EFFECTS OF DIFFERENT COOKING METHODS ON THE ANTIOXIDANT ACTIVITIES OF *Hericium erinaceus* (BULL.: FR.) PERS.

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ABSTRACT

All living organisms require ongoing oxidation process where the carbohydrates, proteins and fats are metabolised by oxygen in order to generate energy. However, this continuous oxidation process in human system generates the production of unfavourable natural by-products called free radicals. Free radicals are highly reactive and are produced in large numbers and when the radicals outnumbered the healthy molecules, the imbalance will affect the antioxidant defence mechanism. Human body has been equipped with antioxidant system that protects the body against free radicals attacks. Apart from fruits, vegetables, whole grains as well as vegetable oil, the consumption of mushrooms in daily diet has become tremendously popular. The increasing awareness of mushrooms benefits among consumer provides significant reason for scientific study to be carried out in terms of their antioxidant properties. One of the mushrooms that are of interest is *Hericium erinaceus* or Lion's Mane Mushroom. The aim of the study was to investigate the effect of different cooking methods (boiling, microwaving, steaming and pressure-cooking) on antioxidant capacities of *H. erinaceus*. Besides total phenolic content determination, the antioxidant assay conducted were 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, reducing power assay, inhibition of lipid peroxidation of buffered egg yolk and Trolox equivalent antioxidant capacity (TEAC) assay. Both fresh and oven-dried samples subjected to 5 minutes of steaming significantly increased the DPPH radical scavenging activity. The reducing powers of all fresh and oven-dried samples increased steadily with the increasing concentrations of 0.5-5.0 mg/ml. In general, the cooking did not significantly enhance the lipid peroxidation inhibition for all tested samples. The same results were depicted in the TEAC assay where cooking did not affect the antioxidant activity significantly except for the samples submitted to 5 minutes of microwaving (fresh) and 5 minutes of pressure-cooking (oven-dried) by which their antioxidant activity was significantly ($p < 0.05$) increased. Most of the cooked samples investigated showed an elevated amount of total phenolic content compared to the respective uncooked sample. A weak correlation between the antioxidant activity and total phenolic content of samples tested indicated that the antioxidant activity of the mushroom was not due to the content of phenolic compounds. Since there were limited numbers of similar studies conducted, further study has to be carried out involving the same species of mushroom cooked under the same conditions for the purpose of data verification.

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ABSTRAK

Semua organisma hidup memerlukan proses pengoksidaan yang berterusan di mana karbohidrat, protein dan lemak dioksidakan oleh oksigen untuk menghasilkan tenaga. Namun, proses pengoksidaan yang berterusan di dalam sistem badan manusia menghasilkan produk sampingan semulajadi yang tidak diingini, iaitu radikal bebas. Radikal bebas mempunyai kereaktifan yang tinggi dan dihasilkan dalam kuantiti yang banyak. Apabila bilangan radikal tersebut mengatasi bilangan molekul sihat, ketidakseimbangan berlaku yang akan memberi kesan kepada mekanisma pertahanan antioksidan manusia. Tubuh badan manusia telah dilengkapi dengan sistem antioksidan yang melindungi tubuh badan daripada serangan radikal bebas. Selain daripada buah-buahan, sayur-sayuran, bijian penuh dan minyak sayuran, pengambilan cendawan dalam diet seharian semakin terkenal. Peningkatan kesedaran tentang kepentingan cendawan dalam kalangan pengguna menyebabkan lebih banyak kajian saintifik dijalankan untuk mengkaji kandungan antioksidanya. Salah satu cendawan yang menarik untuk dikaji adalah *Hericium erinaceus* atau 'Cendawan Berkepala Singa'. Matlamat kajian ini adalah untuk mengkajikesan perbezaan cara memasak (didihan, menggunakan ketuhar gelombang mikro, pengukusan dan menggunakan periuk bertekanan tinggi) terhadap kapasiti antioksidan *H. erinaceus*. Selain daripada penentuan jumlah kandungan fenolik, assai skavengan radikal 1,1-difenil-2-pikrilhidrazil (DPPH), assai kuasa penurunan, perencatan pengoksidaan lemak oleh kuning telur berpenimbal dan assai kapasiti pengoksidaan setara Trolox telah dijalankan. Kedua-dua sampel segar dan sampel kering yang telah dikukus selama 5 minit meningkatkanskavengan radikal 1,1-difenil-2-pikrilhidrazil (DPPH) secara signifikan. Kuasa penurunan bagi semua sampel segar dan sampel kering telah meningkat dengan peningkatan kepekatan sampel pada 0.5-5.0 mg/ml. Secara amnya, kaedah memasak yang dijalankan tidak meningkatkan perencatan pengoksidaan lemak secara signifikan dalam semua sampel yang diuji. Keputusan yang sama diperolehi dalam assai kapasiti pengoksidaan setara Trolox di mana kaedah memasak tidak memberi kesan yang signifikan kecuali terhadap sampel yang dipanaskan di dalam ketuhar gelombang mikro selama 5 minit (sampel segar) dan sampel yang dimasak di dalam periuk bertekanan tinggi selama 5 minit (sampel kering) di mana aktiviti antioksidan sampel tersebut meningkat secara signifikan ($p < 0.05$). Kebanyakan sampel yang telah dimasak menunjukkan pertambahan jumlah kandungan fenolik apabila dibandingkan dengan sampel yang tidak dimasak. Korelasi yang lemah antara aktiviti antioksidan dan jumlah kandungan fenolik menunjukkan bahawa aktiviti antioksidan bagi cendawan ini tidak bergantung kepada kandungan sebatian fenolik. Memandangkan pelaksanaan kajian seumpama ini adalah terhad, kajian seterusnya melibatkan spesis cendawan ini yang dimasak dengan kaedah yang sama perlu dijalankan bertujuan pengesahan data.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
<	Less than
=	Equal to
>	Larger than
±	Plus-minus
°C	Degree Celcius
µ	Micro
µg/ml	Micrograms per milliliter
µM	Micromolar
·OH	Hydroxyl radical
4-HNE	4-hydroxynenal
Abs	Absorbance
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AH	Antioxidant
ANOVA	One-way analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
DMRT	Duncan Multiple Range Test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
e.g	Example
ET	Electron transfer
ETC	Electron transport chain
FC	Folin-Ciocalteu
FRAP	Ferric reducing antioxidant power
g	Gram
GAEs	Gallic acid equivalents
HAT	Hydrogen atom transfer
HEP	*H. erinaceus* polysaccharides
ICRs	Imprinting control regions
LOOH	Lipid hydroperoxide
M	Molar
MDA	Malonaldehyde
mg	Miligram
mg/ml	Miligrams per milliliter
mins	Minutes
ml	Mililiter
mM	Milimolar
mRNA	Messenger ribonucleic acid
MRPs	Maillards's reaction products
NGF	Nerve growth factor
nm	Nanometer
NO·	Nitric oxide
$O_2^{\cdot}$	Superoxide anion
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical antioxidant capacity

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PBS	Phosphate buffer solution
R·	Radical species
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Rotation per minute
SD	Standard deviation
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	*tert*-butylated hydroxyquinone
TCA	Trichloroacetic acid
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TRAP	Total radical trapping antioxidant parameter
W	Watt
w/v	Weight for volume

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CHAPTER 1

INTRODUCTION

Antioxidant is the main focus in conducting this study because it is not just critical as human health-protecting factor, but also as our first line of defence against free radical damage. The main property of antioxidants is they are able to trap or scavenge highly reactive free radicals and oxygen species present in biological systems which existed from a wide variety of sources (Prakash, 2001). In human body, free radicals are by-products of the normal biochemical process. Free radicals are chemical species (atom, molecules or ion) that contain unpaired electrons; they seek out and capture electrons from other substances in order to neutralise themselves (Percival, 1998; Betteridge, 2000). Antioxidants in the human system are responsible in controlling and maintaining free radicals production. The common examples of free radicals being studied are hydroxyl radical, superoxide anion and nitric oxide. Other than ones that are produced in human body, external agents like cigarette smoke, air pollution, ultraviolet light as well as radiation could be sources of free radicals accumulation in human body. Due to excessive exposure to external factors or imbalance production of free radicals in human body, the antioxidant defences may encounter the deficiencies and lead to tissue damage, generally known as oxidative stress. This damage can occur in deoxyribonucleic acid (DNA), proteins and other macromolecules as well as may give rise to wide variety of diseases, for examples cancer, pulmonary dysfunction, diabetes, arthritis and inflammatory diseases, Parkinson's disease and many more (Percival, 1998). This is where antioxidants play an important role in maintaining one's health.

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In general, there are two types of antioxidants; natural antioxidants and synthetic antioxidants. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylated hydroxyquinone (TBHQ) which are usually used in food industry as food stabilizer appeared to be carcinogenic, thus their use has been restricted. Compared to the synthetic antioxidants, the natural antioxidants are much safer and they are abundantly available in our nature. Natural compounds produced by living organisms possess pharmacological and biological activities that are important in the treatment of life-threatening conditions and it is thought that medicines from natural sources may have fewer side effects (Khan *et al*., 2013).

Hericium erinaceus is an edible mushroom containing various medicinal benefits. It is commonly known as lion's mane mushroom, monkey head mushroom oras yamabushitake by Japanese. This mushroom belongs to the class Agaricomycetes in the phylum basidiomycota (Khan *et al*., 2013). This mushroom has been popular for hundreds of years in traditional Chinese and Japanese cuisine and also as herbal medicine to treat various human diseases (Wong *et al*., 2009). As in cuisine, it is normally cooked as a meat substitute and in Malaysia, some prefer to make it as 'satay' (roasted or grilled meat-like dish). Numerous studies were carried out in order to study various health beneficial roles possessed by this particular mushroom. *H. erinaceus* has been reported to have anti-cancer (Wang *et al*., 2001), mild cognitive impairment (Mori *et al*., 2009), anti-microbial (Wong *et al*., 2009) and also neuronal disease protecting activities (Mori *et al*., 2008). The polysaccharide components are believed to be the major effective components for its medicinal properties (Khan *et al*., 2013). Abdullah *et al*. (2012) and Wong *et al*. (2009) had conducted the study on the antioxidant activities of *H. erinaceus*. However, no specific

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research has been done to determine the influence of cooking on the antioxidant activity of *H. erinaceus*.

The effects of cooking towards the total phenolic content and antioxidant activity of edible mushrooms have not been well and extensively studied. The understanding and sensibility among consumer is rising on how the dietary of edible mushrooms may affect their health. This might be due to diverse information available regarding the nutritional contents and their medicinal benefits either from daily newspaper or from electronic media. Moreover, the growing numbers of career women in society contribute to the increasing demand on the information about the faster and effective way of cooking which is beneficial for them to optimise and efficiently manage their cooking time. Still, the proven scientific data is inadequate to educate consumer on how to cook those edible mushrooms without having to lose its nutritional contents and resulting in high retention of the antioxidant capacity. The information is necessary because the mushrooms cannot be eaten raw, which means the mushrooms need to be cooked prior to consumption.

Objectives of this study were to:

(i) evaluate the influence of various cooking methods on the antioxidant activity and total phenolic content of *H. erinaceus*; and

(ii) analyse the optimum mushroom's cooking method and duration resulting in the high retention of antioxidant capacity in the cooked mushroom.

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CHAPTER 2

LITERATURE REVIEW

2.1 Free Radicals and Oxidative Stress

Oxidation is the process where all living organisms (prokaryotic and eukaryotic) utilise oxygen in order to metabolise fats, proteins and carbohydrates for energy production, essentially in order to fuel the biological processes (Percival, 1998; Mau *et al*., 2002; Elmastas *et al*., 2007; Gan *et al*., 2013). This oxidation processes takes place continuously in human biological system, somehow generates the production of unfavourable by-products called free radicals. They are unavoidably formed as natural by-products of biochemical mechanisms and usually are produced in large numbers.

Free radicals can be defined as atom, molecule or ion that lack an electron (Percival, 1998) or contain single, unpaired valence electrons (McCord, 2000; Noori, 2012). Chemically, free radicals are highly reactive due to the existence of the unpaired electrons (Betteridge, 2000). They are capable to go around in human systems, capture electrons from other healthy molecules in order to neutralise themselves. Even they are neutralised in the first attack, a chain reaction mechanism is formed and producing second free radicals and following the process ultimately will generate thousands of free radicals in the systems (Percival, 1998). Free radicals may be divided into two major classes: oxygen-derived (ROS, reactive oxygen species) or nitrogen-derived (RNS, reactive nitrogen species) (Shalaby & Shanab, 2013).

ROS and RNS are the collective terms describing a group of oxidants that are highly reactive (Percival, 1998; Pham-Huy *et al*., 2008; Kunwar & Priyadarsini, 2011), which can be divided into two classes: free radicals and non radicals (Birben *et al*., 2012).

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Free radicals specifically referred to those reactive molecules that contain one or more unpaired electrons whereas non radical forms are created when two free radicals shared their unpaired electrons (Birben *et al*., 2012). Even though the reactivity of free radicals is stronger than the non radical species, they are less stable (Pham-Huy *et al*., 2008). The common examples of ROS are hydroxyl radical ($^{\bullet}OH$) and superoxide anion ($O_2^{\bullet -}$), produced from reduction of molecular oxygen during aerobic respiration (Betteridge, 2000), also induced by radiation (Cheung *et al*., 2003). RNS such as nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) are produced by vascular endothelium and other cells (Betteridge, 2000).

In biological cells, ROS/RNS are formed by several numbers of mechanisms. For instance, they are produced in the mitochondria as a result of oxygen consumption by electron transport chain (ETC). In addition, ROS/RNS are yielded when white blood cells engulf bacteria or viruses during phagocytosis mechanism. In endoplasmic reticulum, ROS/RNS are created by detoxification of toxic substances in the process known as xenobiotic metabolism (Percival, 1998; Noori, 2012). ROS and RNS may exert dual role. They display beneficial roles in physiological cell processes and immune function when they present at low or moderate level but in contrast, when they present at high concentrations, they may exhibit deleterious and adverse effects creating oxidative stress conditions (Pham-Huy *et al*., 2008; Birben *et al*., 2012). Other than free radicals that are overproduced within biological system, environmental and lifestyle factors may also dramatically contribute to the accumulation of free radicals such as exposure to pollution, cigarette smoke, radiation, heavy metal ions, stress and excessive alcohol intake (Percival, 1998; Birben *et al*., 2012; Gan *et al*., 2013).

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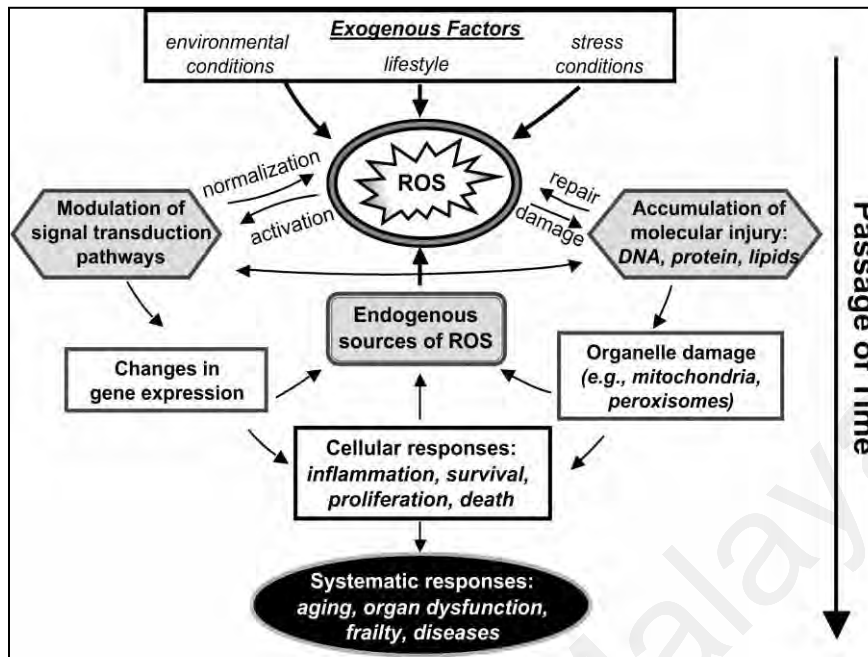

Figure 2.1: A schematic summary of proposed mechanism by which ROS and oxidative stress could contribute to the process of aging (Kregel and Zhang, 2007).

The imbalance in the production of free radicals against the antioxidant defence may lead to pathological condition known as oxidative stress, generally recognised as an important contributor to the serious cell damage (Betteridge, 2000; Shalaby & Shanab, 2013). The cell or tissue damage caused by oxidative stress may lead to the commencement of many chronic and degenerative illnesses namely cancer, rheumatoid arthritis, arteriosclerosis, as well as degenerative diseases associated with aging (Figure 2.1) (Halliwell & Gutteridge, 1984).

These free radicals can oxidise deoxyribonucleic acid (DNA), proteins, lipids, nucleic acids and enzymes resulting in cellular damage and alteration of functions (Percival, 1998; Birben *et al*., 2012). According to Yoshikawa and Naito (2002), oxidative stress can be defined as “a state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of the balance between them”. The oxidant: antioxidant balance may be shifted or disturbed either by the increasing of oxygen species production or when

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levels of antioxidants are diminished (Figure 2.2) (Shalaby & Shanab, 2013). Regulation of reducing and oxidising (redox) mechanisms in biological system is important for cell viability, activation, proliferation and organ function (Birben *et al.*, 2012). The examples of diseases associated with oxidative stress are shown in Figure 2.3.

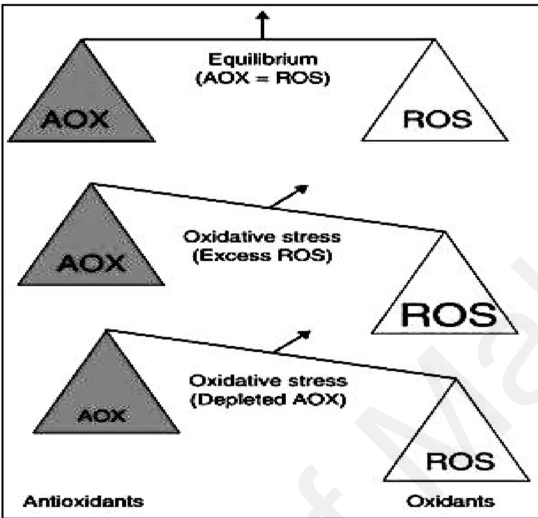

Figure 2.2: Balance between oxidant and antioxidant defines oxidative stress (Kunwar and Priyadarsini, 2011).

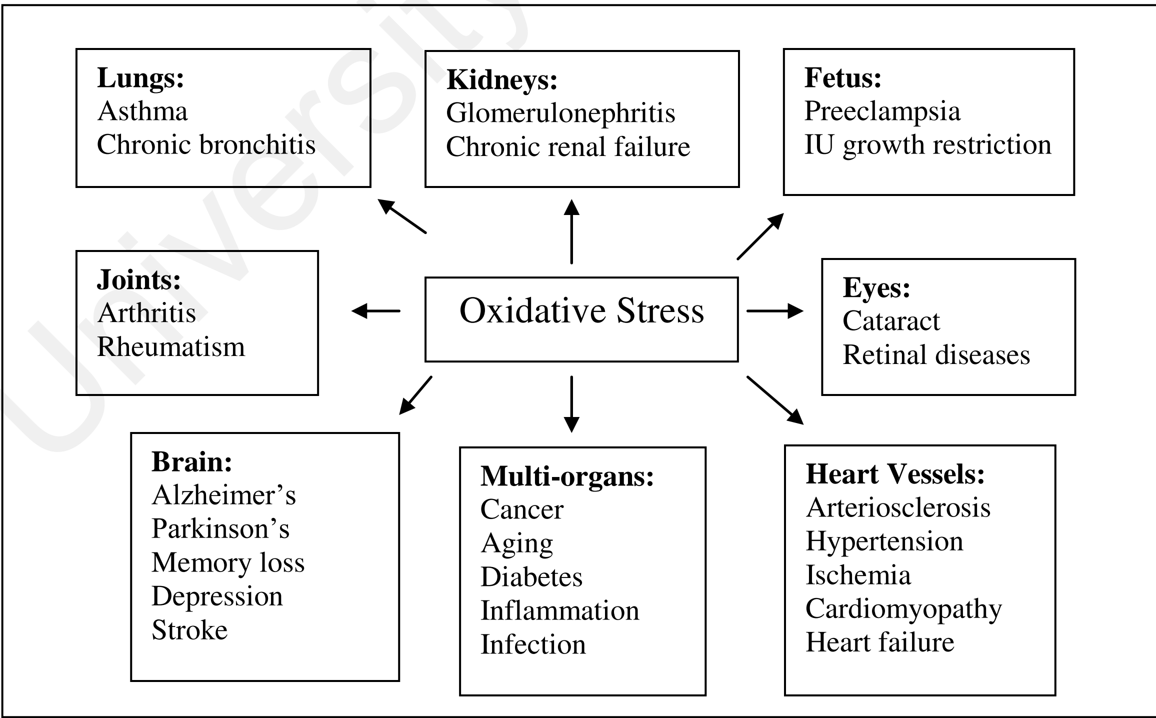

Figure 2.3: Oxidative stress-induced diseases in humans (Pham-Huy *et al.*, 2008).

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2.2 Antioxidants

Naturally, the human body has been equipped with highly sophisticated systems for the protection against the free radicals attack, known as antioxidant system. The system serves to counterbalance the effects of oxidants, performs the function of neutralising the free radicals excess, protects the cells against their toxic effects and also contributes to disease prevention (Pham-Huy *et al*., 2008; Birben *et al*., 2012). According to Noori (2012), antioxidants are chemical compounds that consists monohydroxy/polyhydroxy phenol by which they have low activation energy to donate hydrogen atom, and hence cannot trigger the production of second free radicals which made them stable and ultimately slow down the oxidation step. Further, according to Kohen and Nyska (2002), antioxidants are substances available or present at low concentrations and significantly delay or prevent oxidation of the oxidisable substrate where variety of compounds can act as antioxidants and establish maximum protection for biological sites.

Antioxidants can be classified into three categories based on their mode of mechanism; (i) primary antioxidants involved in the prevention of oxidants formation; (ii) secondary antioxidants displays the scavenging activity of ROS/RNS; and(iii) tertiary antioxidants repairs the oxidised molecules from sources like dietary or consecutive antioxidants (Noori, 2012). Primary and secondary antioxidants fall in the category of endogenous antioxidants, whereas the tertiary antioxidants fall under exogenous antioxidant system. Both endogenous and exogenous systems work synergistically. They function and cooperate to counteract free radical attacks and normalise the redox state during oxidative stress (Kunwar & Priyadarsini, 2011).

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2.2.1 Endogenous Antioxidants

Endogenous or metabolic (molecules that are formed by metabolism in the body) antioxidants, can be classified into antioxidant enzymes (enzymatic) and small antioxidant (non-enzymatic) (Pham-Huy *et al*., 2008; Noori, 2012). The examples of primary antioxidant enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx) through which they work by catalysing free radicals quenching reactions (Percival, 1998; Noori, 2012). Secondary antioxidant enzymes such as glutathione reductase, glucose-6-phosphate dehydrogenase and glutathione-S-transferase, perform directly to detoxify ROS/RNS by lowering the peroxides level. Alternatively, they also constantly supply NADPH and glutathione to ensure and preserve the proper functioning of primary antioxidant enzymes (Noori, 2012). On the contrary, low-molecular-weight compounds like lipoic acid, ubiquinone (coenzyme Q10), melatonin, uric acid and metal binding proteins are some examples of non-enzymatic endogenous antioxidants (Pham-Huy *et al*., 2008; Birben *et al*., 2012). Ferritin, lactoferrin and albumin are some examples of metal binding proteins that have the capability to sequester free iron and copper ions from catalysing oxidative reactions (Percival, 1998).

2.2.2 Exogenous Antioxidants

Exogenous antioxidants are compounds that cannot be produced by metabolic process and can only be catered through foods and supplementation (Pham-Huy *et al*., 2008). Exogenous or nutrient antioxidants are required to restore the redox homeostasis in cells in order to maintain optimal body function (Kunwar & Priyadarsini, 2011). The protective character played by endogenous antioxidant defence systems is usually insufficient to completely prevent oxidative stress-induced damage (Reis *et al*., 2012).

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Moreover when aging processes takes place, this antioxidant protections may disturb and becomes unbalanced leading to the deterioration of physiological functions (Mau *et al*., 2002; Elmastas *et al*., 2007). This is the part where the intake of exogenous antioxidant source from the antioxidant-rich food plays an important role in maintaining the human health and well-being by providing support to the endogenous antioxidant defence system (Yang *et al*., 2002; Barros *et al*., 2007; Reis *et al*., 2012).

Exogenous antioxidants are non-enzymatic antioxidants, mainly derived from daily food intake and other dietary sources (Percival, 1998; Noori, 2012). In this modern day, antioxidant supplementations are abundantly available and their consumption becomes increasingly popular among consumer. The fresh sources of exogenous antioxidants are accessible in several forms, for instance, herbs, spices, vitamins, vegetables, foods and vegetable oil. It is familiar to us that fresh fruits and vegetables are great sources of antioxidants. Many researches had been carried out in determining the antioxidant activity of vegetables like *Brassica* vegetables, artichoke, cauliflower, beetroot, green bean and more (Wachtel-Galor *et al*., 2008; Jiménez-Monreal *et al*., 2009).

The most known and favourite dietary antioxidants are vitamin C, vitamin E and β-carotene which can be found in wide number of fruits, vegetables, whole grains as well as vegetable oil. Vitamin C is a water-soluble antioxidant and functioning by neutralising ROS in the aqueous phase before the commencement of lipid peroxidation. In contrast, vitamin E is a lipid-soluble antioxidant that works by protecting membrane fatty acid from lipid peroxidation (Percival, 1998).

In addition, polyphenolic or phenolic compounds is an exogenous antioxidant compounds which reported to attribute to the antioxidant activity of plant-derived extracts

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(Lugasi *et al*., 2003). Polyphenols is one of the major group of non-essential dietary components (Saad *et al*., 2014). Polyphenols contain multiple hydroxyl groups which made them an effective ROS scavengers and metal chelators (Kunwar & Priyadarsini, 2011). Phenolic compounds are produced as accumulated end products from shikimate and acetate pathways (Pushpa *et al*., 2012). This compound can be divided into two main classes, flavonoids and phenolic acids. Flavonols, flavanones, isoflavonoids and flavones are most common subclasses of flavonoids whereas hydroxy-cinnamic acid and hydroxy-benzoic acid are subclasses of phenolic acid (Shalaby & Shanab, 2013). Flavonoids is the most common and widely distributed subgroup which appears to function as "biological response modifiers" in human while in plants, they serve as protectors against a wide variety of environmental stresses (Percival, 1998). In mushrooms, phenolic acid is reported to be the main phenolic compound that attributes to their antioxidant activities (Ishikawa *et al*., 1984).

2.3 Natural Antioxidants vs. Synthetic Antioxidants

The intake of antioxidants from natural sources is more preferred over the synthetic antioxidants because the synthetic one such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) appeared to possess carcinogenic properties (Cheung *et al*., 2003) and pose several side effects such as hepatotoxicity (Acharya *et al*., 2013). The use of them has been restricted especially in food industry. BHA and BHT as well as *tert*-butylated hydroxyquinone (TBHQ) are phenolic compounds, normally used in food industry to prevent oxidative deterioration of fats and oils in foods (Daker *et al*., 2008). They are petroleum derived and formed as by-product in the petroleum refining process. Research conducted shows that high doses of these ingredients may cause crucial damage

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to lungs, liver and kidney. Besides, they also have toxic effects on the body's blood coagulation system if orally consumed (Shalaby & Shanab, 2013).

Natural products are the chemical compounds or substances produced by living organisms that are found in nature which usually have pharmacological or biological activities (Khan *et al*., 2013). In order to cater the demand of natural antioxidants, numerous studies have been conducted to determine the antioxidant activities of natural products. This includes fruits, vegetables, cereals, tea and mushrooms. The vegetables studied were broccolli (Wachtel-Galor *et al*., 2008; Jiménez-Monreal *et al*., 2009; Chipurura *et al*., 2010), asparagus (Jiménez-Monreal *et al*., 2009), purple skin eggplants (Chumyam *et al*., 2013), cabbage (Wachtel-Galor *et al*., 2008; Chipurura *et al*., 2010) and spinach (Ismail *et al*., 2004; Turkmen *et al*., 2005). Plant foods are good sources of antioxidant substances, mainly polyphenolic compounds (flavonoids and phenolic acids), carotenoids, tocopherol and ascorbic acid which reported to exhibit free radical scavenging activity (Elmastas *et al*., 2007), inhibit lipid peroxidation (Cheung & Cheung, 2005) and able to chelate metals (Saad *et al*., 2014). Natural antioxidants are preferred compared to the laboratory synthesized antioxidants as they carrying fewer side effects in the treatment of life-threatening conditions (Khan *et al*., 2013) and as a less harmful alternative to synthetic antioxidants (Daker *et al*., 2008).

2.4 Mushrooms as Antioxidants

The consumption of mushrooms in daily diet becomes common and popular in our society and tremendously draws much attention to the researchers to study their significance and value in human life. Other than fresh fruits and vegetables, the use of mushrooms in the cuisine is also preferable similarly because they are natural antioxidants.

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In addition to its great texture and flavour, the nutritional properties and its medicinal effects also play an important role in cultivating the great interests among consumer (Cheung *et al*., 2003; Elmastas *et al*., 2007; Sudha *et al*., 2012; Vamanu & Nita, 2013) and because of that, it is considered as functional food (Tan *et al*., 2015). Further, according to Elmastas *et al*. (2007) and Mujić *et al*. (2010), mushrooms also considered as therapeutic food as they were reported to be useful in prevention of some critical illness such as hypertension, cancer and hypercholesterolemia. The investigators also believed that the growing concern among consumer on the natural products or traditional medicines is because they are the promising source of new therapeutics (Khan *et al*., 2013).

It has been indicated that mushrooms are poor in calories and fat while rich in vegetable proteins, chitins, vitamins and minerals (Manzi *et al*., 1999). Mushrooms have long history of usage in traditional Chinese cuisine and medicine (Yang *et al*., 2002). Various secondary metabolites accumulate in the mushrooms are believed to be responsible for their functional properties such as phenolic compounds, polyketides, steroids and terpenes (Cheung *et al*., 2003; Sudha *et al*., 2012; Gan *et al*., 2013). Out of those secondary metabolites, phenolic compounds are most widely distributed and they were found to be an excellent contributor for the antioxidant properties in mushrooms (Cheung *et al*., 2003; Wong *et al*., 2009).

Many studies have reported the antioxidant properties of several wild as well as cultivated edible mushrooms such as *Hericium erinaceus* (Mau *et al*., 2002; Wong *et al*., 2009), *Agaricus bisporus* (Elmastas *et al*., 2007; Reis *et al*., 2012), *Pleurotus* spp. (Sudha *et al*., 2012; Reis *et al*., 2012; Acharya *et al*., 2013), *Lentinula edodes* (Cheung *et al*., 2003; Mujić *et al*., 2010) and *Ganoderma tsugae* Murrill (Mau *et al*., 2005).

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2.5 Lion’s Mane Mushroom (*Hericium erinaceus*)

Hericium erinaceus is an edible mushroom that possesses medicinal properties (Khan *et al*., 2013). This edible fungus has long history of usage in traditional Chinese medicine. This mushroom also known asLion’s Mane Mushroom or Hedgehog Mushroom belongs to the family of Hericeaceae and class of Agaricomycetes under the phylum basidiomycota (Khan *et al*., 2013). In Japan and China, they are called “yamabushitake” and “hóu tóu gū” (monkey head mushroom), respectively (Yang *et al*., 2003). The ecological nature of this mushroom is saprobic and parasitic which fruit from the wounds of living hardwoods and consists of one, unbranched clump with white colour fruit body (Kuo, 2003). Instead of having traditional cap, this particular mushroom appears to have a large clump of teeth, which are spine-like structures a few millimeters long.

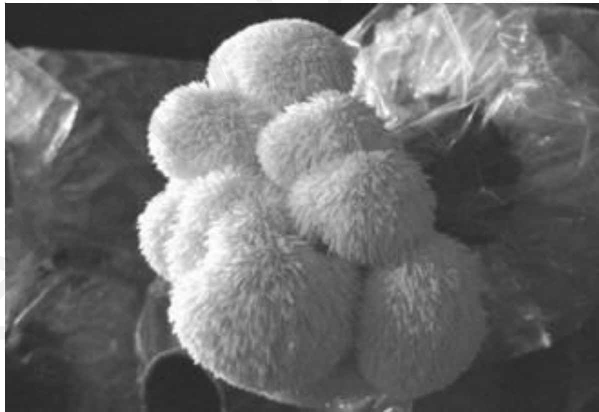

Figure 2.4: Fruit body of *Hericium erinaceus*
(Mushroom Research Centre, University of Malaya).

During ancient days in China, this mushroom was only reserved for royal families, normally prescribed for stomach ailments as well as cancer prevention. Nowadays lion’s mane is often cooked as pork or lamb replacement in Chinese cuisine. Also, when cooked, lion’s mane has a seafood-like flavour and can taste like lobster or shrimp (Stamets, 2005). This special feature could provide an alternative for vegetarian or person who is allergic to

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shellfish to replace the seafood with lion's mane in the meal. The best way to cook this mushroom is by caramelised it in olive oil, deglazed with saké wine and lastly with the butter as finishing. Lion's mane can be bitter if not cooked until crispy along the edges (Stamets, 2012). All edible mushrooms in *Hericium* genus need to be cooked slowly as they are tough and watery mushrooms that require longer periods of heating in order to cook off moisture and make them chewy.

Numerous constituents are present in its fruit body such as polysaccharides, proteins, phenols, hericenones, erinacines and terpenoids (Mizuno, 1999; Wong *et al*., 2009) that beneficially contribute to its properties. Investigations conducted few decades backrevealed that *H. erinaceus* possesses anti-cancer activities by a mechanism called immuno-modulation (Khan *et al*., 2013). In 2001, Wang and co-workers carried out an experiment to evaluate the anti-tumor and immuno-modulation activity of the polysaccharides extracted from the culture broth of *H. erinaceus*and *H. laciniatum* in mice with imprinting control regions (ICRs). The results indicated that both polysaccharides had significant anti-artificial pulmonary metastatic tumor effects. Further, there were a few studies carried out to show the activity of promoting nerve growth factor (NGF) synthesis by the hericenones and erinacines compounds that exist in the fruit body and mycelium (Mori *et al*., 2008; Ma *et al*., 2010). Mori *et al*. (2008) reported that the ethanol extract of *H. erinaceus* promoted nerve growth factor mRNA expression in a concentration-dependent manner via the activation of the JNK pathway. In 2013, Han *et al*. revealed that *H. erinaceus* polysaccharides (HEP) can significantly decrease lipid peroxidation level and increase antioxidant enzymes activities in experimental mice.

In the aspect of antioxidative properties, Wong *et al*. (2009) and Abdullah *et al*. (2012) reported that mycelium extract of *H. erinaceus* is rich in phenolic contentand has

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potential ferric reducing antioxidant power while the fresh fruit body extract was found to have thepotent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Both studies also demonstrated that the total phenolic content and total antioxidant activity in the oven-dried fruit body extract was higher compared to the freeze-dried or fresh fruit body extract.

2.6 Influence of Heat Treatment on Antioxidant Activities of Natural Products

Hericium erinaceus is a high water content mushroom that requires longer period of heating or cooking in order to cook off moisture and make them chewy. The purpose of determining the antioxidant activity of cooked *H. erinaceus* is because the mushroom needs to be processed or heat-treated prior to consumption. This is for the purpose of safety and quality. Cooking can increase their shelf-life because mushrooms are perishables (Tan *et al*., 2015). Mushrooms cannot be eaten raw and many are cooked before being consumed (Sun *et al*., 2014). The cell wall of mushrooms is not very digestible unless they are heat-treated (Wells, 2006). According to Barros *et al*. (2007), cooking affects the mushroom's chemical and nutritional characteristics. Similarly, Choi *et al*. (2006) and Saad *et al*. (2014) stated that the flavours and medicinal properties of mushrooms are well-enhanced by various processing or cooking methods. Antioxidant activities of mushrooms are closely linked not only to species but also to cooking methods (Sun *et al*., 2014).

The research on how heat or cooking procedures may influence the antioxidant activities is largely depends on several factors. For examples, cooking procedure, degree of heating, leaching into the cooking medium, solvent used for extraction, pH and surface area exposed to water and oxygen (Wachtel-Galor *et al*., 2008). In a study by Wachtel-Galor *et al*., 2008, steaming appeared to be the only method retained the total phenolics content in

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the vegetables cooked compared to microwaved and boiled vegetables. It may be that the lower temperature during steaming did not affect the phenolic content. As in microwaving and boiling, the depletion of total phenolics content might be due to the phenolics breakdown during cooking. Additionally, they claimed that cooking in water seems to cause a leakage of vegetables antioxidants into the cooking water and this effect increases with cooking time. This report is supported by Jiménez-Monreal *et al*. (2009) in which they stated that the antioxidant activity of vegetables submitted to microwave heating are higher than those subjected to boiling. This happened because no water was added during microwave treatment, thus the ascorbic acid or other antioxidants were not released from cooked tissue into the cooking water. The particular effects of temperature, pressure and moisture for each cooking methods on the antioxidant activities on natural products are to be explored further as numerous studies conducted before explained these in general manner.

Quite a number of studies have been done to evaluate the effects of heat treatment towards the retention of total phenolic content as well as antioxidant activities of natural products. In 2002, Dewanto and co-workers performed a study of antioxidant activities of processed sweet corn. They stated that vitamin C in apples has been found to contribute <0.4% of total antioxidant activity, indicated that most of the activity comes from the natural combination of phytochemicals. Hence, they suggested that processed fruits and vegetables may retain their antioxidant activity even they lost their vitamin C. The results of the study pointed out that thermal processing at 115°C for 25 minutes significantly raised the total antioxidant activity of sweet corn by 44%. Jeong *et al*. (2004) reported that the heating temperature and duration of treatment significantly increased the antioxidant activity of citrus peels extracts. The heat treatment may liberate the low molecular weight

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phenolic compounds that increase antioxidant activity. Subsequently, they concluded that the heating process can be used as a tool to increase the antioxidant activity of citrus peels.

The influence of heat treatment on the antioxidant activities and polyphenolic contents of Shiitake (*Lentinus edodes*) mushroom was investigated by Choi and co-workers in 2006. After raw Shiitake subjected to heat at 100 and 121°C respectively for 15 and 30 minutes by using an autoclave, the antioxidant activities of the extracts were measured using ABTS radical cation decolourisation assay and DPPH radical scavenging assay. The outcome showed that the ABTS and DPPH radical scavenging activities were increased by 2.0-fold and 2.2-fold in comparison to raw sample, respectively. The results revealed that the polyphenolic content and antioxidant activities of Shiitake mushroom increased as heating temperature and time increased. In contrast, Barros *et al*. (2007) reported that the cooked samples of Portuguese wild edible mushrooms proved to have lower nutrient concentrations and lower antioxidant activities in relation to either dried or frozen samples.

The effects of cooking on antioxidant activities and polyphenol content of edible mushrooms commonly consumed in Thailand were evaluated by Kettawan *et al*. (2011). They analysed the effects of boiling on antioxidant activities of ten edible mushroom varieties through polyphenol content assay together with FRAP, DPPH and ORAC (oxygen radical antioxidant capacity) assay. From the results obtained, it showed that the boiling process significantly decreased both antioxidant activities and polyphenol content in all tested mushrooms. They also stated that the difference of characteristics, structures and shapes of each mushroom variety could affect the loss of antioxidant activities and polyphenol contents. Consuming boiled mushroom tissue along with their broths to retrieve some polyphenol compounds and antioxidant activity is well recommended. The finding by

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Kettawan *et al*. (2011) was supported by a study carried out by Sun *et al*., (2014) by which the boiling significantly decreased the contents of total phenolics of four *Boletus* mushrooms tested. The investigation showed that microwaving was better in retention of total phenolics than other cooking methods (steaming, pressure-cooking, frying and boiling). The effects of different cooking methods on phenolic acid profiles of *Boletus* mushrooms displayed varieties with both the species of mushrooms and the cooking methods.

2.7 Antioxidant Activity Assays

In every studies involving antioxidant activities determination, few assays or methods were carried out in order to quantify the results. Researchers will be able to make the comparison on the antioxidant activities of the multifunctional food or biological antioxidants, resulted from the same assay; thus further evaluation and validation can be made. However, it is difficult to correlate the results from different assays as different assays act through different mechanisms (Frankel & Meyer, 2000).

The antioxidant assays can be characterized into two main categories based on the chemical reactions involved: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET) reactions. HAT-based assays mostly monitor competitive reaction kinetics where the quantitation is derived from the kinetic curves where in contrast, ET-based assays measure the capacity of an antioxidant to reduce the oxidant which can be monitored or observed through colour changes. The examples of HAT-based assays are inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP) (Huang *et al*., 2005).

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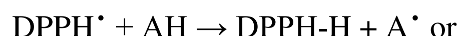
In this study, the assays that will be carried out to evaluate and measure the antioxidant activities of the uncooked and cooked mushrooms (*Hericium erinaceus*) are ET-based assays. The assays involved the monitoring of colour changes where the sample's antioxidant concentrations will influence the degree of colour changes (Huang *et al*., 2005). Likewise, according to Re *et al*. (1999), the influences of both antioxidant concentration and duration of reaction on the inhibition of the radical cation absorption are taken into consideration when determining the antioxidant activity.

2.7.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)Radical Scavenging Activity

This widely used decolourisation assay was first reported in 1995 by Brand-Williams and co-workers. Some extracts known to possess antioxidative properties were allowed to react with a stable radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) in a methanol solution to test their antioxidant activities. DPPH is commercially available long-lived organic nitrogen radical with a deep purple colour and have UV-vis absorption maximum at 515 nm, in its radical form (Brand-Williams *et al*., 1995; Huang *et al*., 2005; Reis *et al*., 2012). This assay is popular for its simplicity and provides a rapid test (Prior *et al*., 2005).The reduction of DPPH radicals is monitored by the decrease in its absorbance where in its radical form, they absorbs at 515 nm and the absorption disappears upon reduction by an antioxidant (AH) or a radical species ($R^{\bullet}$) as illustrated by Brand-Williams *et al*. (1995).



$DPPH^{\bullet} + AH \rightarrow DPPH\text{-}H + A^{\bullet}$ or

$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH\text{-}R$

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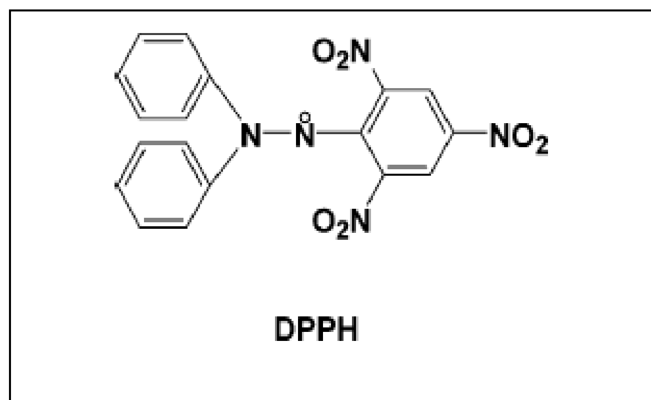

Figure 2.5: Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Huang *et al*., 2005).

2.7.2 Reducing Power Assay

The reducing power ability assay published by Oyaizu (1986) stated that reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. This assay is based on the ability of phenolics to reduce yellow ferric form to blue ferrous form by the action of electron-donating antioxidants (Benzie *et al*., 1999). Antioxidants serve as reductants and cause the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form, Fe^{2+}. Later, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm spectrophotometrically (Öztürk *et al*., 2007). In this assay, the reduction of $Fe^{3+}$to $Fe^{2+}$is caused by the ability of the mushroom extracts to donate the electrons. The increasing absorbance at 700 nm indicates an increase in reductive ability of the tested antioxidant compounds (Mau *et al*., 2002; Mujić *et al*., 2010; Acharya *et al*., 2013).

2.7.3 Inhibition of Lipid Peroxidation of Buffered Egg Yolk

Cellular membranes are vulnerable to the oxidation by ROS due to the presence of high concentration of unsaturated fatty acids in their lipid components. ROS reactions with membrane lipids stimulate lipid peroxidation, develop the generation of lipid hydroperoxide (LOOH) which can further decompose to an aldehyde such as

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malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) (Kunwar & Priyadarsini, 2011). Lipid peroxidation briefly can be defined as the oxidative degradation of lipids. Lipid that contains unsaturated fatty acids with more than one double bond is particularly susceptible to action of free radicals by which free radicals can steal electrons from the lipid in cell membrane. This resulting reaction disrupts biological membrane and therefore is highly deleterious to their structure and function (Devasagayam *et al*., 2003). Noori (2012) stated that cellular macromolecules such as nucleic acids, proteins and lipids can be affected and damaged due to the oxidative stress. Among these targets, the peroxidation of lipids is particularly more damaging because the formation of lipid peroxidation leads to a facile propagation of free radical reactions. According to Cheung and Cheung (2005), lipid peroxidation is a major cause of food deterioration which affects colour, flavour, texture as well as the nutritional value. Other than affecting the food, lipid peroxidation also has been implicated in the pathogenesis of several disease and clinical conditions namely diabetes, premature birth disorders, Parkinson disease, Alzheimer disease and some more (Devasagayam *et al*., 2003).

Since lipid peroxidation has major effects in human health, a lot of researches have been employed to extensively study the relationship of lipid peroxidation with disease, modulation of antioxidants and other contexts. During the process, large numbers of by-products were formed, in which their formation can be measured by various assays. The widely used method to measure the formation of by-products is TBARS (thiobarbituric acid reactive substances) assay, involving the estimation of aldehydic products (MDA - malondialdehyde) by their ability to react with thiobarbituric acid (TBA). The reaction is induced by $Fe^{2+}$and yield TBARS (Devasagayam *et al*., 2003). TBARS is the secondary products of lipid peroxidation (Daker *et al*., 2008). The results will be determined by

measuring the colour intensity of resulted pink coloured species, spectrophotometrically at 532 nm. Devasagayam *et al*. (2003) stated that though this assay is sensitive and widely performed, it is not specific and TBA also reacts with number of components present in biological samples. In this study, egg yolk was used as source of lipid as it was cost effective, readily available and convenient to handle (Kuppusamy *et al*., 2002).

2.7.4 Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox Equivalent Antioxidant Capacity (TEAC) assay or also been known as ABTS radical cation decolourisation assay is a widely used technique for antioxidant activity screening applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds as well as food extracts . TEAC assay was first reported by Miller *et al*. (1993) and later improved by Re *et al*. (1999). The mechanism of this assay is that the pre-formed radical monocation of blue/green $ABTS^{\cdot+}$(2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) chromophore is formed by oxidation of ABTS with potassium persulfate. The antioxidant capacity is determined by measuring the decrease of blue colour intensity of $ABTS^{\cdot+}$ radical after being reduced by hydrogen-donating antioxidants (Re *et al*., 1999). The radical which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm is generated directly in a stable form prior to reaction with putative antioxidants. Upon the addition of antioxidants, the extent of decolourisation as percentage inhibition of the $ABTS^{\cdot+}$radical cation will be determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard, under the same conditions (Re *et al*., 1999). The advantages of this assay are inexpensive, easy to operate and fast reaction (Zulueta *et al*., 2009).

2.8 Determination of Total Phenolic Content

Phenolic content quantification that is commonly carried out is total phenols assay by Folin-Ciocalteu (FC) reagent. This assay is convenient, simple and reproducible and has become a practise in studying phenolic antioxidants (Huang *et al*., 2005). Early developments of this assay were reported by Singleton and Rossi (1965) and Slinkard and Singleton (1977). The total phenolic content assay in actual does not reflect its name but measures a sample's reducing capacity and the reaction occurs through electron transfer (ET) mechanism (Huang *et al*., 2005). FC reagent is the mixture reagents of phosphomolybdic and phosphotungstic acid that is nonspecific to phenolic compounds and can also be reduced by many nonphenolic compounds such as vitamin C (Abdullah *et al*., 2012). Phenolic compounds react with FC reagent only under basic conditions which is adjusted by the addition of sodium carbonate solution (Huang *et al*., 2005) and will give the blue colour to the reaction mixture. Upon the addition of antioxidants or phenolic compounds, the reaction mixture will turn to yellow and the absorbance of the reaction mixture will be recorded spectrophotometrically at 750 nm.

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CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Chemicals and reagents were of analytical grade. Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), phosphate buffer saline (PBS) tablets, trichloroacetic acid (TCA), thiobarbituric acid (TBA), iron (II) sulphate heptahydrate ($FeSO_4$), potassium ferricyanide ($K_3[Fe(CN)_6]$), iron (III) chloride ($FeCl_3$), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) ($C_{18}H_{24}N_6O_6S_4$), potassium persulfate ($K_2S_2O_8$), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox) ($C_{14}H_{18}O_4$) were purchased from Sigma-Aldrich® Inc., USA. Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO) and methanol (CH_3OH) were purchased from Merck.

3.2 Mushroom samples and cooking methods

The fresh fruit bodies of *Hericium erinaceus* (Bull.:Fr) Pers. were purchased from Ganofarm Sdn. Bhd. in Tanjung Sepat, Selangor. The mushrooms were cleaned and washed with tap water to remove dirt and dried on tissue paper prior to weighing. The mushrooms were divided into two parts: fresh fruiting bodies and oven-dried fruiting bodies. The portions for oven-dried fruiting bodies were dried in 45°C oven for 48 hours before being subjected to cooking. Uncooked fruiting bodies were used as a positive control. Each of the 150 g fresh fruiting bodies portion was cut into smaller pieces and subjected to four different cooking methods at different time duration (5 and 10 minutes).

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3.2.1 Boiling

The mushrooms were boiled in distilled water at the ratio of 1:10 (w/v) on hot plate. The beaker was covered with an aluminium foil to minimise the evaporation of water. The procedure was carried out for 5 minutes and 10 minutes, respectively.

3.2.2 Microwaving

The mushrooms were cooked in distilled water at the ratio of 1:10 (w/v) using a commercial 1000 W microwave for 5 minutes and 10 minutes, respectively. The beaker was covered with an aluminium foil to minimise the evaporation of water.

3.2.3 Steaming

The mushrooms were steamed on a tray in a domestic steam cooker. The procedure was carried out under atmospheric pressure for 5 minutes and 10 minutes, respectively.

3.2.4 Pressure-cooking

The mushrooms were pressure-cooked using laboratory autoclave at 121°C and 100 kPa in distilled water at the ratio of 1:10 (w/v). The beaker was covered with an aluminium foil to minimise the evaporation of water. The procedure was performed for 5 minutes and 10 minutes, respectively.

3.3 Preparation of extracts

All the cooked samples in the cooking water were cooled to room temperature. For the uncooked samples, the mushrooms were mixed with distilled water at the ratio of 1:10 (w/v). All samples were then homogenised using Waring Commercial Blender (New Hartford, CT, USA). The residues were removed with a filter and air pump (Model DOA-

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P104-BN, GAST Manufacturing Inc.)and the resulted water filtrate were filtered through Whatman no. 1 filter paper. The filtrates were centrifuged at 5000 rpm for 15 minutes (Beckman J2-MI) at room temperature. The resulting clear supernatants were freeze-dried (Christ Alpha 1-4 LD plus) and the dried water extracts were stored at 4°C prior to assays.

3.4 Antioxidant Activity Assays

3.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)Radical Scavenging Activity

The DPPH free radical scavenging activity of the extracts was measured according to the method described by Brand-Williams *et al*. (1995) with some modifications. The assay was carried out in 96-well plate. Five microlitre of each extract (3 mg/ml) was mixed with 195 µl of freshly prepared 0.06 mM methanolic DPPH solution (0.4728 g of DPPH mixed with 20 ml methanol). All samples were assayed in triplicates. To prevent evaporation, the plate was covered with aluminium foil. The reaction mixtures were shaken and left to stand for 3 hours in the dark condition. The absorbance reading recorded at 515 nm using ELISA Plate Reader (Sunrise Basic Tecan). BHA was used as the standard, methanol as blank and methanolic DPPH solution without the mushroom extract served as negative control. The radical scavenging activities were expressed as percentage of DPPH radical quenched (%), calculated using the following equation:

$$\text{Radical scavenging activity (\%)} = [(A_0 - A_S)/A_0] \times 100,$$

where A_0 is the absorbance of control and A_S is the absorbance of the reaction mixture.

3.4.2 Reducing Power Assay

The reducing power of each extract was determined according to the method of Oyaizu (1986) with slight modifications. All samples were assayed in triplicates. A total of 2.5 ml

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of mushroom extracts (3 mg/ml) were mixed with phosphate buffer solution (2.5 ml, 0.04 M, pH 7.49) and 2.5 ml of 1% potassium ferricyanide. The reaction mixtures were incubated for 20 minutes at 50°C. After incubation, 2.5 ml of 10% TCA was added to the reaction mixtures and followed by centrifugation at 1000 rpm for 10 minutes. Next, 2.5 ml of the obtained supernatant were mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The reaction mixtures (200 µl) were pipetted out into 96-well plate and subjected to absorbance reading at 700 nm using ELISA Plate Reader (Sunrise Basic Tecan). BHA was used as the standard and mixture without mushroom extract as negative control. According to Mau *et al*. (2002) and Mujić *et al*. (2010), higher absorbance reading indicates a higher reducing power.

3.4.3 Inhibition of Lipid Peroxidation of Buffered Egg Yolk

The ability of the mushroom extracts to inhibit lipid peroxidation was measured by procedures described by Kuppusamy *et al*. (2002) with modifications. The fowl egg yolk was emulsified with phosphate buffer (0.1 M, pH 7.4) and 1.0 ml of buffered egg yolk was mixed with 100 µl of each extracts (3 mg/ml) and 100 µl of ferrous sulphate. The test was carried out in triplicate analysis. The reaction mixtures were incubated for 1 hour at 37°C, after which were treated with 500 µl of freshly prepared 15% TCA and 1.0 ml of 1% TBA. The reaction tubes were then incubated in boiling water bath for 15 minutes before being cooled down to room temperature (±27°C). The tubes were centrifuged at 3500 rpm for 15 minutes to remove precipitated protein. Lastly, 100 µl of the clear supernatant was subjected to absorbance reading at 532 nm using ELISA Plate Reader (Sunrise Basic Tecan) to measure the formation of thiobarbituric acid reactive substances (TBARS). BHA was used as the standard and mixture without mushroom extract as negative control. The percentage of inhibition calculated as follows:

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$$\text{Inhibition (\%)} = [(A_0 - A_S)/A_0] \times 100,$$

where A_0 is the absorbance of control and A_S is the absorbance of the samples.

3.4.4 Trolox Equivalent Antioxidant Capacity (TEAC)

The assay was carried out in triplicates with slight modifications. ABTS was dissolved in 5.0 ml of distilled water to get stock concentration of 7 mM. ABTS radical cation was formed by reacting with 88 µl of potassium persulfate (final concentration of 140 mM). The reaction mixture was allowed to stand in the dark at room temperature (±27°C) for 12-16 hours to get dark blue solution. The solution was then diluted with phosphate buffer solution (PBS) until absorbance reached 0.7 (±0.05) at 734 nm. Next, 1.0 ml of diluted ABTS solution (A_{734nm} = 0.700 ± 0.05) was mixed with 100 µl samples (or Trolox as a standard). The reaction mixture was allowed to react for 1 minute before the absorbance reading was taken at 734 nm. The TEAC values calculated based on final concentration and expressed as M Trolox equivalent per 150 g of fresh weight mushroom (M TE/150g).

3.5 Determination of Total Phenolic Content

Total phenolic content of the mushroom extracts was determined using modified methods of Slinkard and Singleton (1977), which was carried out in triplicates. The extracts were prepared at 3 mg/ml and 250 µl of each was mixed with 250 µl of 10% Folin-Ciocalteu reagent. The reaction mixtures were incubated for 3 minutes, followed by the addition of 500 µl of 10% sodium carbonate. The mixtures were left to stand in the dark for 1 hour before the absorbance reading recorded spectrophotometrically (Shimadzu UV Visible Spectrophotometer model UV-mini 1240) at 750 nm. BHA was used as the standard and mixture without mushroom extract served as negative control. Total phenolic content of mushroom extracts were expressed as gallic acid equivalents (GAEs) per 150 g of fresh

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weight mushroom (mg GAE/150g). The concentration of the total phenols in the extracts was derived from gallic acid standard curve prepared ranging from 2 to 10 µg/ml.

3.6 Statistical Analysis

The values obtained were expressed as means ± standard deviation (SD) of triplicate measurements. The data for all investigated samples were analysed using SPSS, as one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$ using Duncan Multiple Range Test (DMRT).

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 Yield of mushroom extracts

The yields for uncooked and cooked *H. erinacues* samples are presented in Table 4.1. Yield percentage of extracts for all samples was obtained by dividing the weight of extracts to the total weight (150 g) of uncooked mushrooms subjected to cooking.

Table 4.1: Extraction efficiency of uncooked and cooked samples of *H. erinacues* subjected to different cooking methods at different cooking time.

	Cooking methods	Cooking time (minutes)	Extraction efficiency	
			Water extract (g)	Percentage (%)
Fresh samples	Uncooked	-	4.18	2.79
	Boiling	5	5.26	3.51
	Microwaving		4.82	3.21
	Steaming		4.91	3.27
	Pressure-cooking		6.95	4.63
	Boiling	10	3.84	2.56
	Microwaving		4.72	3.15
	Steaming		3.72	2.48
	Pressure-cooking		7.42	4.95
Oven-dried samples	Uncooked	-	5.20	3.46
	Boiling	5	3.79	2.53
	Microwaving		4.59	3.06
	Steaming		5.88	3.92
	Pressure-cooking		10.61	7.07
	Boiling	10	4.46	2.97
	Microwaving		7.72	5.15
	Steaming		9.51	6.34
	Pressure-cooking		9.71	6.47

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The extraction efficiencies were in the range of 2 to 7%. Oven-dried sample submitted to pressure-cooking (5 minutes) gave the highest yield (7.07%) while the lowest yield recorded was 2.48%, obtained from the fresh sample subjected to 10 minutes of steaming. According to studies by Mau *et al*. (2002) and Mau *et al*. (2005), the extraction percentage of methanolic extracts of *H. erinaceus* and hot water extracts of *Ganoderma tsugae* were 25.49% and 6.22%, respectively. On the other hand, the yield percentage of various extracts of *Pleurotus eous* were in the range of 1.13 to 41.56%, in the order of hot water extract > methanol extract > ethyl acetate extract (Sudha *et al*., 2012). There were differences in the percentage yield reported in this study compared to others, which might be due to the different processing methods that were carried out. Also, the differences might be because of the lack of the consistency of the extracts of *H. erinaceus* which appeared to be sticky and resinous after they were freeze-dried especially after long exposure to air. Research conducted by Tan *et al*. (2015) also reported low extraction efficiencies of cooked *Pleurotus* spp. which was in the range of 1.49-2.59%.

4.2 Antioxidant Activity Assays

4.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)Radical Scavenging Activity Assay

The antioxidant potential through DPPH free radical scavenging activities of cooked *H. erinacues* samples in comparison to the uncooked sample at the concentration of 3 mg/ml are shown in Table 4.2.

The scavenging activities of fresh and oven-dried samples investigated were in the range of 11.9-27.6%. The lowest scavenging activity (11.94%) was observed in oven-dried sample which was subjected to 5 minutes microwaving with no significant difference ($p < 0.05$) compared to its uncooked sample (13.01%). The oven-dried sample steamed for

Table 4.2: The percentage of DPPH free radical scavenging activities of uncooked and cooked samples of fresh and oven-dried *H. erinacues* subjected to different cooking methods at different cooking time.

	Cooking methods	Cooking time (minutes)	DPPH free radical scavenging activity at 3 mg/ml (%)
Fresh samples	Uncooked	N/A	16.88 ± 1.89[a]
	Boiling	5	18.49 ± 2.42[a,b]
	Microwaving		16.88 ± 3.63[a]
	Steaming		23.12 ± 2.74[b,c]
	Pressure-cooking		22.47 ± 1.43[a,b,c]
	Boiling	10	17.20 ± 1.49[a]
	Microwaving		25.81 ± 1.80[c]
	Steaming		18.39 ± 5.67[a,b]
	Pressure-cooking		18.39 ± 2.64[a,b]
Oven-dried samples	Uncooked	N/A	13.01 ± 0.67[v]
	Boiling	5	23.12 ± 3.26[y]
	Microwaving		11.94 ± 0.32[v]
	Steaming		27.63 ± 1.04[z]
	Pressure-cooking		14.84 ± 1.71[v,w]
	Boiling	10	19.03 ± 3.11[x]
	Microwaving		16.45 ± 1.80[v,w]
	Steaming		19.25 ± 1.22[x]
	Pressure-cooking		26.45 ± 0.85[z]

For each mushroom samples, values were expressed as means ± SD ($n = 3$) and values within the same column followed by different letters (a-c) and (v-z) are significantly different ($p < 0.05$).
N/A = not applicable.
DPPH scavenging activity of BHA = 83.87 ± 0.00 %.

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5 minutes showed the highest scavenging activity of 27.63%, significantly ($p < 0.05$) increased by 2.1-fold compared to the uncooked sample (13.01%).

Both fresh boiling samples (5 minutes and 10 minutes) did not show any significant difference ($p < 0.05$) in their scavenging activities, respectively at 18.49% and 17.20%, in comparison to the uncooked sample (16.88%). Even though the sample heated using

microwave (5 minutes) did not differ significantly compared to uncooked sample, the sample subjected to 10 minutes microwaving showed a significant ($p < 0.05$) increase by 1.5-fold (25.81%) compared to its uncooked sample (16.88%). Hayat *et al*. (2009) revealed that microwave treatment could accelerate the release of high amount of phenolic compounds by thermal destruction of cell wall and sub cellular compartments of citrus peels.

The scavenging activities of fresh samples after 5 minutes of steaming and pressure-cooking increased significantly by 1.4-fold (23.12%) and 1.3-fold (22.47%), compared to the uncooked sample (16.88%). In contrast, both samples subjected to 10 minutes steaming and pressure-cooking did not show significant value of scavenging activities.

For oven-dried samples, when compared to the uncooked (13.01 ± 0.67 %), many samples tested showed significant ($p < 0.05$) increase in scavenging activities of DPPH radicals. The sample subjected to 5 minutes steaming recorded the highest activity (27.63%), significantly ($p < 0.05$) increased by 2.1-fold compared to the uncooked sample. In contrast, 10 minutes steamed sample only showed 1.4-fold (19.25%) increase in the scavenging activity. Similar to the sample subjected to 5 minutes steaming, the sample that was pressure-cooked for 10 minutes also showed higher scavenging activity (26.45%), significantly ($p < 0.05$) increased by 2-fold in comparison to the uncooked.

According to study conducted by Tan *et al*. (2015), *Pleurotus floridanus* showed the highest scavenging activity (>200%) after subjected to pressure-cooking, in comparison to other variety of *Pleurotus* mushroom as well as uncooked. They stated that the improvement in antioxidant activity might be due to the release of active antioxidants from

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the fibrous complexes during pressure-cooking (low moisture level and high temperature). In addition, the sample that was boiled for 5 minutes also displayed the significant ($p < 0.05$) value of scavenging activity (23.12%), 1.7-fold higher than uncooked sample. DPPH scavenging activities for both samples submitted to 5 and 10 minutes of microwaving clearly exhibited insignificant value with respect to the uncooked sample.

Wong *et al*. (2009) reported that fresh fruit body of *H. erinaceus* was the best DPPH scavenger (EC_{50} = 3.75 mg/ml) when compared to the extracts of oven-dried fruit body (EC_{50} = 5.81 mg/ml), freeze-dried fruit body (EC_{50} = 8.67mg/ml) and mycelium (EC_{50} = 13.67 mg/ml). In another study conducted by Mau *et al*. (2002), the scavenging activity of methanolic extracts of *H. erinaceus* was approximately 35% at the concentration of 3 mg/ml. Hot water extracts of *H. erinaceus* showed the EC_{50} value of 25.47 mg/ml in a study investigated by Abdullah *et al*. (2012). From all of the above, Mujić *et al*. (2010) reported the highest scavenging activity of ethanolic *H. erinaceus* extract (EC_{50}= 0.198 mg/ml).

Based on the results obtained, cooking significantly ($p < 0.05$) increased the DPPH radical scavenging activity when compared to the uncooked sample for both fresh and oven-dried mushrooms. This indicates that the cooked *H. erinaceus* is a good DPPH free radical scavenger and could possibly act as primary antioxidants. A study by Sun *et al*. (2014) claimed that when compared to the uncooked sample, microwaving and pressure-cooking significantly ($p < 0.05$) increased the DPPH scavenging activity of *Boletus pinophilus* with EC_{50}valueof 80.5 µg/ml and 66.0 µg/ml, respectively. However in contrast, the samples of *Boletus aereus* cooked by five different methods (steaming, pressure-cooking, microwaving and boiling) showed no significant difference in DPPH scavenging activity in relation to the uncooked sample. Additionally, steaming and boiling did not

significantly enhance the DPPH scavenging activity for *Boletus badius*, *Boletus edulis* and *Boletus pinophilus* when compared to their corresponding uncooked sample.

Elmastas *et al*. (2007) revealed that the scavenging effects of methanolic extracts from mushroom species on DPPH radicals increased with increasing concentrations. At the concentration of 180 µg/ml, the scavenging effects of methanolic extracts from mushroom species and standards increased in the order of *Boletus badius* (68.7%) > *Verpa conica* (75.7%) > *Agaricus bisporus* (77.5%) > *Pleurotus ostreatus* (81.3%) > *Polysporus squamosus* (82.8%) > *Russula delica* (86.1%) > *Lepista nuda* (91.3%) > α-tocopherol (95.4%) > BHA (97.4%).

Wong *et al*. (2009) reported that the DPPH radical scavenging of the fresh, oven-dried and freeze-dried fruit body of *H. erinaceus* increased up to 87.35, 87.78 and 89.24% with the increasing concentrations of extracts at 7, 10 and 14 mg/ml, respectively. This observation indicated that the DPPH radical scavenging of the extracts was also dose-dependent.

From the results obtained, the best three cooking methods were 5 minutes steaming of oven-dried sample, 10 minutes pressure-cooking of oven-dried sample and 10 minutes microwaving of fresh sample, in terms of high capability to scavenge DPPH free radicals.

4.2.2 Reducing Power Assay

The reducing power ability of uncooked and cooked samples of *H. erinaceus* extracts are shown in Table 4.3 (a) and Table 4.3 (b).

This reducing power assay was performed in order to measure the ability of the sample extracts (antioxidants) to reduce Fe^{3+}-ferricyanide complex to the ferrous form,

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Fe^{2+}, which denotes the capability of the antioxidants to donate electrons. The higher the absorbance value indicates greater reducing power when the resulting ferrous forms, Fe^{2+} were monitored through the formation of Perl's Prussian blue spectrophotometrically at 700 nm. At each concentration tested (0.5 mg/ml, 1.0 mg/ml, 3.0 mg/ml and 5.0 mg/ml), BHA clearly displayed significant high reducing capacity of 1.27, 2.15, 2.21 and 2.45, respectively, which were absolutely higher than all the samples examined. BHA is a synthetic compound and serve as a positive control, hence the high absorbance reading (>1.5) is considered acceptable as this study is semi-quantitative determination (Skoog *et al*., 1997).

The tested sample extracts demonstrated variable reducing capacities and overall, the reducing capacity of fresh *H. erinaceus* samples as shown in Table 4.3 (a) were increased with increasing concentrations. The reducing power ability of uncooked sample of fresh *H. erinaceus* showed an increase by 11-fold from the concentrations of 0.5 to 5.0 mg/ml (absorbance = 0.03 to 0.29). At the concentration of 0.5 mg/ml, the reducing capacities of all cooked samples were not significantly ($p < 0.05$) different in comparison to those uncooked. The reducing capacity of fresh samples subjected to 5 minutes of pressure-cooking at 1.0, 3.0 and 5.0 mg/ml were 0.18, 0.52 and 0.68, respectively. The reducing capacity detected were significant ($p < 0.05$) when compared to the uncooked sample. Likewise, with respect to the uncooked, the same trend was observed for the sample submitted to pressure-cooking for 10 minutes, where the reducing capacity of the samples was significantly ($p < 0.05$) increased with increasing concentrations. In a study by Sun *et al*. (2014), when compared to other cooking methods, the reducing power ability of pressure-cooked *Boletus pinophilus* (EC_{50} = 49.5 µg/ml) significantly ($p < 0.05$) increased relative to the raw sample (EC_{50} = 66.5 µg/ml).

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Table 4.3 (a): Reducing power of uncooked and cooked samples of fresh *H. erinaceus* at different concentrations.

	Cooking methods	Cooking time (minutes)	Absorbance values (700 nm) of samples at different extract concentrations (mg/ml)			
			0.50	1.00	3.00	5.00
Fresh samples	Uncooked	N/A	0.03 ± 0.00[a]	0.08 ± 0.00[a,b]	0.21 ± 0.00[a,b]	0.29 ± 0.01[a,b]
	Boiling	5	0.04 ± 0.00[a]	0.10 ± 0.00[a,b]	0.25 ± 0.01[b,c]	0.27 ± 0.00[a,b]
	Microwaving		0.02 ± 0.00[a]	0.07 ± 0.00[a]	0.20 ± 0.00[a,b]	0.30 ± 0.01[a,b]
	Steaming		0.03 ± 0.01[a]	0.10 ± 0.00[a,b]	0.30 ± 0.02[c]	0.31 ± 0.01[a,b]
	Pressure-cooking		0.09 ± 0.01[a]	0.18 ± 0.00[c]	0.52 ± 0.02[e]	0.68 ± 0.01[d]
	Boiling	10	0.02 ± 0.00[a]	0.07 ± 0.00[a]	0.15 ± 0.00[a]	0.18 ± 0.01[a]
	Microwaving		0.04 ± 0.01[a]	0.11 ± 0.01[a,b]	0.33 ± 0.01[c]	0.36 ± 0.00[b,c]
	Steaming		0.03 ± 0.00[a]	0.09 ± 0.01[a,b]	0.26 ± 0.01[b,c]	0.31 ± 0.02[a,b]
	Pressure-cooking		0.06 ± 0.00[a]	0.13 ± 0.00[b,c]	0.43 ± 0.02[d]	0.47 ± 0.02[c]
Standard antioxidant	Butylated hydroxyanisole (BHA)		1.27 ± 0.14[b]	2.15 ± 0.09[d]	2.21 ± 0.15[f]	2.45 ± 0.23[e]

For each mushroom samples, values were expressed as means ± SD (n = 3) and values within the same column followed by different letters are significantly different ($p < 0.05$).
BHA was used as standard antioxidant for comparison.
N/A = not applicable.

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Table 4.3 (b): Reducing power of uncooked and cooked samples of oven-dried *H. erinaceus* at different concentrations.

	Cooking methods	Cooking time (minutes)	Absorbance values (700 nm) of samples at different extracts concentrations (mg/ml)			
			0.50	1.00	3.00	5.00
Oven-dried samples	Uncooked	N/A	0.07 ± 0.00^{y}	$0.14 \pm 0.00^{w,x}$	0.34 ± 0.00^{v}	0.47 ± 0.01^{x}
	Boiling	5	0.04 ± 0.00^{y}	0.10 ± 0.01^{w}	$0.35 \pm 0.02^{v,w}$	0.48 ± 0.00^{x}
	Microwaving	5	0.06 ± 0.00^{y}	$0.13 \pm 0.00^{w,x}$	$0.44 \pm 0.03^{w,x,y}$	0.45 ± 0.03^{x}
	Steaming	5	0.08 ± 0.01^{y}	0.16 ± 0.00^{x}	$0.36 \pm 0.02^{v,w,x}$	0.51 ± 0.01^{x}
	Pressure-cooking	5	0.05 ± 0.00^{y}	0.10 ± 0.01^{w}	$0.35 \pm 0.01^{v,w}$	0.47 ± 0.01^{x}
	Boiling	10	0.08 ± 0.00^{y}	$0.15 \pm 0.01^{w,x}$	0.46 ± 0.03^{y}	0.51 ± 0.02^{x}
	Microwaving	10	0.07 ± 0.00^{y}	$0.15 \pm 0.00^{w,x}$	$0.35 \pm 0.02^{v,w}$	0.47 ± 0.03^{x}
	Steaming	10	0.10 ± 0.01^{y}	0.24 ± 0.00^{y}	$0.45 \pm 0.04^{x,y}$	0.75 ± 0.01^{y}
	Pressure-cooking	10	0.05 ± 0.01^{y}	$0.11 \pm 0.01^{w,x}$	$0.34 \pm 0.01^{v,w}$	0.41 ± 0.01^{x}
Standard antioxidant	Butylated hydroxyanisole (BHA)		1.27 ± 0.14^{z}	2.15 ± 0.09^{z}	2.21 ± 0.15^{z}	2.45 ± 0.23^{z}

For each mushroom samples, values were expressed as means ± SD ($n = 3$) and values within the same column followed by different letters are significantly different ($p < 0.05$).
BHA was used as standard antioxidant for comparison.
N/A = not applicable.

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Similarly, the reducing power of oven-dried *H. erinaceus* samples as depicted in Table 4.3 (b) were increased steadily with increasing concentrations. The reducing power ability of the uncooked sample of oven-dried *H. erinaceus* showed a dramatic increase (6-fold) which was 0.07 to 0.47 as the concentration hiked from 0.5 to 5.0 mg/ml. At the concentration of 0.5 mg/ml, as in comparison to the uncooked sample, all investigated oven-dried *H. erinaceus* samples showed no significant difference ($p < 0.05$) in terms of the reducing capacities, which followed the same pattern with the fresh samples. On the other hand, with respect to the uncooked sample (absorbance = 0.14), the samples subjected to 5 and 10 minutes of steaming showed the significant ($p < 0.05$) increase in the reducing capacity of 0.16 and 0.24, respectively, at the concentration of 1 mg/ml. The value recorded was comparable with the finding from Mujić *et al*. (2010) where they stated that the reducing capacity of *H. erinaceus* dry extracts has been determined as 0.185 at the same concentration.

Besides, at 3 mg/ml, the samples submitted to 5 minutes microwaving, 10 minutes boiling and 10 minutes steaming demonstrated significant ($p < 0.05$) increase in the reductive capabilities when compared to the uncooked sample. However, compared to the uncooked (absorbance = 0.47), only sample subjected to 10 minutes of steaming showed the significant ($p < 0.05$) increase in reducing power ability of 0.75 in the tested concentration of 5 mg/ml. Overall results showed that the reducing power for some of the samples were decreased, however with no significant difference ($p < 0.05$) when compared to the uncooked sample at respective concentration. Study by Mujić *et al*. (2010) determined that the reducing capacity of ethanolic *H. erinaceus* extract was better compared to ethanolic *Lentinula edodes* extract. At 3 mg/ml, the reducing capacity of

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H. erinaceus was in the range of 0.50-0.60 and 0.80-0.90 at 5 mg/ml, which was slightly higher compared to the results obtained above.

The reducing power of *H. erinaceus* extract was also increased with increasing concentrations as represented by Abdullah *et al*. (2012) where the reducing capacity were 0.023 at 0.5 mg/ml and 0.077 at 1 mg/ml. In the same study, of all the mushrooms investigated, *Ganoderma lucidum* demonstrated the highest reducing capacity of 0.023 and 0.453, respectively, at the tested concentrations of 0.5 mg/ml and 1 mg/ml. Research carried out by Mau *et al*. (2002) revealed that the reducing power of the investigated sample (*Dictyophora indusiata, Grifola frondosa*, *H. erinaceus* and *Tricholoma gigateum*) increased as the concentrations increased. At the tested concentration of 9 mg/ml, the reducing power of methanolic extract from *H. erinaceus* was 1.01 while 1.78 at 24 mg/ml, in which it was comparable to oyster mushroom. In the same study, they claimed basket stinkhorn (*Dictyophora indusiata*) having the most excellent reducing capacity where the reducing power recorded was 1.09 at 3 mg/ml, abruptly increased to 2.51 at the concentration of 9 mg/ml and later reached plateau at 9-24 mg/ml.

Based on the results obtained, the reducing power ability of fresh and oven-dried *H. erinaceus* extracts exhibited dose-dependent manner at various concentrations. Thorough results showed that the reducing power abilities of oven-dried samples were better in comparison to the fresh samples. This indicates that the reducing power of both samples might be due to their hydrogen-donating ability; however oven-dried samples might contain higher amounts of reductones which could react with free radicals to stabilise and terminate radical chain reactions (Mau *et al*., 2002; Abdullah *et al*., 2012; Sudha *et al*., 2012). The best three cooking methods to in ferric reducing capacities were in the order of

10 minutes steaming of oven-dried sample > 5 minutes pressure-cooking of fresh sample > 10 minutes pressure cooking of fresh sample.

4.2.3 Inhibition of Lipid Peroxidation of Buffered Egg Yolk

The ability of the uncooked and cooked samples of *H. erinaceus* extracts to inhibit peroxidation of phospholipids in egg yolk, at the concentration of 3 mg/ml are presented in Table 4.4.

Table 4.4: The percentage of lipid peroxidation inhibition of uncooked and cooked samples of fresh and oven-dried *H. erinacues* subjected to different cooking methods at different cooking time.

	Cooking methods	Cooking time (minutes)	Inhibition of lipid peroxidation at 3 mg/ml (%)
Fresh samples	Uncooked	N/A	22.93 ± 5.54[a]
	Boiling	5	13.03 ± 7.15[a]
	Microwaving		17.67 ± 5.06[a]
	Steaming		20.18 ± 6.57[a]
	Pressure-cooking		20.05 ± 4.41[a]
	Boiling	10	15.79 ± 4.34[a]
	Microwaving		18.17 ± 3.91[a]
	Steaming		11.78 ± 7.67[a]
	Pressure-cooking		13.16 ± 7.88[a]
Oven-dried samples	Uncooked	N/A	14.23 ± 1.52[y]
	Boiling	5	8.77 ± 6.47[y]
	Microwaving		29.07 ± 5.12[z]
	Steaming		9.65 ± 8.91[y]
	Pressure-cooking		4.76 ± 1.56[y]
	Boiling	10	11.91 ± 8.58[y]
	Microwaving		11.15 ± 6.58[y]
	Steaming		12.28 ± 4.02[y]
	Pressure-cooking		8.75 ± 6.15[y]

For each mushroom samples, values were expressed as means ± SD (n = 3) and values within the same column followed by different letters (a) and (y-z) are significantly different ($p < 0.05$).
N/A = not applicable.
Lipid peroxidation inhibition of BHA = 53.63 ± 6.15 %.

The levels of lipid peroxidation inhibition of all cooked samples of fresh *H. erinaceus* were insignificantly lower compared to the uncooked sample (22.93 ± 5.54 %). The sample subjected to 10 minutes of steaming showed the lowest percentage of inhibition which was at 11.78% (reduced by 1.9-fold). This indicated that all cooking processes applied were not significantly enhance the lipid peroxidation inhibition of fresh *H. erinaceus*. Overall results of uncooked and cooked samples of fresh *H. erinaceus* are comparable to the results obtained by Abdullah *et al*. (2012) where the percentage of lipid peroxidation inhibition of *H. erinaceus* extract was 47.52% at the concentration of 10 mg/ml. In the same study, the highest percentage of inhibition was exhibited by *Ganoderma lucidum* (57.18%), followed by *Pleurotus florida* and *Auricularia auricular-judae*, respectively at 56.58% and 56.41% of lipid peroxidation inhibition.

The results obtained from oven-dried samples were different as compared to the fresh samples. The sample submitted to 5 minutes microwaving demonstrated the highest peroxidation inhibition (29.07 ± 5.12 %), significantly ($p < 0.05$) increased by 2-fold compared to the inhibition percentage of uncooked sample (14.29 ± 1.52 %). This indicated that the oven-dried sample treated to 5 minutes of microwaving has a capacity to inhibit lipid peroxidation significantly ($p < 0.05$) than other treated samples. The rest of the cooked oven-dried samples showed insignificant difference in comparison to the uncooked. The sample treated at 5 minutes of pressure-cooking showed the lowest percentage of peroxidation inhibition (4.76%), reduced by 3-fold with respect to the uncooked sample.

Vamanu and Nita (2013) investigated the ability of four different *Boletus edulis* extracts to inhibit peroxidation of phospholipids in egg yolk. The percentages of peroxidation inhibition of the extracts were demonstrated at different levels by which at the concentration of 1 mg/ml, the ethanol extract exhibited the highest level of lipid

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peroxidation (57.61 ± 0.29 %). The level of inhibition of the extracts decreased in the order: ethanolic extract > methanolic extract > hot water extract > cold water extract. In another study, *Agaricus bisporus* (brown) and *Lentinula edodes* were the mushroom species that showed to have the highest lipid peroxidation inhibition by TBARS assay, where both species showed 50% of peroxidation inhibition (EC_{50}) at 1.45 mg/ml and 1.64 mg/ml, respectively. This indicated that both mushroom species were excellent in inhibiting the lipid peroxidation. However, the fruiting body and mycelium of *Pleurotus eryngii* samples presented the lowest level of peroxidation inhibition (EC_{50} = 3.95 mg/ml and 21.03 mg/ml, respectively) (Reis *et al*., 2012).

In contrasts, when comparing to the study reported above, the peroxidation inhibition of four different dried mushroom extracts investigated by Barros *et al*. (2007) showed lower level of inhibition. The EC_{50} values of peroxidation inhibition level for each mushroom species were in the order of *Macrolepiota mastoidea* (24.20 mg/ml) > *Lactarius deliciosus* (26.40 mg/ml) > *Sarcodon imbricatus* (38.17 mg/ml) > *Macrolepiota procera* (>50 mg/ml). In another study conducted by Sudha *et al*. (2012), hot water extracts of *P. eous* demonstrated good peroxidation inhibition which inhibits brain lipid peroxidation at the percentage of 7.72-76.54% in a concentration range of 0.04-0.2 mg/ml. At 2-10 mg/ml, hot water extracts of *P. eous* exhibited lipid peroxidation inhibition in liver homogenate with inhibition percentage of 9.57-74.78%.

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4.2.4 Trolox Equivalent Antioxidant Capacity (TEAC) Assay

The capacity of the uncooked and cooked samples of *H. erinaceus* (antioxidant) to decolourise oxidant (ABTS) was determined by the reduction of radical cation. Calculated relative to the reactivity of Trolox as a standard, TEAC values of all investigated samples at the concentration of 3 mg/ml are included in Table 4.5.

Table 4.5: TEAC values of uncooked and cooked samples of fresh and oven-dried *H. erinacues* subjected to different cooking methods at different cooking time.

	Cooking methods	Cooking time (minutes)	TEAC (M TE/150 g of fruit bodies)
Fresh samples	Uncooked	N/A	0.44 ± 0.02^{e}
	Boiling	5	$0.34 \pm 0.03^{c,d}$
	Microwaving	5	0.47 ± 0.01^{e}
	Steaming	5	0.29 ± 0.02^{c}
	Pressure-cooking	5	0.12 ± 0.06^{a}
	Boiling	10	0.34 ± 0.01^{d}
	Microwaving	10	0.24 ± 0.00^{b}
	Steaming	10	0.16 ± 0.01^{a}
	Pressure-cooking	10	0.16 ± 0.03^{a}
Oven-dried samples	Uncooked	N/A	$0.18 \pm 0.01^{w,x}$
	Boiling	5	0.06 ± 0.00^{u}
	Microwaving	5	0.12 ± 0.03^{v}
	Steaming	5	$0.15 \pm 0.05^{v,w}$
	Pressure-cooking	5	0.47 ± 0.04^{z}
	Boiling	10	0.11 ± 0.00^{v}
	Microwaving	10	$0.22 \pm 0.04^{x,y}$
	Steaming	10	$0.18 \pm 0.02^{w,x}$
	Pressure-cooking	10	0.26 ± 0.02^{y}

For each mushroom samples, values were expressed as means ± SD (n = 3) and values within the same column followed by different letters (a-e) and (u-z) are significantly different ($p < 0.05$).
N/A = not applicable.
TEAC values of BHA = 1.97×10^{5} µM TE/g BHA.

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In most published studies, this antioxidant capacity assay has also been reported as ABTS radical scavenging capacity assay. This particular assay has been conducted in many research laboratories in order to measure antioxidant capacity of many compounds and food samples (Huang *et al*., 2005). In this study, the TEAC values are expressed in mol Trolox equivalent per 150 g of uncooked fruit bodies (M TE/150 g) due to the simplicity of the figures and easier to make the comparison.

Same as other assays conducted before, this procedure was carried out at the sample's concentrations of 3 mg/ml. According to the results obtained, the TEAC value of uncooked fresh sample was 0.44 ± 0.02 M TE/150 g of fruit bodies. Except for the fresh sample subjected to 5 minutes of microwaving, all other tested samples showed significant ($p < 0.05$) decrease in TEAC levels which in the range of 0.12-0.34 M TE/150 g with respect to the uncooked sample. Both samples submitted to pressure-cooking at 5 and 10 minutes showed the lowest TEAC levels of 0.120 M TE/150 g (decreased by 3.6-fold) and 0.160 M TE/150 g (decreased by 2.7-fold), respectively, which were significantly ($p < 0.05$) different with respect the uncooked sample. As indicated from the results, cooking did not significantly enhance the TEAC levels of fresh samples of *H. erinaceus*.

Research conducted by Tan *et al*. (2015) indicated that all cooking methods that were applied significantly ($p < 0.05$) decreased the TEAC values of *P. cyctidiosus* (abalone mushroom), *P. flabellatus* (pink oyster), *P. floridanus* (white oyster) as well as *P. pulmonarius* (grey oyster) when compared to their respective uncooked sample. However, the TEAC value of the boiling sample of *P. citrinopileatus* (123.21 ± 3.62 μM TE/150 g) was higher than those uncooked (114.43 ± 5.48 μM TE/150 g) but with no significant difference at $p < 0.05$. The same trend was observed for sample of *P. eryngii*

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subjected to microwaving where the TEAC value was 85.98 μM TE/150 g, insignificantly increased with respect to the uncooked sample (73.30 μM TE/150 g).

As for cooked oven-dried *H. erinaceus*, the TEAC values for samples subjected to pressure-cooking both at 5 and 10 minutes respectively increased by 2.6-fold and 1.5-fold (0.47 M TE/150 g and 0.26 M TE/150 g) which significantly ($p < 0.05$) different relative to the uncooked sample (0.18 M TE/150 g). In contrasts, as compared to the uncooked sample, the samples subjected to boiling showed the lowest TEAC levels for both cooking duration, respectively at 0.06M TE/150 g (decreased by 2.9-fold) and 0.11M TE/150 g (decreased by 0.6-fold) with significant difference at $p < 0.05$. Meanwhile, the TEAC values for samples steamed at both cooking duration showed lower but insignificant values when compared to the uncooked. From the results depicted, pressure-cooking significantly enhanced the antioxidant activity in terms of TEAC values of oven-dried samples of *H. erinaceus*.

In 2013, Chumyam *et al*. revealed that the antioxidant capacities of four cultivars of purple skin eggplants were significantly ($p < 0.05$) increased when submitted to three heating process, relative to their respective uncooked samples. All cultivars submitted to boiling, steaming and microwaving showed the excellent ABTS radical scavenging capacity by which sample heated by microwaving had the highest antioxidant capacities in all varieties tested.

The scavenging abilities of ethyl acetate, methanolic and hot water extracts of *P. eous* on ABTS radical at the samples concentration of 10-50 mg/ml were increased from 22.87-81.52%, 35.58-97.21% and 26.62-90.65%, respectively (Sudha *et al*., 2012). They concluded that the ABTS radical scavenging activity of various extract indicates its ability

to scavenge free radicals, thereby preventing lipid oxidation via a chain-breaking reaction. The best samples that showed the high levels of TEAC values were 5 minutes microwaving of fresh sample and 5 minutes pressure-cooking of oven-dried sample.

4.3 Determination of Total Phenolic Content (TPC)

Effects of different cooking procedures on the total phenolic content (TPC) of uncooked and cooked samples of *H. erinaceus* are presented in Table 4.6. The investigation was carried out at the sample's concentrations of 3 mg/ml and expressed as mg gallic acid equivalents (GAEs) per 150 g of uncooked fruit bodies.

Folin-Ciocalteu assay was carried out in order to investigate the effects of different cooking procedures on the TPC of *H. erinaceus*. This method widely employed by many researchers despite the fact that Folin-Ciocalteu reagent does not react exclusively with phenolics, but also with other reducing agents such as ascorbic acid (Abdullah *et al*., 2012). Many researches reported that the antioxidant activity of plant materials was well correlated with the content of phenolic compounds with phenolic acids being the most common compound found in mushrooms (Cheung *et al*., 2003; Pushpa *et al*., 2012).

The TPC of uncooked fresh *H. erinaceus* was recorded at 28.10 ± 0.76 mg GAE/150 g. From all fresh samples tested, only sample subjected to 10 minutes of boiling exhibited the significant decrease ($p < 0.05$) in TPC (24.53 mg GAE/150 g) when compared to the uncooked sample. As conducted by Kettawan *et al*. (2011), boiling reduced the TPC content in all mushroom samples tested. For instance, phenolic content of boiled *L. edodes* decreased significantly ($p < 0.01$) to 41.5 mg GAE/100 g compared to the uncooked sample (129.1 mg GAE/100 g). Likewise, boiling significantly reduced ($p < 0.05$) the phenolic content of *Agaricus hygrometricus* with respect to its uncooked sample.

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Table 4.6: Total phenolic content (TPC) of uncooked and cooked samples of fresh and oven-dried *H. erinacues* subjected to different cooking methods at different cooking time.

	Cooking methods	Cooking time (minutes)	TPC (mg GAE/150 g of fruitbodies)
Fresh samples	Uncooked	N/A	28.10 ± 0.76[b]
	Boiling	5	45.15 ± 2.74[d]
	Microwaving		31.62 ± 2.53[c]
	Steaming		48.67 ± 1.16[e]
	Pressure-cooking		85.52 ± 0.29[g]
	Boiling	10	24.53 ± 0.55[a]
	Microwaving		50.41 ± 1.92[e]
	Steaming		33.79 ± 1.08[c]
	Pressure-cooking		78.25 ± 1.65[f]
Oven-dried samples	Uncooked	N/A	54.36 ± 0.60[u]
	Boiling	5	42.18 ± 0.40[s]
	Microwaving		56.61 ± 0.85[u]
	Steaming		64.57 ± 1.39[v]
	Pressure-cooking		90.28 ± 2.22[y]
	Boiling	10	50.15 ± 1.06[t]
	Microwaving		86.89 ± 0.63[x]
	Steaming		114.69 ± 0.15[z]
	Pressure-cooking		79.66 ± 3.87[w]

For each mushroom samples, values were expressed as means ± SD (n = 3) and values within the same column followed by different letters (a-g) and (s-z) are significantly different ($p < 0.05$). N/A = not applicable.
Total phenolic content of BHA = 43.81± 0.59g GAE/g BHA.

Instead, the rest of the samples displayed increases in TPC after heat-treated and were significant ($p < 0.05$) in comparison to the uncooked. The TPC of pressure-cooked samples at both 5 and 10 minutes of cooking were the highest, respectively at 85.52 mg GAE/150 g (increased by 3-fold) and 78.25 mg GAE/150 g (increased by 2.8-fold). This result is comparable with the finding of Tan *et al*. (2015) which of all the *Pleurotus* mushroom tested using various cooking methods, only the pressure-cooked sample of

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P. cyctidiosus showed an increase in total phenolic content (9.21 mg GAE/150 g) with respect to its uncooked sample.

As reported by Wong *et al*. (2009), the TPC for the mycelium of *H. erinaceus* had the highest ($p < 0.05$) value at 31.20 mg GAE/g of mycelium as compared to oven-dried, freeze-dried and fresh fruit body extract. They stated that phenolic compounds have been identified as important antioxidants in mushrooms as other compounds such as vitamin C, E and β-carotene. Phenolic compounds in plants are powerful free radical scavengers which can inhibit lipid peroxidation by neutralising peroxyl radicals generated during the oxidation of lipids (Shahidi *et al*., 1994). In other study conducted by Abdullah *et al*. (2012), the TPC of *H. erinaceus* was 10.20 ± 2.25 mg GAE/g extract, while *G. lucidum* gave the highest phenolic content of 63.51 mg GAE/g extract.

The total polyphenols of methanolic extract of *H. erinaceus* as reported by Mau *et al*. (2002) was 12.05 mg GAE/g which the value was comparable with the polyphenols content of *Grifola frondosa* sample (12.31 mg GAE/g). In 2010, Mujić*et al*.recorded 7.80 mg GAE/g for the TPC of investigated *H. erinaceus*, which considered as the lowest value as to compare with the phenolic content of *L. edodes* (11.70 mg GAE/g) and *Agrocybe aegerita* (23.07 mg GAE/g).

The TPC of uncooked oven-dried sample was much higher (54.36 ± 0.61 mg GAE/150 g) when compared to uncooked fresh sample (28.10 mg GAE/150 g). This could be due to the formation of compounds such as Maillard's reaction products (MRPs) that have antioxidant activity during heat treatment or thermal processing as explained by Choi *et al*. (2006) and Wong *et al*. (2009). The highest TPC after 5 minutes of cooking was obtained from pressure-cooked sample with the value of 90.28 ± 2.22 mg GAE/150 g.

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The 10 minutes steamed sample showed significantly ($p < 0.05$) highest value of phenolic content of 114.69 mg GAE/150 g (increased by 2.1-fold) compared to the uncooked sample. The TPC of cabbage, choy-sum and broccoli were markedly decreased after 5 minutes of steaming, boiling and microwaving as reported by Wachtel-Galor *et al*. (2008) in relative to the fresh sample. As for cauliflower, only sample that was steamed showed an increase in phenolic content in comparison with other cooking procedures.

In contrast, the boiled samples exhibited the lowest value of phenolic content for both cooking durations, respectively at 42.18 and 50.15 mg GAE/150 g. This finding resembles the results reported by Tan *et al*. (2015) which stated that the phenolic content for the boiled samples of *P. eryngii* (7.91 mg GAE/150 g), *P. floridanus* (11.29 mg GAE/150 g) and *P. pulmonarius* (13.36 mg GAE/150 g) showed reduction but with no significant difference ($p < 0.05$) when compared to their respective uncooked samples.

Study reported by Sun *et al*. (2014) reported that all cooking methods that were applied (steaming, pressure-cooking, microwaving, frying and boiling) decreased the total phenolic content of four *Boletus* mushrooms as compared to the uncooked sample. Of all cooking procedures, all boiling samples for each mushroom variety (*B. aereus*, *B. badius*, *B. pinophilus* and *B. edulis*) in the study displayed the lowest value of phenolic content. For example, boiling significantly ($p < 0.05$) decreased the phenolic content in *B. aereus* sample (70.5 µg GAE/g of fresh weight) when compared to its uncooked (209.5 µg GAE/g of fresh weight).

The results obtained showed that oven-dried samples subjected to 10 minutes steaming, 5 minutes pressure-cooking and 10 minutes microwaving were the best three

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cooking methods recommended in order to enhance the TPC of *H. erinaceus*. In addition, for fresh sample, pressure-cooking both at 5 and 10 minutes was the best cooking methods to be suggested.

Though many previous researches indicated that the TPC may contribute significant effects on total antioxidant activity, this study showed a difference. For fresh samples, the 5 minutes pressure-cooked sample had the highest total phenolic content with an excellent antioxidant activity in DPPH radical scavenging assay and reducing power assay. However, after 10 minutes of pressure-cooking, sample tested using reducing power assay was well correlated with the sample undergone TPC determination test. This could be explained that the accumulation of phenolic compounds in pressure-cooked samples did significantly enhance the antioxidant activity. In contrasts, other cooked samples had lower antioxidant activity which might be due to lower amount of total phenolic content. On the other hand, all antioxidant capacity assays showed no correlation in terms of the markedly highest value with the total phenolic content of oven-dried samples.

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CHAPTER 5

CONCLUSION

Steaming and pressure-cooking may be the best cooking methods to be applied when preparing Lion's Mane Mushroom as a dish. This is due to the highest antioxidant levels obtained from these two cooking methods in all assays conducted. As in DPPH radical scavenging activity assay, oven-dried sample steamed for 5 minutes gave the highest activity followed by oven-dried sample submitted to 10 minutes of pressure-cooking. Fresh sample treated to 10 minutes of microwaving also showed a significant increased by 1.5-fold compared to fresh uncooked sample.

Both fresh and oven-dried samples worked in dose-dependent manner in terms of reducing power abilities. The most excellent abilities of cooked samples to reduce Fe^{3+}-ferricyanide complex to Fe^{2+} were 10 minutes steaming of oven-dried sample followed by 5 and 10 minutes pressure-cooking of fresh sample. Here, the duration of cooking for pressure-cooked sample did not play a significant role as both duration works best to enhance the antioxidant activity. Hence, as time-efficient suggestion, 5 minutes of pressure-cooking is sufficient.

However, cooking did not enhance the antioxidant activity of the mushroom in terms of lipid peroxidation inhibition. Only oven-dried sample treated to 5 minutes of microwaving significantly increased the peroxidation inhibition by 2-fold.

Similarly, TEAC levels did not increase in fresh cooked samples as in comparison to the uncooked. In contrast, both pressure-cooked samples (oven-dried) significantly enhanced the TEAC levels by 2.6-fold (5 minutes) and 1.5-fold (10 minutes). Boiling procedures decreased the TEAC levels in oven-dried sample.

Samples submitted to steaming and pressure-cooking also showed the highest level of total phenolic content, both for fresh and oven-dried samples. The phenolic content of fresh sample subjected to pressure-cooking exhibited an increase by 3-fold (5 minutes) and 2.8-fold (10 minutes). 10 minutes of steaming was the best method for oven-dried sample as phenolic content was the highest compared for both fresh and oven-dried samples.

The combination of various assays conducted in this study gave beneficial and relevant information in order to assess the antioxidant activity of uncooked and cooked *H. erinaceus* since there was no similar study conducted before. The results demonstrated in this study could provide useful scientific data and report if similar study were to be conducted in the future.

Overall results revealed that the level of antioxidant capacity and total phenolic content of oven-dried *H. erinaceus* was higher compared to the raw one. Oven-dried fruit body could be used by food industries specifically food manufacturing company as food ingredients or as replacement for synthetic antioxidants. This is because the dried extract of the mushroom can be stored and preserved for a longer period of time compared to the fresh extract. However, the correct technique and procedure of heating and drying should be further investigated if it were to be used on a large scale. On the other hand, the antioxidant capacity of the fresh samples presented in this study could also cater the demand for information by consumer especially woman that always do the cooking.

In conclusion, cooking procedures especially steaming and pressure-cooking significantly enhanced and increased the antioxidant capacity as well as the total phenolic content of *H. erinaceus* compared to the uncooked sample. Since the duration of cooking was just in minimal differences, the optimum cooking duration could not be determined

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from this study. Hence, further study investigating the effects of cooking procedures on the total antioxidant capacity of *H. erinaceus* is highly recommended with larger scope such as the addition of other cooking methods (e.g: frying and baking) as well as other antioxidant assays so that the verification of the data could be done. The identification of the active compounds present in the sample extract should also be taken into account so that this particular species of mushroom can be largely used by nutraceutical and pharmaceutical industry in the near future. Hence, the use and intake of this mushroom can be widely introduced to the consumer which might serve as possible protective agents to help lower the risk of oxidative damages as well as a good source of natural antioxidants.

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APPENDIX

Appendix A: Experimental and statistical data for DPPH radical scavenging activity.

(A1) DPPH radical scavenging activity of BHA and samples.

	Cooking methods	Cooking time (mins)	Absorbance Reading (515 nm)			Scavenging Activity (%)			Average	Std
			R1	R2	R3	R1	R2	R3		
Fresh samples	Uncooked	-	0.260	0.251	0.262	16.129	19.032	15.484	16.88	1.89
	Boiling	5	0.245	0.260	0.253	20.968	16.129	18.387	18.49	2.42
	Microwaving		0.248	0.255	0.270	20.000	17.742	12.903	16.88	3.63
	Steaming		0.235	0.248	0.232	24.194	20.000	25.161	23.12	2.74
	Pressure-cooking		0.245	0.242	0.234	20.968	21.935	24.516	22.47	1.83
	Boiling	10	0.254	0.254	0.262	18.065	18.065	15.484	17.20	1.49
	Microwaving		0.224	0.235	0.231	27.742	24.194	25.484	25.81	1.80
	Steaming		0.273	0.240	0.246	11.935	22.581	20.645	18.39	5.67
	Pressure-cooking		0.255	0.244	0.260	17.742	21.290	16.129	18.39	2.64
Oven-dried samples	Uncooked	-	0.272	0.268	0.269	12.258	13.548	13.226	13.01	0.67
	Boiling	5	0.250	0.232	0.233	19.355	25.161	24.839	23.12	3.26
	Microwaving		0.273	0.272	0.274	11.935	12.258	11.613	11.94	0.32
	Steaming		0.228	0.222	0.223	26.452	28.387	28.065	27.63	1.04
	Pressure-cooking		0.268	0.258	0.266	13.548	16.774	14.194	14.84	1.71
	Boiling	10	0.247	0.262	0.244	20.323	15.484	21.290	19.03	3.11
	Microwaving		0.258	0.265	0.254	16.774	14.516	18.065	16.45	1.80
	Steaming		0.253	0.252	0.246	18.387	18.710	20.645	19.25	1.22
	Pressure-cooking		0.231	0.226	0.227	25.484	27.097	26.774	26.45	0.85
BHA			0.050	0.050	0.050	83.871	83.871	83.871	83.87	0.00

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(A2) Statistical analysis for DPPH scavenging activity of samples.

a) Analysis of variance of fresh samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	251.001	8	31.375	3.618	.011
Within Groups	156.095	18	8.672		
Total	407.096	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT) of fresh samples.

Fresh Samples (3 mg/ml)	N	Subset for alpha = 0.05		
		a	b	c
Uncooked	3	16.88167		
Microwaving(5)	3	16.88167		
Boiling(10)	3	17.20467		
Steaming(10)	3	18.38700	18.38700	
Pressure-cooking(10)	3	18.38700	18.38700	
Boiling(5)	3	18.49467	18.49467	
Pressure-cooking(5)	3	22.47300	22.47300	22.47300
Steaming(5)	3		23.11833	23.11833
Microwaving(10)	3			25.80667
Sig.		.054	.092	.205

N = No. of replicates, a-c = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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c)Analysis of variance of oven-dried samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	769.927	8	96.241	28.573	.000
Within Groups	60.628	18	3.368		
Total	830.555	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

d) Duncan Multiple Range Test (DMRT) of oven-dried samples.

Oven-driedSamples (3 mg/ml)	N	Subset for alpha = 0.05				
		v	w	x	y	z
Microwaving(5)	3	11.93533				
Uncooked	3	13.01067				
Pressure-cooking(5)	3	14.83867	14.83867			
Microwaving(10)	3		16.45167	16.45167		
Boiling(10)	3			19.03233		
Steaming(10)	3			19.24733		
Boiling(5)	3				23.11833	
Pressure-cooking(10)	3					26.45167
Steaming(5)	3					27.63467
Sig.		.082	.296	.093	1.000	.440

N = No. of replicates, v-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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Appendix B: Experimental and statistical data for reducing power assay.

(B1) Experimental data of BHA and fresh samples (0.50 mg/ml).

a) Reducing power abilities of BHA and fresh samples (0.50 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Fresh samples	Uncooked	N/A	0.093	0.090	0.088	0.029	0.026	0.024	0.03	0.00
	Boiling	5	0.109	0.102	0.106	0.045	0.038	0.042	0.04	0.00
	Microwaving		0.088	0.083	0.092	0.024	0.019	0.028	0.02	0.00
	Steaming		0.100	0.090	0.096	0.036	0.026	0.032	0.03	0.01
	Pressure-cooking		0.159	0.142	0.152	0.095	0.078	0.088	0.09	0.01
	Boiling	10	0.086	0.087	0.082	0.022	0.023	0.018	0.02	0.00
	Microwaving		0.108	0.104	0.099	0.044	0.040	0.035	0.04	0.00
	Steaming		0.094	0.088	0.092	0.030	0.024	0.028	0.03	0.00
	Pressure-cooking		0.125	0.126	0.130	0.061	0.062	0.066	0.06	0.00
BHA			1.451	1.179	1.372	1.387	1.115	1.308	1.27	0.14

Ctrl = Control (0.064).

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(B2) Statistical analysis of BHA and fresh samples (0.50 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.095	9	.455	230.289	.000
Within Groups	.040	20	.002		
Total	4.135	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Fresh Samples (0.50 mg/ml)	N	Subset for alpha = 0.05	
		a	b
Boiling(10)	3	.02100	
Microwaving(5)	3	.02367	
Uncooked	3	.02633	
Steaming(10)	3	.02733	
Steaming(5)	3	.03133	
Microwaving(10)	3	.03967	
Boiling(5)	3	.04167	
Pressure-cooking(10)	3	.06300	
Pressure-cooking(5)	3	.08700	
BHA	3		1.27000
Sig.		.130	1.000

N = No. of replicates, a-b = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B3) Experimental data of BHA and oven-dried samples (0.50 mg/ml).

a) Reducing power abilities of BHA and oven-dried samples (0.50 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Oven-dried samples	Uncooked	N/A	0.133	0.131	0.139	0.069	0.067	0.075	0.07	0.00
	Boiling	5	0.105	0.111	0.107	0.041	0.047	0.043	0.04	0.00
	Microwaving		0.125	0.126	0.128	0.061	0.062	0.064	0.06	0.00
	Steaming		0.146	0.140	0.155	0.082	0.076	0.091	0.08	0.01
	Pressure-cooking		0.117	0.112	0.110	0.053	0.048	0.046	0.05	0.00
	Boiling	10	0.140	0.135	0.143	0.076	0.071	0.079	0.08	0.00
	Microwaving		0.132	0.127	0.132	0.068	0.063	0.068	0.07	0.00
	Steaming		0.174	0.157	0.154	0.110	0.093	0.090	0.10	0.01
	Pressure-cooking		0.115	0.108	0.118	0.051	0.044	0.054	0.05	0.01
BHA			1.451	1.179	1.372	1.387	1.115	1.308	1.27	0.14

Ctrl = Control (0.064).

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(B4) Statistical analysis of BHA and oven-dried samples (0.50 mg/ml).

a)Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.919	9	.435	219.416	.000
Within Groups	.040	20	.002		
Total	3.959	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Oven-dried Samples (0.50 mg/ml)	N	Subset for alpha = 0.05	
		y	z
Boiling(5)	3	.04367	
Pressure-cooking(5)	3	.04900	
Pressure-cooking(10)	3	.04967	
Microwaving(5)	3	.06233	
Microwaving(10)	3	.06633	
Uncooked	3	.07033	
Boiling(10)	3	.07533	
Steaming(5)	3	.08300	
Steaming(10)	3	.09767	
BHA	3		1.27000
Sig.		.211	1.000

N = No. of replicates, y-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B5) Experimental data of BHA and fresh samples (1.00 mg/ml).

a) Reducing power abilities of BHA and fresh samples (1.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Fresh samples	Uncooked	N/A	0.127	0.127	0.134	0.077	0.077	0.084	0.08	0.00
	Boiling	5	0.146	0.142	0.148	0.096	0.092	0.098	0.10	0.00
	Microwaving		0.126	0.123	0.120	0.076	0.073	0.070	0.07	0.00
	Steaming		0.145	0.139	0.146	0.095	0.089	0.096	0.09	0.00
	Pressure-cooking		0.227	0.233	0.233	0.177	0.183	0.183	0.18	0.00
	Boiling	10	0.119	0.118	0.118	0.069	0.068	0.068	0.07	0.00
	Microwaving		0.161	0.157	0.170	0.111	0.107	0.120	0.11	0.01
	Steaming		0.143	0.130	0.137	0.093	0.080	0.087	0.09	0.01
	Pressure-cooking		0.185	0.184	0.183	0.135	0.134	0.133	0.13	0.00
BHA			2.089	2.247	2.251	2.039	2.197	2.201	2.15	0.09

Ctrl = Control (0.05).

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(B6) Statistical analysis of BHA and fresh samples (1.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11.300	9	1.256	1445.496	.000
Within Groups	.017	20	.001		
Total	11.318	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Fresh Samples (1.00 mg/ml)	N	Subset for alpha = 0.05			
		a	b	c	d
Boiling(10)	3	.06833			
Microwaving(5)	3	.07300			
Uncooked	3	.07933	.07933		
Steaming(10)	3	.08667	.08667		
Steaming(5)	3	.09333	.09333		
Boiling(5)	3	.09533	.09533		
Microwaving(10)	3	.11267	.11267		
Pressure-cooking(10)	3		.13400	.13400	
Pressure-cooking(5)	3			.18100	
BHA	3				2.14567
Sig.		.121	.056	.065	1.000

N = No. of replicates, a-d = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B7) Experimental data of BHA and oven-dried samples (1.00 mg/ml).

a) Reducing power abilities of BHA and oven-dried samples (1.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Oven-dried samples	Uncooked	N/A	0.194	0.193	0.195	0.144	0.143	0.145	0.14	0.00
	Boiling	5	0.155	0.153	0.141	0.105	0.103	0.091	0.10	0.01
	Microwaving		0.182	0.182	0.176	0.132	0.132	0.126	0.13	0.00
	Steaming		0.213	0.213	0.216	0.163	0.163	0.166	0.16	0.00
	Pressure-cooking		0.153	0.147	0.158	0.103	0.097	0.108	0.10	0.01
	Boiling	10	0.187	0.201	0.197	0.137	0.151	0.147	0.15	0.01
	Microwaving		0.200	0.203	0.204	0.150	0.153	0.154	0.15	0.00
	Steaming		0.285	0.283	0.289	0.235	0.233	0.239	0.24	0.00
	Pressure-cooking		0.169	0.165	0.159	0.119	0.115	0.109	0.11	0.01
BHA			2.089	2.247	2.251	2.039	2.197	2.201	2.15	0.09

Ctrl = Control (0.05).

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(B8) Statistical analysis of BHA and oven-dried samples (1.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.869	9	1.208	1383.018	.000
Within Groups	.017	20	.001		
Total	10.886	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Oven-dried Samples (1.00 mg/ml)	N	Subset for alpha = 0.05			
		w	x	y	z
Boiling(5)	3	.09967			
Pressure-cooking(5)	3	.10267			
Pressure-cooking(10)	3	.11433	.11433		
Microwaving(5)	3	.13000	.13000		
Uncooked	3	.14400	.14400		
Boiling(10)	3	.14500	.14500		
Microwaving(10)	3	.15233	.15233		
Steaming(5)	3		.16400		
Steaming(10)	3			.23567	
BHA	3				2.14567
Sig.		.069	.082	1.000	1.000

N = No. of replicates, w-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B9) Experimental data of BHA and fresh samples (3.00 mg/ml).

a) Reducing power abilities of BHA and fresh samples (3.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Fresh samples	Uncooked	N/A	0.265	0.265	0.266	0.205	0.205	0.206	0.21	0.00
	Boiling	5	0.303	0.303	0.314	0.243	0.243	0.254	0.25	0.01
	Microwaving		0.258	0.262	0.258	0.198	0.202	0.198	0.20	0.00
	Steaming		0.349	0.362	0.378	0.289	0.302	0.318	0.30	0.01
	Pressure-cooking		0.584	0.563	0.598	0.524	0.503	0.538	0.52	0.02
	Boiling	10	0.212	0.208	0.216	0.152	0.148	0.156	0.15	0.00
	Microwaving		0.381	0.383	0.400	0.321	0.323	0.340	0.33	0.01
	Steaming		0.326	0.308	0.324	0.266	0.248	0.264	0.26	0.01
	Pressure-cooking		0.470	0.486	0.510	0.410	0.426	0.450	0.43	0.02
BHA			2.173	2.443	2.206	2.113	2.383	2.146	2.21	0.15

Ctrl = Control (0.06).

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(B10) Statistical analysis of BHA and fresh samples (3.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.290	9	1.143	499.557	.000
Within Groups	.046	20	.002		
Total	10.336	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Fresh Samples (3.00 mg/ml)	N	Subset for alpha = 0.05					
		a	b	c	d	e	f
Boiling(10)	3	.15200					
Microwaving(5)	3	.19933	.19933				
Uncooked	3	.20533	.20533				
Boiling(5)	3		.24667	.24667			
Steaming(10)	3		.25933	.25933			
Steaming(5)	3			.30300			
Microwaving(10)	3			.32800			
Pressure-cooking(10)	3				.42867		
Pressure-cooking(5)	3					.52167	
BHA	3						2.21400
Sig.		.211	.174	.069	1.000	1.000	1.000

N = No. of replicates, a-f = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B11) Experimental data of BHA and oven-dried samples (3.00 mg/ml).

a) Reducing power abilities of BHA and oven-dried samples (3.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Oven-dried samples	Uncooked	N/A	0.399	0.398	0.405	0.339	0.338	0.345	0.34	0.00
	Boiling	5	0.388	0.418	0.436	0.328	0.358	0.376	0.35	0.02
	Microwaving		0.475	0.502	0.528	0.415	0.442	0.468	0.44	0.03
	Steaming		0.417	0.405	0.436	0.357	0.345	0.376	0.36	0.02
	Pressure-cooking		0.393	0.415	0.411	0.333	0.355	0.351	0.35	0.01
	Boiling	10	0.504	0.504	0.557	0.444	0.444	0.497	0.46	0.03
	Microwaving		0.387	0.396	0.431	0.327	0.336	0.371	0.34	0.02
	Steaming		0.529	0.466	0.533	0.469	0.406	0.473	0.45	0.04
	Pressure-cooking		0.390	0.408	0.415	0.330	0.348	0.355	0.34	0.01
BHA			2.173	2.443	2.206	2.113	2.383	2.146	2.21	0.15

Ctrl = Control (0.06).

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(B12) Statistical analysis of BHA and oven-dried samples (3.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9.122	9	1.014	383.419	.000
Within Groups	.053	20	.003		
Total	9.175	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Oven-dried Samples (3.00 mg/ml)	N	Subset for alpha = 0.05				
		v	w	x	y	z
Uncooked	3	.34067				
Pressure-cooking(10)	3	.34433	.34433			
Microwaving(10)	3	.34467	.34467			
Pressure-cooking(5)	3	.34633	.34633			
Boiling(5)	3	.35400	.35400			
Steaming(5)	3	.35933	.35933	.35933		
Microwaving(5)	3		.44167	.44167	.44167	
Steaming(10)	3			.44933	.44933	
Boiling(10)	3				.46167	
BHA	3					2.21400
Sig.		.697	.052	.055	.658	1.000

N = No. of replicates, v-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B13) Experimental data of BHA and fresh samples (5.00 mg/ml).

a) Reducing power abilities of BHA and fresh samples (5.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Fresh samples	Uncooked	N/A	0.357	0.343	0.349	0.295	0.281	0.287	0.29	0.01
	Boiling	5	0.330	0.324	0.329	0.268	0.262	0.267	0.27	0.00
	Microwaving		0.371	0.353	0.368	0.309	0.291	0.306	0.30	0.01
	Steaming		0.380	0.356	0.373	0.318	0.294	0.311	0.31	0.01
	Pressure-cooking		0.751	0.733	0.727	0.689	0.671	0.665	0.68	0.01
	Boiling	10	0.242	0.236	0.245	0.180	0.174	0.183	0.18	0.00
	Microwaving		0.430	0.425	0.422	0.368	0.363	0.360	0.36	0.00
	Steaming		0.382	0.350	0.382	0.320	0.288	0.320	0.31	0.02
	Pressure-cooking		0.515	0.522	0.560	0.453	0.460	0.498	0.47	0.02
BHA			2.643	2.252	2.652	2.581	2.190	2.590	2.45	0.23

Ctrl = Control (0.062).

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(B14) Statistical analysis of BHA and fresh samples (5.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	12.434	9	1.382	257.827	.000
Within Groups	.107	20	.005		
Total	12.542	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Fresh Samples (5.00 mg/ml)	N	Subset for alpha = 0.05				
		a	b	c	d	e
Boiling(10)	3	.17900				
Boiling(5)	3	.26567	.26567			
Uncooked	3	.28767	.28767			
Microwaving(5)	3	.30200	.30200			
Steaming(5)	3	.30767	.30767			
Steaming(10)	3	.30933	.30933			
Microwaving(10)	3		.36367	.36367		
Pressure-cooking(10)	3			.47033		
Pressure-cooking(5)	3				.67500	
BHA	3					2.45367
Sig.		.066	.161	.090	1.000	1.000

N = No. of replicates, a-e = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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(B15) Experimental data of BHA and oven-dried samples (5.00 mg/ml).

a) Reducing power abilities of BHA and oven-dried samples (5.00 mg/ml).

	Cooking methods	Cooking time (mins)	Absorbance Reading (700 nm)			R1-Ctrl	R2-Ctrl	R3-Ctrl	Average	Std
			R1	R2	R3					
Oven-dried samples	Uncooked	N/A	0.543	0.541	0.524	0.481	0.479	0.462	0.47	0.01
	Boiling	5	0.544	0.543	0.537	0.482	0.481	0.475	0.48	0.00
	Microwaving		0.524	0.473	0.534	0.462	0.411	0.472	0.45	0.03
	Steaming		0.583	0.579	0.563	0.521	0.517	0.501	0.51	0.01
	Pressure-cooking		0.535	0.538	0.526	0.473	0.476	0.464	0.47	0.01
	Boiling	10	0.566	0.554	0.593	0.504	0.492	0.531	0.51	0.02
	Microwaving		0.505	0.510	0.565	0.443	0.448	0.503	0.46	0.03
	Steaming		0.823	0.817	0.804	0.761	0.755	0.742	0.75	0.01
	Pressure-cooking		0.475	0.457	0.480	0.413	0.395	0.418	0.41	0.01
BHA			2.643	2.252	2.652	2.581	2.190	2.590	2.45	0.23

Ctrl = Control (0.062).

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(B16) Statistical analysis of BHA and oven-dried samples (5.00 mg/ml).

a) Analysis of variance.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.516	9	1.168	211.470	.000
Within Groups	.111	20	.006		
Total	10.626	29			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT).

Oven-dried Samples (5.00 mg/ml)	N	Subset for alpha = 0.05		
		x	y	z
Pressure-cooking(10)	3	.40867		
Microwaving(5)	3	.44833		
Microwaving(10)	3	.46467		
Pressure-cooking(5)	3	.47100		
Uncooked	3	.47400		
Boiling(5)	3	.47933		
Boiling1(0)	3	.50900		
Steaming(5)	3	.51300		
Steaming(10)	3		.75267	
BHA	3			2.45367
Sig.		.149	1.000	1.000

N = No. of replicates, x-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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Appendix C: Experimental and statistical data for lipid peroxidation inhibition.

(C1) Lipid peroxidation inhibition of BHA and samples.

	Cooking methods	Cooking time (mins)	Absorbance Reading (532 nm)			Peroxidation Inhibition (%)			Average	Std
			R1	R2	R3	R1	R2	R3		
Fresh samples	Uncooked	-	0.202	0.221	0.192	24.060	16.917	27.820	22.93	5.54
	Boiling	5	0.250	0.212	0.232	6.015	20.301	12.782	13.03	7.15
	Microwaving		0.230	0.204	0.223	13.534	23.308	16.165	17.67	5.06
	Steaming		0.227	0.217	0.193	14.662	18.421	27.444	20.18	6.57
	Pressure-cooking		0.208	0.226	0.204	21.805	15.038	23.308	20.05	4.41
	Boiling	10	0.212	0.235	0.225	20.301	11.654	15.414	15.79	4.34
	Microwaving		0.226	0.221	0.206	15.038	16.917	22.556	18.17	3.91
	Steaming		0.257	0.217	0.230	3.383	18.421	13.534	11.78	7.67
	Pressure-cooking		0.249	0.236	0.208	6.391	11.278	21.805	13.16	7.88
Oven-dried samples	Uncooked	-	0.253	0.262	0.259	15.947	12.957	13.953	14.29	1.52
	Boiling	5	0.255	0.250	0.223	4.135	6.015	16.165	8.77	6.47
	Microwaving		0.184	0.204	0.178	30.827	23.308	33.083	29.07	5.12
	Steaming		0.262	0.244	0.215	1.504	8.271	19.173	9.65	8.91
	Pressure-cooking		0.252	0.258	0.250	5.263	3.008	6.015	4.76	1.57
	Boiling	10	0.259	0.230	0.214	2.632	13.534	19.549	11.90	8.58
	Microwaving		0.219	0.254	0.236	17.669	4.511	11.278	11.15	6.58
	Steaming		0.239	0.240	0.221	10.150	9.774	16.917	12.28	4.02
	Pressure-cooking		0.158	0.176	0.156	11.732	1.676	12.849	8.75	6.15
BHA			0.083	0.094	0.072	53.631	47.486	59.777	53.63	6.15

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(C2) Statistical analysis for lipid peroxidation inhibition of samples.

a) Analysis of variance of fresh samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	346.841	8	43.355	1.201	.352
Within Groups	649.765	18	36.098		
Total	996.606	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically no significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT) of fresh samples.

Fresh Samples (3 mg/ml)	N	Subset for alpha = 0.05
		1
Steaming(10)	3	11.77933
Boiling(5)	3	13.03267
Pressure-cooking(10)	3	13.15800
Boiling(10)	3	15.78967
Microwaving(5)	3	17.66900
Microwaving(10)	3	18.17033
Pressure-cooking(5)	3	20.05033
Steaming(5)	3	20.17567
Uncooked	3	22.93233
Sig.		.063

N = No. of replicates, a = Letters representing significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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c) Analysis of variance of oven-dried samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1126.860	8	140.858	3.923	.008
Within Groups	646.348	18	35.908		
Total	1773.209	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

d) Duncan Multiple Range Test (DMRT) of oven-dried samples.

Oven-dried Samples		Subset for alpha = 0.05	
(3 mg/ml)	N	y	z
Pressure-cooking(5)	3	4.76200	
Pressure-cooking(10)	3	8.75233	
Boiling(5)	3	8.77167	
Steaming(5)	3	9.64933	
Microwaving(10)	3	11.15267	
Boiling(10)	3	11.90500	
Steaming(10)	3	12.28033	
Uncooked	3	14.28567	
Microwaving(5)	3		29.07267
Sig.		.105	1.000

N = No. of replicates, y-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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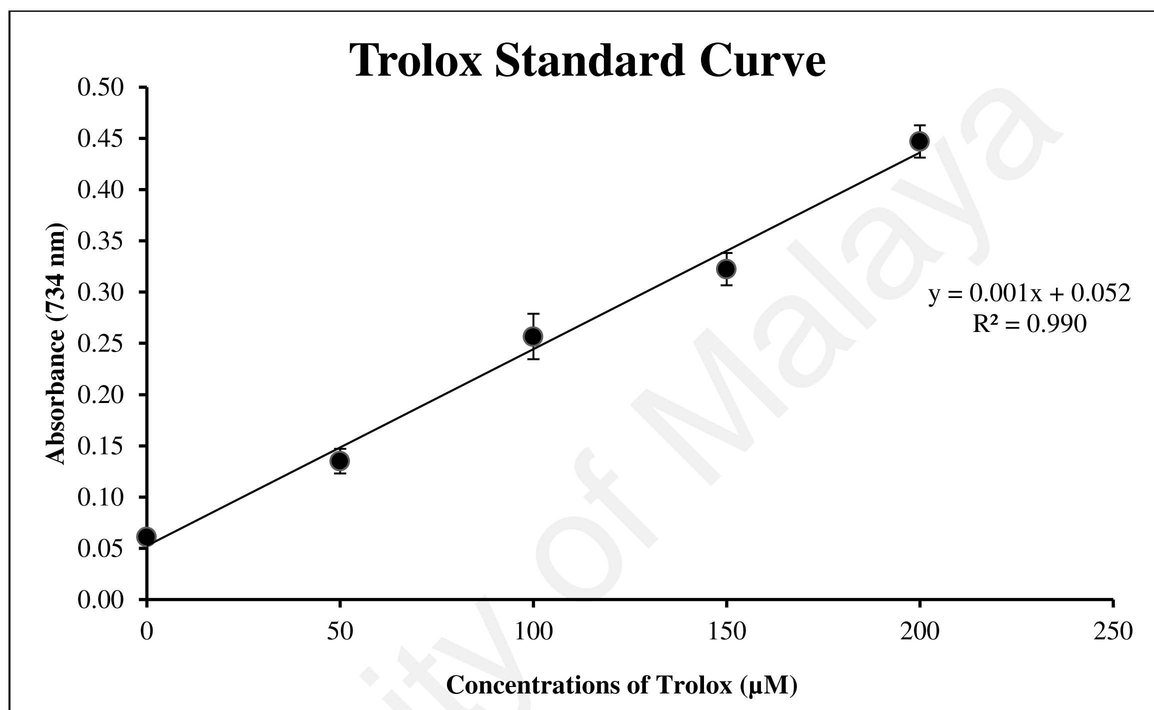
Appendix D: Experimental and statistical data for Trolox Equivalent Antioxidant Capacity (TEAC).

(D1) Trolox standard curve.

a) Absorbance at 734 nm against concentrations of Trolox (µM).



*Each value is expressed as mean ± standard deviation (*n*=3).

(D2) TEAC values of BHA.

BHA Concentration: 3 mg/ml

BHA	Absorbance Reading (734 nm)			Blank-Abs	Blank-Abs	Blank-Abs	µM Trolox (Average)	µM Trolox/g BHA	Std
	R1	R2	R3	R1	R2	R3			
	0.056	0.065	0.068	0.649	0.64	0.637	590.00	1.97×10^5	2.08×10^3

Blank = 0.705

Calculation:

3 mg/ml BHA = 3 mg of BHA in 1 ml distilled water/reaction medium
= 0.003 g of BHA in 1 ml distilled water/reaction medium

µM Trolox = (Absorbance – 0.052)/0.001 [Standard curve equation]

µM Trolox/g BHA = µM Trolox/0.003 g BHA

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(D3) TEAC values of fresh and oven-dried samples.

	Cooking methods	Cooking time (mins)	Crude extracts (mg) in 150 g fruit bodies	Absorbance Reading (734 nm)			M Trolox in 0.3 mg extract			M Trolox/150 g of fruit bodies			Average	Std
				R1	R2	R3	R1	R2	R3	R1	R2	R3		
Fresh samples	Uncooked	-	4180.0	0.381	0.349	0.363	0.00003	0.00003	0.00003	0.458	0.414	0.433	0.44	0.02
	Boiling	5	5260.0	0.235	0.233	0.266	0.00002	0.00002	0.00002	0.321	0.317	0.375	0.34	0.03
	Microwaving		4820.0	0.340	0.350	0.344	0.00003	0.00003	0.00003	0.463	0.479	0.469	0.47	0.01
	Steaming		4910.0	0.228	0.248	0.218	0.00002	0.00002	0.00002	0.288	0.321	0.272	0.29	0.02
	Pressure-cooking		6950.0	0.099	0.131	0.081	0.00000	0.00001	0.00000	0.109	0.183	0.067	0.12	0.06
	Boiling	10	3840.0	0.317	0.319	0.327	0.00003	0.00003	0.00003	0.339	0.342	0.352	0.34	0.01
	Microwaving		4720.0	0.207	0.208	0.207	0.00002	0.00002	0.00002	0.244	0.245	0.244	0.24	0.00
	Steaming		3720.0	0.178	0.192	0.182	0.00001	0.00001	0.00001	0.156	0.174	0.161	0.16	0.01
	Pressure-cooking		7420.0	0.129	0.108	0.113	0.00001	0.00001	0.00001	0.190	0.139	0.151	0.16	0.03
Oven-dried samples	Uncooked	-	5200.0	0.160	0.150	0.155	0.00001	0.00001	0.00001	0.187	0.170	0.179	0.18	0.01
	Boiling	5	3790.0	0.104	0.097	0.101	0.00001	0.00000	0.00000	0.066	0.057	0.062	0.06	0.00
	Microwaving		4590.0	0.110	0.151	0.128	0.00001	0.00001	0.00001	0.089	0.151	0.116	0.12	0.03
	Steaming		5880.0	0.111	0.157	0.116	0.00001	0.00001	0.00001	0.116	0.206	0.125	0.15	0.05
	Pressure-cooking		10610.0	0.184	0.197	0.173	0.00001	0.00001	0.00001	0.467	0.513	0.428	0.47	0.04
	Boiling	10	4460.0	0.130	0.129	0.125	0.00001	0.00001	0.00001	0.116	0.114	0.109	0.11	0.00
	Microwaving		7720.0	0.153	0.132	0.122	0.00001	0.00001	0.00001	0.260	0.206	0.180	0.22	0.04
	Steaming		9510.0	0.113	0.099	0.110	0.00001	0.00000	0.00001	0.193	0.149	0.184	0.18	0.02
	Pressure-cooking		9710.0	0.127	0.140	0.134	0.00001	0.00001	0.00001	0.243	0.285	0.265	0.26	0.02

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Calculation:

Sample concentration = 3 mg/ml

Sample load = 100 µl (0.1 ml)/cuvette

Total volume for assay mixture = 1000 µl (1.0 ml)

If 1 mg/ml = 1 mg extract/1 ml distilled water, then in 0.1 ml = 0.1 mg extract

For 3 mg/ml extract: 0.1 mg extract x 3 = **0.3 mg extract/cuvette**

In 0.3 mg extract:

For example uncooked fresh sample (R1)

Crude extract = 4180.0 mg/150 g fruit bodies

Gram (g) of fruit bodies in a cuvette (0.3 mg extract) = (1 g/4180.0 mg) x 0.3 mg

= **0.0000718 g of fruit bodies/cuvette**

Based on Trolox standard curve: y = 0.001*x* + 0.052

Thus, µM Trolox = (Absorbance (R1) - 0.052)/0.001

= (0.381-0.052)/0.001

= **329 µM Trolox**

Hence, based on working concentration:

3 mg/ml = 329 µM Trolox

0.3 mg extract in 0.1 ml = 329 µM Trolox

0.3 mg extract = 329 µM Trolox x 0.1 ml

= 32.9 µM Trolox in 1.0 ml (total volume in a cuvette)

= 0.0329 mMTrolox

= **0.0000329 M Trolox**

Thus, M Trolox/150 g fruit bodies = 0.0000329 M Trolox/0.0000718 g

= **0.458 M Trolox/150 g fruit bodies**

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(D4) Statistical analysis for TEAC values of samples.

a) Analysis of variance of fresh samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.368	8	.046	63.444	.000
Within Groups	.013	18	.001		
Total	.381	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT) of fresh samples.

Fresh Samples (3 mg/ml)	N	Subset for alpha = 0.05				
		a	b	c	d	e
Pressure-cooking(5)	3	.11967				
Pressure-cooking(10)	3	.16000				
Steaming(10)	3	.16367				
Microwaving(10)	3		.24433			
Steaming(5)	3			.29367		
Boiling(5)	3			.33767	.33767	
Boiling(10)	3				.34433	
Uncooked	3					.43500
Microwaving(5)	3					.47033
Sig.		.073	1.000	.061	.765	.125

N = No. of replicates, a-e = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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c) Analysis of variance of oven-dried samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.341	8	.043	47.993	.000
Within Groups	.016	18	.001		
Total	.357	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

d) Duncan Multiple Range Test (DMRT) of oven-dried samples.

Oven-dried Samples (3 mg/ml)	N	Subset for alpha = 0.05					
		u	v	w	x	y	z
Boiling(5)	3	.06167					
Boiling(10)	3		.11300				
Microwaving(5)	3		.11867				
Steaming(5)	3		.14900	.14900			
Steaming(10)	3			.17533	.17533		
Uncooked	3			.17867	.17867		
Microwaving(10)	3				.21533	.21533	
Pressure-cooking(10)	3					.26433	
Pressure-cooking(5)	3						.46933
Sig.		1.000	.177	.263	.136	.059	1.000

N = No. of replicates, u-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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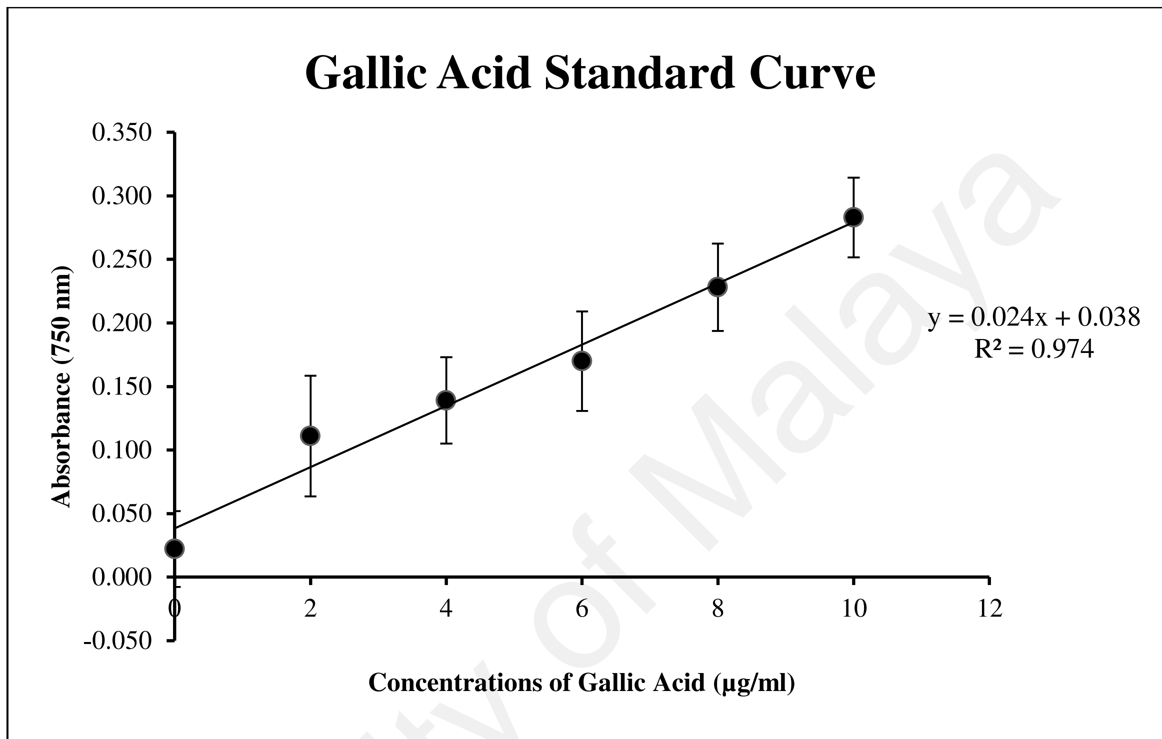
Appendix E: Experimental and statistical data for total phenolic content.

(E1) Gallic acid standard curve.

a) Absorbance at 750 nm against concentrations of Gallic Acid (µg/ml).



*Each value is expressed as mean ± standard deviation (*n*=3).

(E2) Total phenolic content of BHA.

BHA Concentration: 3 mg/ml

BHA	Absorbance Reading (750 nm)			mg GAE			mg GAE/ g BHA (Average)	g GAE/ g BHA	Std
	R1	R2	R3	R1	R2	R3			
	3.24	3.159	3.179	133.417	130.042	130.875	43814.81	43.81	0.59

3 mg/ml BHA = 3 mg of BHA in 1 ml distilled water/reaction medium
= 0.003 g of BHA in 1 ml distilled water/reaction medium
mg GAE = (Absorbance – 0.038)/0.024 [Standard curve equation]
mg GAE/g BHA = mg GAE/0.003 g BHA
g GAE/g BHA = (mg GAE/0.003 g BHA)/1000

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(E3) Total phenolic content of fresh and oven-dried samples.

	Cooking methods	Cooking time (mins)	Crude extracts (mg) in 150 g fruit bodies	Absorbance Reading (750 nm)			mg GAE in 0.5 mg extract			mg GAE/150 g of fruit bodies			Average	Std
				R1	R2	R3	R1	R2	R3	R1	R2	R3		
Fresh samples	Uncooked	-	4180.0	0.531	0.528	0.507	0.0103	0.0102	0.0098	28.621	28.447	27.228	28.10	0.76
	Boiling	5	5260.0	0.630	0.699	0.639	0.0123	0.0138	0.0125	43.249	48.290	43.906	45.15	2.74
	Microwaving		4820.0	0.486	0.554	0.491	0.0093	0.0108	0.0094	29.991	34.543	30.326	31.62	2.54
	Steaming		4910.0	0.771	0.745	0.739	0.0153	0.0147	0.0146	49.987	48.213	47.804	48.67	1.16
	Pressure-cooking		6950.0	0.924	0.927	0.921	0.0185	0.0185	0.0184	85.524	85.813	85.234	85.52	0.29
	Boiling	10	3840.0	0.493	0.491	0.510	0.0095	0.0094	0.0098	24.267	24.160	25.173	24.53	0.56
	Microwaving		4720.0	0.774	0.830	0.817	0.0153	0.0165	0.0162	48.249	51.920	51.068	50.41	1.92
	Steaming		3720.0	0.716	0.678	0.682	0.0141	0.0133	0.0134	35.030	33.067	33.273	33.79	1.08
	Pressure-cooking		7420.0	0.781	0.813	0.798	0.0155	0.0161	0.0158	76.570	79.868	78.322	78.25	1.65
Oven-dried samples	Uncooked	-	5200.0	0.796	0.795	0.781	0.0158	0.0158	0.0155	54.744	54.672	53.661	54.36	0.61
	Boiling	5	3790.0	0.846	0.841	0.831	0.0168	0.0167	0.0165	42.532	42.269	41.743	42.18	0.40
	Microwaving		4590.0	0.931	0.911	0.936	0.0186	0.0182	0.0187	56.929	55.654	57.248	56.61	0.84
	Steaming		5880.0	0.839	0.809	0.838	0.0167	0.0161	0.0167	65.415	62.965	65.333	64.57	1.39
	Pressure-cooking		10610.0	0.641	0.668	0.643	0.0126	0.0131	0.0126	88.859	92.838	89.153	90.28	2.22
	Boiling	10	4460.0	0.842	0.867	0.834	0.0168	0.0173	0.0166	49.803	51.352	49.308	50.15	1.07
	Microwaving		7720.0	0.844	0.855	0.846	0.0168	0.0170	0.0168	86.421	87.601	86.636	86.89	0.63
	Steaming		9510.0	0.907	0.907	0.905	0.0181	0.0181	0.0181	114.780	114.780	114.516	114.69	0.15
	Pressure-cooking		9710.0	0.636	0.653	0.597	0.0125	0.0128	0.0116	80.647	82.940	75.387	79.66	3.87

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Calculation:

Sample concentration = 3 mg/ml

Sample load = 500 µl (0.5 ml)/cuvette

Total volume for assay mixture = 2000 µl (2.0 ml)

If 1 mg/ml = 1 mg extract/1 ml distilled water, then in 0.5 ml = 0.5 mg extract

For 3 mg/ml extract: 0.5 mg extract x 3 = **1.5 mg extract/cuvette**

In 1.5 mg extract:

For example uncooked fresh sample (R1)

Crude extract = 4180.0 mg/150 g fruit bodies

Gram (g) of fruit bodies in a cuvette (1.5 mg extract) = (1 g/4180.0 mg) x 1.5 mg

= **0.0003589 g of fruit bodies/cuvette**

Based on Gallic Acid standard curve: y = 0.024*x* + 0.038

Thus, µg GAE = (Absorbance (R1) - 0.052)/0.001

= (0.531-0.038)/0.024

= **20.542 µg GAE**

Hence, based on working concentration:

3 mg/ml = 20.542 µg GAE

0.3 mg extract in 0.5 ml = 20.542 µg GAE

0.3 mg extract = 20.542 µg GAE x 0.5 ml

= 10.271 µg GAE in 2.0 ml (total volume in a cuvette)

= **0.01027 mg GAE**

Thus, mg GAE/150 g fruit bodies = 0.01027 mg GAE/0.0003589 g

= **28.62 mg GAE/150 g fruit bodies**

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(E4) Statistical analysis for total phenolic content of samples.

a) Analysis of variance of fresh samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11251.172	8	1406.396	531.196	.000
Within Groups	47.657	18	2.648		
Total	11298.829	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

b) Duncan Multiple Range Test (DMRT) of fresh samples.

Fresh Samples (3 mg/ml)	N	Subset for alpha = 0.05						
		a	b	c	d	e	f	g
Boiling(10)	3	24.53333						
Uncooked	3		28.10000					
Microwaving(5)	3			31.62000				
Steaming(10)	3			33.79000				
Boiling(5)	3				45.15000			
Steaming(5)	3					48.66667		
Microwaving(10)	3					50.41333		
Pressure-cooking(10)	3						78.25333	
Pressure-cooking(5)	3							85.52000
Sig.		1.000	1.000	.120	1.000	.205	1.000	1.000

N = No. of replicates, a-g = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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c) Analysis of variance of oven-dried samples.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13196.608	8	1649.576	602.680	.000
Within Groups	49.267	18	2.737		
Total	13245.875	26			

The ANOVA table shows the F-ratio and P-value. Since the P-value of the F-test is less than 0.05, there is statistically significant difference between the mean absorbance from one sample to another at 95% confidence level.

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d) Duncan Multiple Range Test (DMRT) of oven-dried samples.

Oven-dried Samples (3 mg/ml)	N	Subset for alpha = 0.05							
		s	t	u	v	w	x	y	h
Boiling(5)	3	42.18000							
Boiling(10)	3		50.15333						
Uncooked	3			54.35667					
Microwaving(5)	3			56.61000					
Steaming(5)	3				64.57333				
Pressure-cooking(10)	3					79.66000			
Microwaving(10)	3						86.88667		
Pressure-cooking(5)	3							90.28333	
Steaming(10)	3								114.69333
Sig.		1.000	1.000	.113	1.000	1.000	1.000	1.000	1.000

N = No. of replicates, s-z = Letters representing different significant values.
(5) = 5 minutes of cooking duration, (10) = 10 minutes of cooking duration.

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