapped in a filter paper. The samples were then placed in a 30 mm – 80 mm cellulose thimber and inserted into the extraction beaker. The extraction beakers were placed into the Soxtherm® extraction unit for digestion and the Soxtherm® Manager program was set to run with following parameters (Table 3.2):

Table 3.2: Parameters for the Soxtherm® Manager program.

Parameters	Setting
T-classification	200ºC
Extraction temperature	150ºC
Reduction interval	3 min 30 s
Reduction pulse	1 s
Hot evaporation	30 min
Evaporation A	5 × interval
Extraction time	1 h 20 min
Evaporation B	3.0 × interval
Evaporation C	10 min
Program length	2 h 28 min

The weights of the extraction beaker before digestion (M1) and after digestion (M2) were measured and the crude fat content was calculated using the following formula: $\%\, Fat = \frac{M2-M1}{M0} \times 100$ where the M0 is the weight of sample.

Figure 3.7: Crude fat extraction using Soxtherm® rapid extraction system.

3.7 Mineral Analysis

3.7.1 Sample and Equipment Preparation

Before the mineral analysis, all glasswares and apparatuses were pre-treated by soaking in 0.5 M nitric acid (HNO_3) for 24 hours. For the sample preparation, a total of 1.5 g each of the dried raw and boiled samples were acid-digested using the method of Uddin et al. (2016). In brief, 15 mL of 65% HNO_3 and 45 mL of 37% HCl (1 part of 65% HNO_3 to 3 parts of 37% HCl) were introduced to the powdered sample. The mixture was then boiled at 95°C in a water bath for 6 hours until the samples were fully dissolved. The solution was then topped up to 100 mL with deionised water in a volumetric flask. The filtration was done using 0.22 μm nylon membrane syringe filters (Minisart NY 25, Sartorius Stedim Biotech GmbH, Goettingen, Germany) before analysis.

3.7.2 Analysis of the Mineral Content

The mineral contents of the sample include Aluminium (Al), Calcium (Ca), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Magnesium (Mg), Manganese (Mn), Nickel (Ni), Lead (Pb) and Zinc (Zn) were quantified using Flame Atomic Absorption Spectrometer (FAAS) (AAnalystTM 200, PerkinElmer Inc.). The acetylene gas and oxidant airflow were set at 2.50 and 13.30 L/min respectively for all the minerals. The analysis was done in triplicate against a calibration curve constructed by a 5-point of standard solution for each mineral. The standard concentration for each element was listed in Table 3.3.

Table 3.3: Standard concentration for each mineral.

Minerals	Standard Concentration (ppm)
Aluminium (Al)	30, 60, 90, 120, 150
Calcium (Ca)	0.5, 1.0, 1.5, 2.0, 2.5
Cadmium (Cd)	0.4, 0.8, 1.2, 1.6, 2.0
Cobalt (Co)	2, 4, 6, 8, 10
Chromium (Cr)	8, 16, 24, 32, 40
Copper (Cu)	1, 2, 3, 4, 5
Iron (Fe)	2, 4, 6, 8, 10
Magnesium (Mg)	3, 7, 11, 15, 19
Manganese (Mn)	0.5, 1.25, 2, 2.75, 3.5
Nickel (Ni)	1.5, 3, 4.5, 6, 7.5
Lead (Pb)	3, 7, 11, 15, 19
Zinc (Zn)	0.2, 0.4, 0.6, 0.8, 1.0

3.8 Anti-inflammatory Properties

3.8.1 Protein Denaturation Assay

The method used by Gunathilake et al. (2018a) was adapted with slight modifications. Five concentrations of the daikon radish root extracts (125 μg/mL – 2000 μg/mL) with two-fold dilution were used. A 500 μL of 1% bovine serum albumin was mixed with the raw and boiled radish root extract (50 μL). The mixture was incubated at 37°C for 20 minutes, followed by heating to 57°C for 3 minutes in a water bath. After heating, 250 μL of phosphate-buffered saline (PBS) (pH 6.3) was added to the mixture and mixed well using a vortex mixer (Bibby Scientific Ltd., UK). Then, 100 μL of the reaction mixture was

transferred into a flat-bottom 96-well microplate and added with a 1:1 ratio of copper-alkaline solution (100 μL) and 5% Folin-Ciocalteu's solution (100 μL). The microplate was incubated at 55°C for 10 minutes and the absorbance was read at 650 nm after incubation using a 96-well microplate reader (FLUOstar® Omega, BMG Labtech, Mornington, VIC, Australia). Aspirin was used as positive control while PBS was used as a blank. The results were interpreted in percentage (%) of inhibition using the following formula: $\% \, inhibition = \left(1 - \frac{Ae}{Ao}\right) \times 100$, where A_e = absorbance of the sample; A_0 = absorbance of the blank. The assay was performed thrice to obtain an average value.

3.9 Antioxidant Properties

DPPH radical scavenging activity, total phenolic content (TPC) and ferric-reducing antioxidant power (FRAP) were performed to examine the antioxidant properties. All three assays were done in triplicate to obtain reliable data.

3.9.1 DPPH Radical Scavenging Activity

One hundred microlitres of 12 different concentrations (2-fold downward dilutions from 2000 to 0.9766 μg/mL) of the raw and boiled extracts were prepared and incubated in the dark at room temperature for 20 minutes with 100 μL of 0.2 mM DPPH dissolved in methanol. The absorbance of the mixture was examined under 515 nm using a 96-well microplate reader, according to Kong et al. (2020). Ascorbic acid with the same dilution as the sample extracts (2000 – 0.9766 μg/mL) acted as the positive control in this experiment. A blank was

prepared by substituting the sample extracts with 100 μL of 2:1 methanol-water. The results were then expressed in % inhibition using the following formula (Gunathilake et al., 2018a) and the half-maximal inhibitory concentration (IC_{50}) were determined from the graph of % inhibition against sample concentrations. The IC_{50} represented the concentration needed for the sample extracts to scavenge 50% of the DPPH radicals.

$$\%\ inhibition = \left(1 - \frac{Ae - As}{Ao}\right) \times 100$$

A_e = absorbance of the sample; A_s = absorbance of the sample blank;

A_0 = absorbance of the negative control

3.9.2 Total Phenolic Content (TPC)

The total phenolic content (TPC) was examined using the method of Chan et al. (2013) with slight modifications. Overally, 25 μL of 50% Folin-Ciocalteau's phenol reagent were added to 25 μL of sample extracts (4 concentrations with two-fold dilution from 1000 μg/mL to 125 μg/mL) followed by 75 μL of deionised water into a flat-bottom 96-well microplate. The microplate was then shaken on a see-saw rocker (Cole-Parmer™ Stuart™, Fischer Scientific, UK) at room temperature in the dark for 6 minutes. Then, 100 μL of 700 mM Na_2CO_3 was then added to further incubate the mixture at room temperature in a dark environment for 90 minutes. The absorbance was measured at 765 nm using the microplate reader and the results were collected in gallic acid equivalent (GAE)/g sample. The gallic acid (5 μg/mL to 80 μg/mL) was used to construct a standard calibration curve in this assay.

3.9.3 Ferric-reducing Antioxidant Power (FRAP) Assay

FRAP reagent was prepared fresh by mixing 10 mL of 0.3 M acetate buffer (pH 3.6), 1 mL of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and 1 mL of 20 mM $FeCl_3$ solution at 10:1:1 ratio (v/v) according to Benzie and Strain (1996). A volume of 270 μL of the FRAP reagent was warmed to 37ºC and the reagent blank reading was taken at 593 nm. Subsequently, 30 μL of the raw and boiled sample extracts at different concentrations (125, 250, 500 and 1000 μg/mL) was added to the reagent and the absorbance was recorded again at 593 nm after incubation at 37°C for 4 minutes. The results were analysed by obtaining the difference between the absorbance (ΔA_{593nm}) and a standard curve with $FeSO_4$ (0.2 – 1.6 mM) was constructed. The results obtained were expressed in mmol Fe^{2+} equivalent (E)/g sample.

3.10 Data Analysis

All the analyses performed were done in triplicate. The mean ± standard deviation for all the data was calculated using Microfost Office Excel 365. The results obtained were analysed with IBM® SPSS® version 22. Student's T-test was performed to analyse the significant differences between the raw and boiled daikon radish roots data. A p-value lower than 0.05 denoted statistical significance.

CHAPTER 4

RESULTS

4.1 Nutritional Composition

The moisture, ash, crude protein, crude fat, and crude fibre contents of raw and boiled daikon radish roots were determined and tabulated in Table 4.1. Other than the moisture content, the findings are expressed in percentages based on dry weight (DW). According to the results obtained, there were significant differences in the moisture, ash, crude protein, and crude fibre contents of raw and boiled daikon radish roots. Although the boiled daikon radish roots have a significantly higher moisture content, the moisture content determined from both the raw and boiled samples exceeded 95%. Therefore, daikon radish roots were determined to have a high moisture content. Meanwhile, the boiled daikon radish roots contained more ash and crude fibre than the raw daikon radish roots, which accounted for 25.10% and 16.41% of the dried radish mass, respectively. In comparison to boiled daikon radish roots, the raw dried daikon radish roots had a higher crude protein level. There were no significant differences between the raw and boiled daikon radish roots in the crude fat content. Overall, the boiled daikon radish roots had higher moisture, ash and crude fibre content, which can be said to have a higher nutritional value than the raw daikon radish roots.

Table 4.1: Nutritional composition of the raw and boiled roots of *Raphanus sativus* subsp. *longipinnatus.*

Composition	Raw	Boiled
Moisture (% FW)	95.89 ± 0.16*	96.64 ± 0.05*
Ash (% DW)	19.55 ± 0.60*	25.10 ± 1.10*
Crude Protein (% DW)	12.69 ± 0.44*	10.07 ± 0.44*
Crude Fat (% DW)	0.20 ± 0.20	0.13 ± 0.12
Crude Fibre (% DW)	13.36 ± 2.66*	16.41 ± 1.60*

The data are shown as mean ± standard deviation (n=3). Asterisk mark denotes significant differences ($p < 0.05$) between raw and boiled samples.

4.2 Mineral Analysis

The mineral composition of the raw and boiled daikon radish roots is summarised in Table 4.2. Twelve minerals were analysed in this study. The top five of the most abundant minerals in the raw daikon radish sample in descending order were magnesium, calcium, aluminium, zinc and iron (Mg > Ca > Al > Zn > Fe); whereas calcium, magnesium, iron, chromium and aluminium (Ca > Mg > Fe > Cr > Al) were 5 of the most abundantly present minerals in the boiled daikon radish sample. However, Mg and Ca remained the most abundant minerals in both samples.

Among the 12 minerals, aluminium, cadmium, cobalt and lead showed no significant differences between the raw and boiled daikon radishes. On the other hand, a higher magnesium level was recorded in the raw daikon radishes compared to the boiled sample. As for calcium, chromium, copper, iron, manganese, nickel and zinc, higher mineral contents were detected in the boiled

samples. All the minerals were analysed based on their respective 5-point standard curves, in which the regression coefficient (R^2) of all linear curves was higher than 0.99.

Table 4.2: Mineral composition of the raw and boiled roots of *Raphanus sativus* subsp. *longipinnatus.*

Mineral (mg/100g DW)	Raw	Boiled	Linear equation	R^2
Aluminium (Al)	17.54 ± 2.51	19.21 ± 3.83	y = 0.00266x	0.9920
Calcium (Ca)	153.59 ± 3.45*	447.14 ± 20.76*	y = 0.13369x	0.9997
Cadmium (Cd)	0.10 ± 0.02	0.09 ± 0.01	y = 0.00266x	0.9973
Cobalt (Co)	0.32 ± 0.04	0.31 ± 0.02	y = 0.00266x	0.9950
Chromium (Cr)	0.54 ± 0.20*	23.98 ± 0.69*	y = 0.00266x	0.9978
Copper (Cu)	1.31 ± 0.03*	1.45 ± 0.02*	y = 0.00266x	0.9901
Iron (Fe)	3.27 ± 0.11*	53.44 ± 1.33*	y = 0.00266x	0.9960
Magnesium (Mg)	192.09 ± 6.70*	136.47 ± 0.01*	y = 0.00266x	0.9971
Manganese (Mn)	1.09 ± 0.04*	2.28 ± 0.09*	y = 0.00266x	0.9997
Nickel (Ni)	0.46 ± 0.03*	8.08 ± 0.24*	y = 0.00266x	0.9991
Lead (Pb)	1.80 ± 0.10	1.64 ± 0.07	y = 0.00266x	0.9947
Zinc (Zn)	4.90 ± 0.08*	6.43 ± 0.08*	y = 0.00266x	0.9983

The data are shown as mean ± standard deviation (n=3). Asterisk marks denote significant differences ($p < 0.05$) between raw and boiled samples.

4.3 Anti-inflammatory Activity

The anti-inflammatory activity of the raw and boiled daikon radish roots was determined by the protein denaturation assay (PDA). The results were expressed in IC_{50} (μg/mL) as a mean of three replicates (Table 4.3), in which the raw daikon radish roots had a higher IC_{50} value. This denotes that the raw daikon radish had lower anti-inflammatory activity compared to the boiled daikon

radish, as it required 985.17 ± 56.37 μg/mL to inhibit the protein denaturation by half.

According to Figure 4.1, a sigmoid dose-dependent curve illustrated the relationship between the percentage of protein denaturation inhibition and the concentration of the sample extract. As the concentration of the sample extract increased, the percentage of inhibition elevated. The sigmoid curve represented an exponential increase in the percentage of inhibition at moderate concentration subsequently reaching a plateau at a higher concentration. However, the positive control, acetylsalicylic acid (aspirin) was reaching its maximum inhibitory activity starting from 250 μg/mL, having 35.57% - 46.27% of protein denaturation inhibition at 1250 - 2000 μg/mL. Therefore, it can be observed from Figure 4.1 that the anti-inflammatory activity was recorded highest in boiled daikon radish roots, followed by raw daikon radish roots.

Table 4.3: Anti-inflammatory activity of the raw and boiled roots of *Raphanus sativus* subsp. *longipinnatus.*

Sample	Protein Denaturation Assay IC_{50} (μg/mL)
Raw	1061.53 ± 105.38*
Boiled	664.79 ± 27.25*

The data are shown as mean ± standard deviation (n=3). Asterisk marks denote significant differences ($p < 0.05$) between raw and boiled samples.

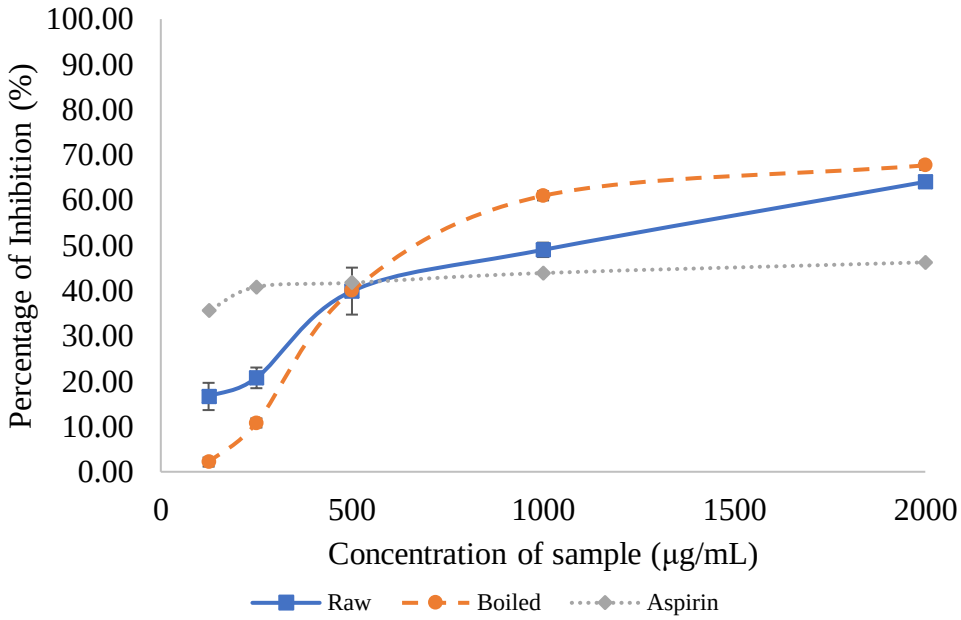

Figure 4.1: Percentage inhibition of protein denaturation of the raw and boiled daikon radish roots.

4.4 Antioxidant Activities

The antioxidant activities of the 80% methanolic raw and boiled daikon radish roots extracts are recorded in Table 4.4. According to Figure 4.2, the IC_{50} of the DPPH scavenging activity of the raw daikon radish roots could not be determined due to the maximum percentage of inhibition at the highest sample concentration (2000 µg/mL) was lower than 50%. On the other hand, the boiled daikon radish roots required 1.59 mg/mL with a standard deviation of 0.09 mg/mL to scavenge 50% of the free DPPH radicals, giving an IC_{50} of 1.59 mg/mL. From the result obtained, it can be concluded that the boiled daikon radish had a higher DPPH scavenging activity compared to the raw daikon radish. The ascorbic acid was used as a positive control in this assay which gave an IC_{50} of 4.07 µg/mL.

For the total phenolic content (TPC), a calibration curve (Figure 4.3) with a linear equation and R^2 of y = 0.0338x + 0.1331 and 0.9978 respectively was constructed with gallic acid from 5 to 80 μg/mL in two-fold dilutions. The calibration curve was used to calculate the gallic acid equivalent (GAE) in the raw and boiled daikon radish sample. The absorbances for the raw and boiled daikon radish samples at the concentration of 1000 μg/mL were used to calculate the GAE, as the sample extracts at lower concentrations were below the limit of quantification (< LOQ). Results showed the raw daikon radish had a statistical significantly higher total phenolic content (p = 0.002) compared to the boiled sample.

The ferric-reducing antioxidant power (FRAP) of the raw and boiled daikon radish roots were not significantly different statistically. The ferrous sulphate calibration curve (Figure 4.4) was constructed with a linear equation of y = 0.2142x + 0.0516 and a R^2 of 0.9937. The result was expressed in mmol Fe^{2+} equivalent per gram sample, which can be determined from Figure 4.5.

Table 4.4: Antioxidant activity of the raw and boiled roots of *Raphanus sativus* subsp. *longipinnatus.*

Sample	DPPH	TPC	FRAP
	IC_{50} (mg/mL)	(μg GAE/g sample)	mmol Fe^{2+} E/g sample
Raw	-	6.36 ± 1.52*	0.36 ± 0.09
Boiled	1.59 ± 0.09	0.22 ± 0.30*	0.47 ± 0.03

The data are shown as mean ± standard deviation (n=3). Asterisk marks denote significant differences ($p < 0.05$) between raw and boiled samples. DPPH: DPPH radical scavenging assay; TPC: total phenolic content; FRAP: ferric-reducing antioxidant power assay; GAE: gallic acid equivalent; Fe^{2+} E: Fe^{2+} equivalent.

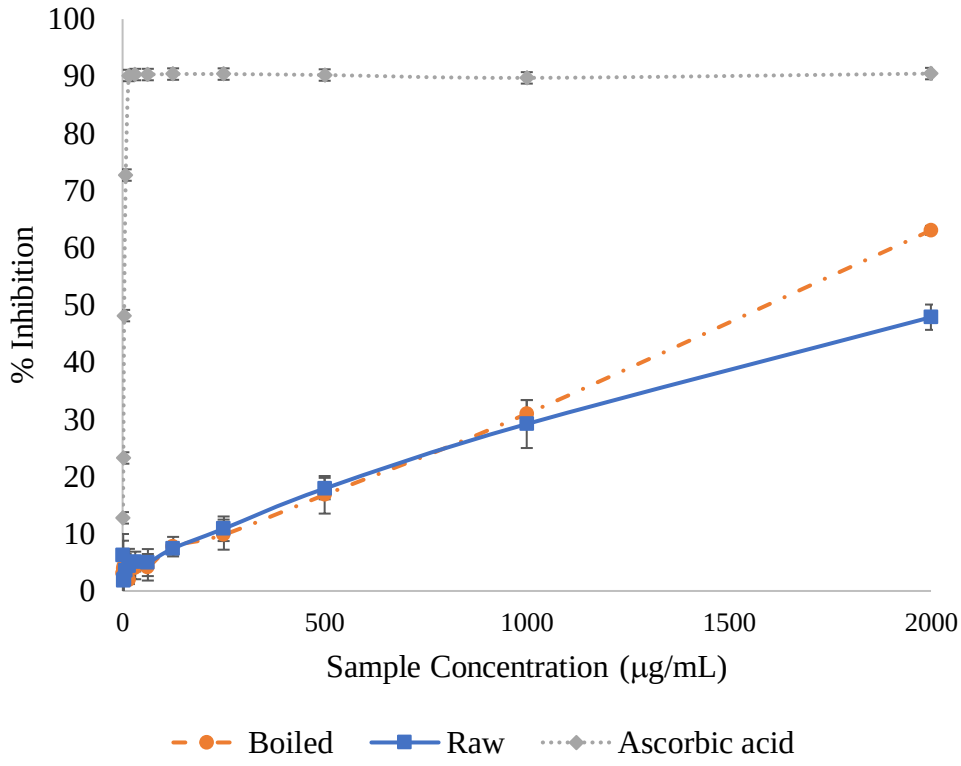

Figure 4.2: Percentage inhibition of DPPH radicals for raw and boiled daikon radish roots.

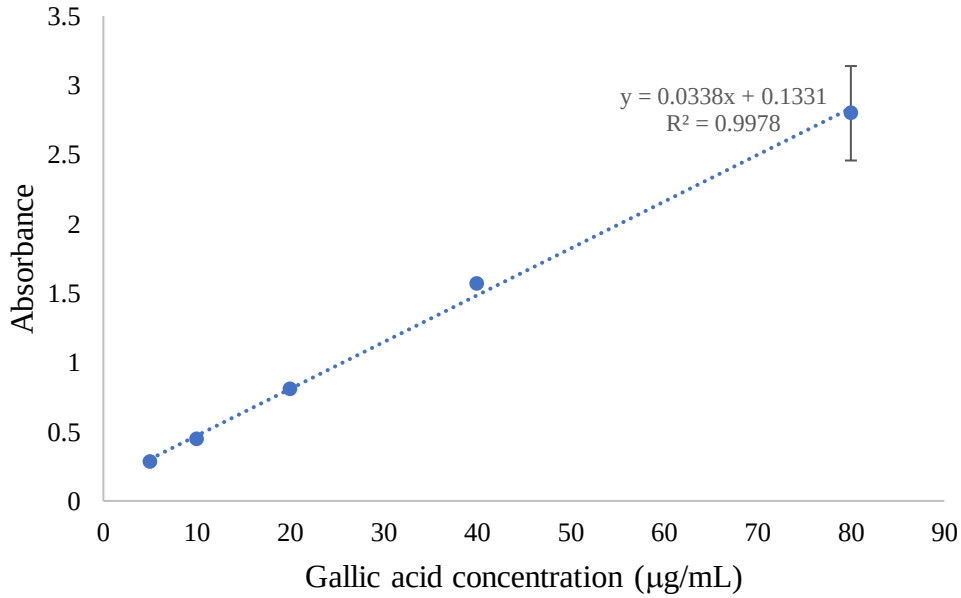

Figure 4.3: Calibration curve constructed using gallic acid.

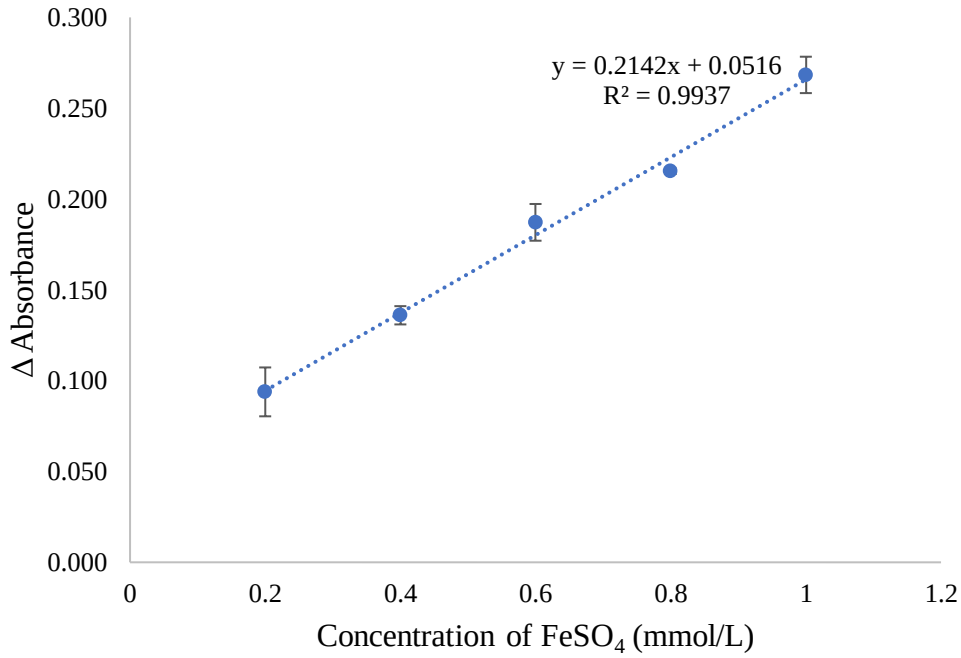

Figure 4.4: Ferrous sulphate calibration curve.

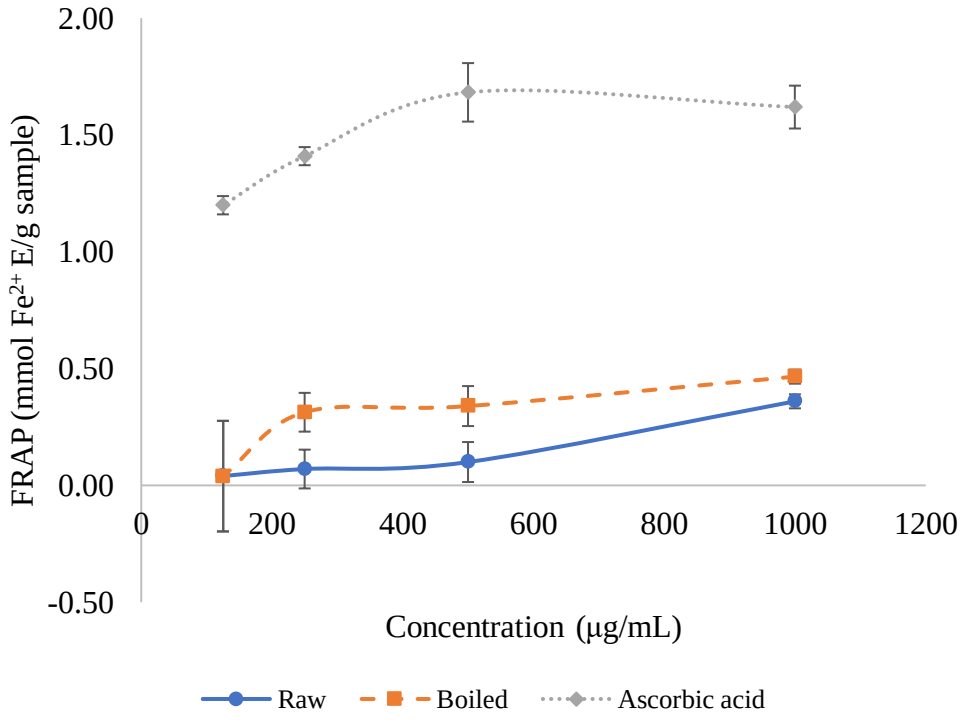

Figure 4.5: Ferric-reducing antioxidant power of the 80% methanol extracts from raw and boiled daikon radish roots.

CHAPTER 5

DISCUSSION

5.1 Nutritional Composition

The nutritional compositions of the raw and boiled daikon radishes were analysed and compared. According to Table 4.1, the moisture contents of the raw and boiled daikon radishes were recorded at 95.89 ± 0.16% and 96.64 ± 0.05% per fresh weight sample. This is comparable with the research done by Goyeneche et al. (2015) who measured the moisture content of the red radish (*Raphanus sativus* L.) at 95.24 ± 0.29 g/100 g sample. A significant difference between the raw and boiled samples was observed, in which the boiled daikon radish had higher moisture content than the raw one. This may be attributed to the increase of permeability (Song et al., 2018) where water diffused into the radish sample when it is fully submerged. As the deionised water was used to boil the sample which is considered a hypotonic solution, the water diffused into the radish and caused a higher moisture content.

Apart from that, ash analysis examines the inorganic residue that remains in the sample after the complete oxidation of an organic matter (Ismail, 2017). The ash analysis is important to evaluate the nutritional value including mineral content contributed by the inorganic compound of the food. In this study, the ash content was analysed based on the dry weight (DW). The ash content of the raw and boiled daikon radish roots was recorded at 19.55 ± 0.60 and 25.10 ± 1.10% DW respectively, while the raw sample had a significantly lower ash content. This

can be explained by the higher mineral contents in the boiled daikon radish roots, as the inorganic residues represented the amount of minerals that reside in the daikon radishes (Popov et al., 2011). Karmakar et al. (2013) reported 20.5 ± 0.20% DW of ash content was recorded in radish leaves. However, no data on the daikon radish roots can be obtained to support the results obtained in this study.

The crude protein obtained was at 12.69 ± 0.44% DW and 10.07 ± 0.44% DW for the raw and boiled daikon radish respectively. This is equivalent to 0.52 ± 0.02% FW and 0.34 ± 0.01% FW in raw and boiled radish respectively after conversion of dry weight into fresh weight. According to Cresson et al. (2017), the formula used in converting dry to fresh weight was: $\frac{100-water\ content\ (\%)}{100} \times \%\ dry\ weight$. As Goyeneche et al. (2015) recorded the crude protein content in the raw *Raphanus sativus* L. roots to be 0.57 ± 0.09 g/100g fresh sample, it supported the results obtained in this experiment. However, the crude protein content is prone to variation between subspecies (Gamba et al., 2021). In this study, the boiled daikon radish had a significantly lower crude protein content. This may be due to the leaching of protein into the boiling water during the process, leaving less protein retained in the analysing sample (Huda and Mohd, 2014).

Crude fat was detected in the raw and boiled daikon radish samples based on the dry weight. The raw daikon radish was recorded at 0.20 ± 0.20% DW, whereas the boiled daikon radish had 0.13 ± 0.12% DW of crude fat. This can be

supported by the research done by Eveline and Pasau (2019) who used different subspecies of *Raphanus sativus* (*Raphanus sativus* L.) and recorded a 0.68% of fat content. However, the results in this study did not show any significant difference between the raw and boiled daikon radish samples. Therefore, it can be deduced that the crude fat content of daikon radish was not altered by the boiling process.

On the other hand, crude fibre content was detected in the raw and boiled daikon radish. There was a significantly higher crude fibre content (16.41 ± 1.60% DW) in the boiled daikon radish than in the raw daikon radish (13.36 ± 2.66). Ashraf et al. (2016) reported the crude fibre content of the *Raphanus sativus* L. to be 17.00 ± 0.35 g/100g DW, which is comparable to this study. The rationale for the elevation of the fibre content in the boiled daikon radish is currently unclear. However, it is deduced that boiling may alter the chemical structure of the cell, causing an increase in the detectable fibre content in the boiled sample. Nonetheless, this hypothesis requires scientific proof from further studies.

5.2 Mineral Analysis

In this study, all the minerals were analysed based on standard curves with $R^2 > 0.99$. Essential minerals including calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn) were analysed. Among the other minerals, nickel (Ni) is important as an enzymatic component (Nielsen, 2017); whereas chromium (Cr) may contribute to health benefits by playing a

role in insulin action (Vincent and Lukaski, 2018), promoting it to be an essential nutrient. Meanwhile, high dietary intake of trace minerals such as aluminium (Al), cadmium (Cd) and lead (Pb) may pose risks to health. Although no reports of toxicity at a lower level to date, dietary aluminium may be neurotoxic at a higher level (Soni et al., 2001). Cadmium is considered a food contaminant that may contribute to gastrointestinal tract, liver, kidney and heart damage (Centre for Food Safety, 2017); while lead can accumulate in the human body to cause toxicity (U.S. Food and Drug Administration, 2020).

Among the analysed minerals, Mg had the highest level in the raw daikon radish. For a better analysis of the result, the data had been converted into a fresh weight basis by using the formula: $\frac{100-water\ content\ (\%)}{100} \times dry\ weight$ according to Cresson et al. (2017). The data obtained in fresh weight were 7.90 ± 0.28 mg/100g FW and 4.58 ± 0.00 mg/100g FW for the raw and boiled daikon radish roots respectively. The results observed in this study recorded a lower Mg level compared to the study by Gupta et al. (2003) who obtained 15 mg/100g FW in the raw *Raphanus sativus* L. ssp. *salivus*. As the variation of the radish samples used were different, a different result was obtained. Comparing the raw and boiled samples, the Mg level in the raw daikon radish was significantly higher. This may be due to the mineral loss during the boiling process (Kimura and Itokawa, 1990; Ando et al., 2015).

As for Ca, Goyeneche et al. (2015) found 147.87 ± 1.31 mg/100g DW in the raw *Raphanus sativus* L., which is comparable to the results obtained in this study

(153.59 ± 3.45 mg/100g DW for raw; 447.14 ± 20.76 mg/100g DW for boiled samples). The boiled daikon radish roots had a statistically higher Ca level compared to the raw daikon radish. An approximate 3-fold higher Ca level was detected in the boiled sample. The increment is supported by the finding of Kimura and Itokawa (1990) who concluded a higher Ca level can be obtained in food after boiling. This can be explained by the high temperature during boiling released the protein-bound calcium, contributing to a higher calcium level after boiling (Wu et al., 2018). Calcium is vital for bone and teeth structure. According to the National Institutes of Health (2020), the recommended daily intake is set at 1000 mg for adults. By this statement, consumption of 200g of the boiled daikon radish roots provided approximately 900 mg of Ca, which satisfy 90% of the recommended daily intake. As a high calcium level was detected in the boiled daikon radish, it served as a good source of dietary calcium.

Gamba et al. (2021) stated that the *Raphanus sativus* is a good source of copper. This study detected 1.31 ± 0.03 and 1.45 ± 0.02 mg/100g DW for the raw and boiled daikon radish roots respectively. The boiled daikon radish exhibited a slightly higher Cu level, with a p-value of 0.013. According to Zhang et al. (2016), Cu is an important micronutrient to plants, which is crucial as a cofactor for proteins and enzymes to form Cu-binding proteins. As there is the presence of bound Cu in plants, it is hypothesised that boiling denatured proteins, leads to the release of bound Cu. This causes a higher detectable Cu in the daikon radish roots after boiling.

Azam et al. (2013) recorded 11.4 mg/100g DW of Cr in the raw *Raphanus sativus* L. cv. Mino, which is much higher than the result of raw daikon radish roots obtained in this study (0.54 ± 0.20 mg/100g DW). Nonetheless, an approximately 40-fold higher Cr level was detected in the boiled daikon radish sample (23.98 ± 0.69 mg/100g DW). There is no data regarding the effects of boiling on the chromium level in food to date to support the results obtained in this study. According to Harvard T.H. Chan School of Public Health (2021), dietary chromium is in its trivalent form, which is non-toxic to humans. A high level of Cr intake has not been associated with adverse effects. However, the hexavalent chromium (VI) from environmental pollution has been proven to cause various toxicity. Tiwari et al (2013) mentioned that the high level of chromium in the soil causes accumulation of chromium in the radish roots, and may cause health issues to humans. Since a significant amount of chromium is present in the boiled daikon radish roots, it is important to identify the form of chromium present for safe consumption.

For the iron level, Goyeneche et al. (2015) obtained 0.15 ± 0.01 mg/100g DW in the raw *Raphanus sativus* L., whereas Azam et al. (2013) reported 37.41 ± 0.297 μg/g DW in the raw *Raphanus sativus* L. cv. Mino. The results obtained in this study corresponded to the findings of Azam et al. (2013) at 3.27 ± 0.11 mg/100g DW in the raw sample, which is higher than those of Goyeneche et al. (2015). A huge increase in the iron level was detected in the boiled daikon radish samples, giving a result of 53.44 ± 1.33 mg/100g DW. Hoppler et al. (2008) discovered that boiling releases plant ferritin-iron in legumes. As the ferritin-iron consisted of a huge part of the plant, boiling may dissociate the iron from

the plant ferritin, causing a higher iron level in the boiled samples. Iron is an important element in haemoglobin to transport oxygen throughout the body. The Institute of Medicine (US) Panel on Micronutrients (2001) listed that the recommended dietary allowance (RDA) of iron for postmenopausal women and men is at 8 mg/day, whereas 18 mg/day of iron is recommended for premenopausal women. From the results obtained in this study, the iron level in the boiled daikon radish samples had exceeded the RDA, representing a high iron content present in the boiled daikon radish roots.

In addition, according to the study by Goyeneche et al (2015), the Mn and Zn values for the raw and boiled *Rapahnus sativus* L. were reported to be 0.07 ± 0.01 mg/100g DW and 0.24 ± 0.00 mg/100g DW respectively. However, 37.90 ± 0.120 μg/g DW and 50.87 ± 1.362 μg/g DW for Mn and Zn respectively were reported in *Raphanus sativus* L. cv. Mino from Azam et al. (2013). These variations in the mineral content can be attributed to different subspecies used in the analysis. In this study, the values for Mn in the raw and boiled daikon radish roots were 1.09 ± 0.04 mg/100g DW and 2.28 ± 0.09 mg/100g DW. The Zn values were at 4.90 ± 0.08 mg/100g DW and 6.43 ± 0.08 mg/100g DW in raw and boiled daikon radish roots respectively. A slightly higher Mn and Zn content is detected in the boiled samples. These results contradicted the findings of Dugo et al. (2005) and Hummel et al. (2020), who reported that boiling encouraged the loss of Mn and Zn in plants.

On the other hand, Cd was the lowest mineral content in both raw and boiled daikon radish roots. It can be observed from Table 4.2 that Al, Cd and Pb which

may cause toxicity to humans has a low concentration in both raw and boiled daikon radish samples. There are no significant differences observed between the raw and boiled daikon radish roots. According to the Centre for Food Safety (2017), the tolerable daily intake for a 70-kg adult for Al, Cd and Pb were set to 210 mg (European Union, 2017), 62 μg (Satarug et al., 2017) and 93 mg (Nag and Cummins, 2021) respectively. Therefore, the daikon radish cultivated in Cameron Highlands, Malaysia is said to be safe to consume as the analysed Al, Cd and Pb level fell below the tolerable limits.

5.3 Anti-inflammatory Activity

The anti-inflammatory activity of the raw and boiled daikon radish roots extracts was evaluated using a protein denaturation assay (PDA). During inflammation, leucocytes secrete proteases to cause protein degradation. Protein degradation occurs during tissue necrosis, which promotes inflammation through releasing damage-associated molecular patterns (DAMP) (Opie, 1962; Davidovich et al., 2014). Since protein denaturation has been associated with inflammatory diseases (Osman et al., 2016), the ability to inhibit albumin denaturation reflects the anti-inflammatory capacity of the sample extracts. The PDA is a common *in vitro* anti-inflammatory assay with acetylsalicylic acid as a positive control (Sarveswaran et al., 2017).

Table 4.3 summarises the IC_{50} of the 80% methanol raw and boiled daikon radish roots extracts. The raw daikon radish has an IC_{50} of 1061.53 ± 105.38 μg/mL while only 664.79 ± 27.25 μg/mL was recorded for the boiled daikon radish. This represented that the raw and boiled daikon radish required these values to inhibit 50% of the protein from being denatured. The presence of anti-inflammatory activities of the daikon radish roots can be supported by the findings from Choi et al. (2016) and Park and Song (2017), who confirmed the anti-inflammatory properties in the seeds and leaves of the red radish *Raphanus sativus* L., respectively.

A significantly higher anti-inflammatory activity was recorded in the boiled sample extract. This is backed by Gunathilake et al. (2018b) who recorded a higher protein denaturation inhibiting ability in boiled *Centella asiatica* and *Passiflora edulisthe.* A possible explanation is that the compound that participates in anti-inflammation may be bounded and was only released after boiling as the precise mechanism of the inhibition is currently not known (Gunathilake et al., 2018b). However, Gunathilake et al. (2018b) also observed a reduction in anti-inflammatory activity in boiled samples of *Olax zeylanica*, *Cassia auriculata, Sesbania grandiflora,* and *Gymnema lactiferum*. This leads to the conclusion that the effect of boiling in anti-inflammatory activity varies between plant species.

5.4 Antioxidant Activity

5.4.1 DPPH Radical Scavenging Assay

The antioxidant activities of the raw and boiled daikon radish roots extracts were determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay mentioned by Gunathilake et al. (2018a). The results obtained from the DPPH assay were expressed in IC_{50}, which denotes the concentration in mg/mL needed for the sample extract to scavenge the DPPH molecules by half. The IC_{50} for the raw daikon radish roots could not be determined due to the low maximum concentration examined.

As for the results obtained for the boiled daikon radish roots, an IC_{50} of 1.59 ± 0.09 mg/mL was obtained. Research by Jamuna et al. (2015) obtained an IC_{50} range of 920 μg/mL to 3859.5 μg/mL in *Raphanus sativus* L. using different extraction methods, i.e. fresh material cold extraction, dried material cold extraction and Soxhlet extraction. The values reported in this study fell within the range of the results from Jamuna et al. (2015), which shows similar DPPH scavenging activities between the boiled daikon radish roots and *Raphanus sativus* L.. On the other hand, Im et al. (2010) reported a lower DPPH radical scavenging activity in ethanolic extract of *Raphanus sativus* L. (15.43-18.71% DPPH inhibition activity at 800 μg/mL). The variation of DPPH radical scavenging activity obtained may be attributed to several reasons. Atik and Mohammedi (2011) mentioned that the types of solvent used in sample extractions can significantly alter the results. Besides, the temperature and the

duration of the sample extraction were proven to contribute to the differences in the scavenging activity (Thoo et al., 2010; Wong et al., 2014).

5.4.2 Total Phenolic Content (TPC) Assay

Phenolic compounds are the secondary metabolites that can be found in plants which comprise an aromatic ring with a hydroxyl group to participate in antioxidation (Tungmunnithum et al., 2018). This makes it possible for dietary antioxidant intake to help maintain homeostasis (Aryal et al., 2019). In this study, the raw 80% methanol daikon radish roots extract exhibited 6.36 ± 1.52 μg GAE/g sample, whereas the boiled extract reported only 0.22 ± 0.30 μg GAE/g sample. Bors et al. (2015) mentioned that the total phenolic content (TPC) in different varieties of radish roots ranged between 4 – 5 mg GAE/g sample. On the other hand, Tsouvaltzis and Brecht (2014) discovered that the TPC in *Raphanus sativus* L. was at 0.14 – 0.21 mg GAE/g sample. Although the results in this study were lower compared to Bors et al. (2015) and Tsouvaltzis and Brecht (2014), the values corresponded with the findings of Hanlon and Barnes (2011), who discovered the phenolic contents of *Raphanus sativus* L. roots ranged between 2.0 – 22.6 μg GAE/g DW.

The differences in the results compared to the previous studies can be explained by different variations of the radishes used, which can significantly alter the result (Bors et al., 2015). Another possible explanation is the differences in the

solvents used for the sample extractions. Researchers have shown that the type of solvent greatly influences the phenolic compound extraction (Li et al., 2006; Do et al., 2014). Besides, the cultivating (Im et al., 2010) and storing (Tsouvaltzis and Brecht, 2014) conditions may also affect the TPC of a plant.

On the other hand, the boiled daikon radish showed a significantly lower value of TPC ($p = 0.002$). This may be due to the prolonged exposure of phenolic compounds to high temperatures during boiling. Hwang et al. (2012) measured the phenolic compound extracted from red pepper at different boiling times and reported a high loss of TPC at a longer boiling duration. Ismail et al. (2004) recorded a significant decrease in the phenolic compound after 1 minute of blanching using kale, spinach, cabbage, swamp cabbage and shallots as the samples. This shows that the phenolic compounds in plants are sensitive to thermal treatment. Hwang et al. (2012) further mentioned that a higher loss was recorded in the moist-heating method compared to dry-heating, which leads to a hypothesis that the phenolic compounds may leach into the water, causing a lower TPC to remain in the daikon radish. This result supported the findings of Francisco et al. (2010) who recorded a lower TPC in the Brassicaceae family after boiling.

The total phenolic content is strongly correlated with the antioxidant activities (Aryal et al., 2019). From the results obtained, a higher TPC value was reported in the raw daikon radish root extracts, which denotes a higher antioxidant activity. However, in the DPPH radical scavenging assay, the raw daikon radish

root extracts exhibited a lower antioxidant activity, which contradicted the higher TPC in the raw extract. As such, the antioxidant activities of the daikon radish may not be heavily dependent on the presence of phenolic compounds. Instead, the antioxidant activities are mostly accounted by other active components, which requires further analysis.

5.4.3 Ferric-reducing Antioxidant Power (FRAP) Assay

In the FRAP assay, a colourimetric redox reaction occurs when the antioxidant present in the daikon radish sample extracts reduces the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by electron transfer (Guo et al., 2003). The reaction produces a blue colour which can be measured at the absorbance of 593 nm (Shahidi and Zhong, 2015).

In this experiment, raw daikon radish roots extract possessed 0.36 ± 0.09 mmol Fe^{2+} E/g sample while boiled sample shown 0.47 ± 0.03 mmol Fe^{2+} E/g sample. There were no significant differences between the raw and boiled daikon radish roots extracts when the results were evaluated with the Student's T-test. This denotes that raw and boiled daikon radish contributed similar antioxidant activities in the FRAP assay. Furthermore, the reducing power of the sample extract is relatively low when compared to ascorbic acid which acted as a positive control. This shows the mild activity of FRAP in *Raphanus sativus*, and the results are in-line with the findings from Charoonratana et al. (2014), who

reported the high IC_{50} of the *Raphanus sativus* L. var. *caudatus* Alef in FRAP assay, ranging between 1225.12 – 6079.83 μg/mL.

5.5 Limitations of the Study

According to the National Center for Complementary and Integrative Health (2018), the essential minerals important to maintain good health includes calcium, chloride, cobalt, copper, fluoride, iodine, iron, magnesium, manganese, phosphorus, potassium, selenium, sodium, sulphur, and zinc. However, due to the unavailability of the appropriate analyser, there are some essential minerals that were left unanalysed in this study, including sodium, potassium, chloride, etc.

In addition, only one anti-inflammatory assay was used in this study. This is because of the unavailability of the reagent, which the ordering and delivery processes were delayed due to the COVID-19 pandemic. Besides the protein denaturation assay, there are other simpler and easier assays developed to examine the anti-inflammatory properties of the sample extracts to allow better analysis of the anti-inflammatory properties in different pathways. As this study only covered an assay, the results may not be conclusive for the anti-inflammatory properties of daikon radish.

Besides, the maximum concentration of the raw daikon radish roots extract used in the DPPH radical scavenging assay was 2000 μg/mL, which had its maximum activity at 47.87 ± 0.773%. The percentage of inhibition for the designed

concentration was not reaching 50%, which the half-maximal inhibitory concentration (IC_{50}) could not be determined. Hence, no comparison between the IC_{50} for the raw and boiled daikon radish roots extracts can be made.

Last but not least, 80% methanol was used as the only solvent to prepare the raw and boiled daikon radish roots extracts. This is prone to result variations as a single solvent may not be able to fully extract the bioactive compounds. The common solvent used in plant extraction includes polar, intermediate polar and nonpolar solvents (Abubakar and Haque, 2020) to maximise the extraction of the bioactive compounds. This aids in a more comprehensive evaluation of biological activities. Thus, multiple solvent extraction systems can be used to obtain a more comprehensive result.

5.6 Future Study

This study has allowed the understanding of the nutritional composition, anti-inflammatory activity and antioxidant properties of the raw and boiled *Raphanus sativus* subsp. *longipinnatus* roots. For future directions, liquid chromatography can be used to analyse the vitamin profile to expand the study of the health benefits of daikon radish.

Apart from that, further studies focusing on anti-inflammatory or antioxidant activities should be conducted to provide a more extensive study of the biological activities of the daikon radish. Examples of the anti-inflammatory assays include membrane lysis assay, cyclooxygenase and 5-lipooxygenase

inhibition assay (Sarveswaran et al., 2017). Other antioxidant assays can also be performed, such as the Oxygen Radical Absorbance Capacity (ORAC) assay, 2,2′-Azinobis-(3- ethylbenzothiazoline-6-sulfonic acid (ABTS) assay and more (Munteanu and Apetrei, 2021).

Furthermore, the assays could be repeated with different solvent extraction systems for a more comprehensive study. This allows comparable data between different solvents to be obtained. Multiple solvent extractions using hexane, and ethyl acetate can be used to extract the non-polar and polar compounds (Wilkinson, 1998).

Besides, to solve the problem that the IC_{50} for the raw daikon radish roots extracts could not be determined in the DPPH radical scavenging assay, a higher concentration range should be designed. For example, two-fold serial dilution downwards from 4000 μg/mL can be performed. This enables a higher scavenging activity to be recorded, hence the percentage of inhibition could exceed 50% for the determination of the IC_{50} for the raw daikon radish roots extracts.

Last but not least, as the boiling water may contain the leached protein and the liberated phenolic compounds, there may be some health benefits when consumed. As the boiled daikon radishes are commonly consumed together with soup, the boiling water of the daikon radish roots can be analysed to examine the nutritional values.

CHAPTER 6

CONCLUSIONS

In this study, the nutritional composition, anti-inflammatory and antioxidant properties of the raw and boiled daikon radish (*Raphanus sativus* subsp. *Longipinnatus*) roots were analysed. The boiled daikon radish roots had significantly higher ($p < 0.05$) moisture, ash and crude fibre contents, whereas the raw daikon radish roots had a statistically higher crude protein content. These showed that boiling significantly affects the moisture, ash, crude fibre and crude protein contents in the daikon radish. On the other hand, the crude fat content for both raw and boiled daikon radish roots did not show statistical differences, representing no effect of boiling on the crude fat content.

Among the 12 minerals analysed, 8 of them showed significant differences between raw and boiled daikon radish samples. In general, the boiled daikon radish has higher mineral contents including calcium, chromium, copper, iron, manganese, nickel and zinc. These may mostly be attributed to the bound properties of the minerals to protein in plants. Only magnesium was reported to be statistically higher in the raw sample. The presence of aluminium, cadmium, cobalt, and lead were not significant different between the raw and boiled daikon radish roots samples.

On the other hand, both raw and boiled daikon radishes exhibited anti-inflammatory and antioxidant properties. The boiled daikon radishes gave a higher anti-inflammatory and antioxidant capacities in PDA and DPPH radical scavenging assays although raw daikon radishes had a higher total phenolic content. This suggested that the main bioactive compound that participated in the antioxidant activities was not contributed by the phenolic compounds. In conclusion, the boiled daikon radish was considered a better source of dietary nutrition, anti-inflammatory agent and antioxidant compared to the raw daikon radish.

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APPENDIX A

CHEMICAL AND REAGENT PREPARATION

0.13 M Sulphuric Acid (H_2SO_4)

Approximately 500 mL of deionised water was added into a 1 L volumetric flask. A total of 6.92 mL of 98% concentrated H_2SO_4 was pipetted into the volumetric flask and the final volume was topped up to 1 L.

0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH)

One mL of 10 mM DPPH (39.4 mg of DPPH powder dissolved into 10 mL of methanol) was diluted to 50 mL in methanol. The reagent was kept in the dark by wrapping aluminium foil around the schott bottle.

0.23 M Sodium Hydroxide (NaOH)

Approximately 500 mL of deionised water was added to a 1 L volumetric flask. A total of 19.2 g of NaOH pellets were added into the bottle and stirred until fully dissolved. The solution is then topped up to 1 L.

1 M Hydrochloric Acid (HCl)

Approximately 200 mL of deionised water was added into a 500 mL volumetric flask. A total of 41.5 mL of 37% concentrated HCl was pipetted into the volumetric flask and the final volume was topped up to 500 mL with deionised water.

1% Bovine Serum Albumin (BSA)

The 1% BSA was prepared by dissolving 1 g of BSA powder into 100 mL of deionised water.

10 mM 2,4,6-tripyridyl-s-triazine (TPTZ)

The TPTZ solution was prepared by adding 0.31 g of TPTZ to 100 mL of 40 mM HCl.

100 mM Phosphate-buffered Saline (PBS) (pH6.3)

The PBS was prepared by adding 4 g of sodium chloride (NaCl), 0.1 g of potassium chloride (KCl), 0.72 g of sodium hydrogen phosphate and 0.12 g of potassium dihydrogen phosphate into 500 mL of deionised water. The pH was adjusted to 6.3 by using 1 M HCl.

2 mM Ferrous Sulphate Heptahydrate ($FeSO_4 \cdot 7H_2O$) Stock Solution

The $FeSO_4.7H_2O$ was weighed to 0.0278 g and dissolved in 50 mL of deionised water.

20 mM Iron (III) Chloride Hexahydrate ($FeCl_3.6H_2O$) Solution

The $FeCl_3.6H_2O$ was weighed to 0.054 g and dissolved in 10 mL of deionised water.

300 mM Acetate buffer (pH 3.6)

A total of 20.41 g of sodium acetate 3-hydrate powder was measured and dissolved in 200 mL of deionised water. The solution was adjusted to pH 3.6 by gradually adding glacial acetic acid. The solution is then topped up to 500 mL.

32% Sodium Hydroxide (NaOH)

Approximately 500 mL of deionised water was added to a schott bottle. A total of 320 g of NaOH pellets were added into the bottle and stirred until fully dissolved. The solution is then topped up to 1 L.

4% Boric Acid

The boric acid powder was weighed to 20 g and dissolved in 500 mL of deionised water.

700 mM Sodium Carbonate (Na_2CO_3)

The Na_2CO_3 powder was weighed to 3.7 g and dissolved in 50 mL of deionised water.

Copper-alkaline Reagent

The copper-alkaline reagent was prepared by mixing 10 mL of 0.16% potassium sodium tartrate in 1 N NaOH with 0.1 mL of 5% copper (II) sulfate-5- hydrate.