



UNIVERSITI PUTRA MALAYSIA

***ANTIOXIDANT PROPERTIES OF FRESH AND FROZEN PEEL OF
LEMON, KEY LIME AND MUSK LIME AS A POTENT SOURCE OF
ANTIOXIDANT***

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BY

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LIST OF ABBREVIATION

ANOVA	Analysis of variance
DPPH	1,1 Diphenyl-2-picrylhydrazyl
DW	Dry weight
EC ₅₀	Efficient Concentration at 50%
FRAP	Ferric ion reducing antioxidant power
TPTZ	2,4,6-tri-2(2-pyridyl)-s-triazine
FW	Fresh weigh
g	Gram
GAE	Gallic acid equivalent
QE	Quercetin equivalent
ROS	Reactive oxygen species
SD	Standard deviation
TPC	Total phenolic content
TFC	Total flavonoid content
nm	Nano meter
mg	Milli gram
ml	Mililiter
%	Percentage

ABSTRACT

ANTIOXIDANT PROPERTIES OF FRESH AND FROZEN LEMON, KEY LIME AND MUSK LIME AS A SOURCE OF POTENT ANTIOXIDANT.

Nur Fatin Inazlina NA

Citrus belong to family *Rutaceae* appear as promising sources of beneficial components as it contains high phenolic and flavonoid compound. However, citrus peel often being discarded as waste which contain high polyphenol and believed able to protect from chronic diseases. The freezing process may offer better antioxidant yield, polyphenol content and activity based on previous literature. This study aimed to determine antioxidant content and activity of fresh and frozen selected citrus peels. Frozen and fresh peels of lemon, key lime and musk lime were subjected to Folin-Ciocalteu reagent for phenolic content (TPC) determination and aluminium chloride calorimetric assay for total flavonoid content (TFC) determination. The antioxidant activity determined by DPPH free radical scavenging activity assay and ferric ion reducing antioxidant power assay (FRAP). The TPC of frozen samples were significantly higher than fresh samples ($p<0.05$) and ranged from 72.01 ± 0.67 mg GAE/g to 136.48 ± 0.58 mg GAE/g. Similarly, TFC were also significantly different ($p<0.05$) ranging from 50.51 ± 1.36 mg QE/g to 178.32 ± 2.13 mg QE/g. The EC_{50} values obtained from DPPH free radical scavenging assay ranged from 0.823 ± 0.1 mg/ml to 3.16 ± 0.92 mg/ml with frozen citrus peels having a lower value indicating its higher antioxidant activity as compared to the fresh citrus peels. Furthermore, frozen citrus peels exhibited higher value in FRAP assay compared to fresh citrus peels sample that range from 0.38 ± 0.003 mM Fe^{2+} /g to 0.53 ± 0.009 mM Fe^{2+} /g with significant different between the fresh and frozen citrus peel samples ($p<0.05$). For Pearson correlation, there is no correlation between DPPH with TPC and TFC. However, there is positive correlation exist between FRAP with TPC and TFC ($r=0.783$, $r=0.681$, respectively). Overall, the present study showed frozen citrus peels possess higher antioxidant activity and content compared to fresh citrus peels. Suggesting frozen citrus peel as more potent source of natural antioxidant.

ABSTRAK

SIFAT ANTIOKSIDAN DALAM KULIT LEMON, LIMAU NIPIS DAN LIMAU KASTURI SEGAR DAN BEKU SEBAGAI SUMBER ANTIOKSIDAN YANG KUAT.

Nur Fatin Inazlina NA

Sitrus berasal daripada keluarga *Rutaceae* muncul sebagai sumber yang menjanjikan komponen bermanfaat kerana mengandungi kandungan fenolik dan flavonoid yang tinggi. Kandungan polifenol yang tinggi di dalam kulit dan dipercayai dapat melindungi daripada penyakit kronik namun begitu ia sering kali dibuang sebagai sisa. Proses pembekuan menawarkan hasil antioksidan yang lebih baik, kandungan polifenol dan aktiviti berdasarkan kajian terdahulu. Kajian ini bertujuan untuk menentukan kandungan antioksidan dan aktiviti pada kulit sitrus dalam keadaan segar dan beku. Kulit lemon, limau nipis dan limau kasturi segar dan beku telah diuji reaksi Folin-Ciocalteu untuk penentuan kandungan fenolik (TPC) dan assay kalorimetri aluminium klorida untuk penentuan kandungan flavonoid (TFC). Aktiviti antioksidan yang ditentukan oleh DPPH aktiviti pengurangan radikal bebas dan ujian pengurangan ion ferric dan potensi antioksida (FRAP). Sampel beku mempunyai kandungan fenolik lebih tinggi daripada sampel segar ($p < 0.05$) dan berkisar dari 72.01 ± 0.67 mg GAE/g kepada 136.48 ± 0.58 mg GAE/g. Seterusnya, sampel beku juga turut mempunyai kandungan flavonoid lebih tinggi ($p < 0.05$) berkisar dari 50.51 ± 1.36 mg QE/g hingga 178.32 ± 2.13 mg QE/g. Nilai EC_{50} yang diperolehi dari ujian radikal bebas DPPH berkisar daripada 0.823 ± 0.1 mg/ml kepada 3.16 ± 0.92 mg/ml. Kulit sitrus beku mempunyai nilai lebih rendah menunjukkan aktiviti antioksidannya yang lebih tinggi berbanding dengan kulit sitrus segar. Tambahan pula, kulit sitrus beku menunjukkan nilai yang lebih tinggi dalam ujian FRAP berbanding dengan sampel kulit sitrus segar yang berkisar antara 0.38 ± 0.003 mM Fe^{2+} /g hingga 0.53 ± 0.009 mM Fe^{2+} /g dengan perbezaan yang signifikan antara sampel kulit jeruk segar dan beku ($p < 0.05$). Bagi korelasi Pearson, tiada korelasi antara DPPH dengan TPC dan TFC. Walau bagaimanapun, terdapat hubungan positif yang kuat antara FRAP dengan TPC dan TFC ($r = 0.783$, $r = 0.681$). Secara keseluruhannya, kajian ini menunjukkan kulit sitrus beku mempunyai aktiviti antioksidan yang lebih tinggi dan kandungan berbanding kulit sitrus segar. Kulit seruk beku dicadangkan sebagai sumber antioksidan semula jadi yang lebih kuat.

CHAPTER 1

INTRODUCTION

1.1 Research background

Recently, there is significant shifting in disease pattern from under nutrition and infectious diseases toward chronic and non-communicable disease. This clearly shown by National Health and Morbidity Survey clearly show the increase prevalence of chronic and non-communicable diseases (Institute for Public Health, 2015). Dietary pattern is a major cause affecting development of these disease. Today's lifestyle, exposed to many types of free radical and environmental pollution in air, water and soil which lead to development of chronic diseases. Free radical or reactive oxygen species (ROS) is the result from normal essential metabolic processes and from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. The implication of free radicals and ROS exposure exert a multitude of biological effects covering many diseases that may have implication in physiological regulatory function such as cardiovascular diseases, atherosclerosis, chronic inflammation, and neurodegenerative (Datta, Sinha, & Chattopadhyay, 2000).

Apart from this food had become a main concern as one of main contributor to maintain a good health. Food with high antioxidant can lower the chances to get disease. It was reported that natural antioxidant from plant sources have a protective effect against development of chronic disease (Wootton-Beard & Ryan, 2011). Thus, antioxidant is important as protectant to shield human body from damaging oxidation reaction by reducing the free radical and other reactive oxygen species and inhibit the process of oxidation (Shafekh et al., 2012).

Generally, citrus fruit production about 88 million tons worldwide annually (Negro, Mancini, Ruggeri, & Fino, 2016) while Malaysia also had grown a lot of citrus fruits as a dietary sources and income generator. Citrus fruits had been planted for domestic and export use which account 6,208 hectare around this country (Mohamed & Rokiah, 2010). Citrus fruits had been proven as one of key source of antioxidant. Citrus fruits in genus *Citrus L.* belong to family Rutaceae which include fruits such as lemon, orange, lime, mandarin, sour lime orange and grapefruits. These fruits appear as promising sources rich in multiple beneficial components for human as it is a good source of phenolic compound and dietary fibre with the large portion of its peel often being discarded as a waste into the environment. Total yield of peel is around 44% as by-product from a single citrus fruits (Li et al., 2006). There are studies reported that citrus provide ample supply of antioxidant such as flavonoid in citrus peel that able to increase serum antioxidant capacity against lipid peroxidation (Assini et. al., 2013). The citrus peel also found as a rich source of health beneficial compound such as ascorbic-acid, carotenoids and poly-phenolic antioxidant (Anwar et at., 2008). Thus, processing the citrus by-products can act as potential nutraceutical resources as it possesses health potential properties (Rafiq et al., 2016).

Besides, public formally believe that fresh fruits have better effect of antioxidant. Previous studies on fruits and vegetables sample on the antioxidant content and activity had shown that both frozen and fresh sample are the similar in antioxidant content and activity (Hunter & Fletcher, 2002). Nevertheless, the antioxidant in the fresh sample had the highest content of ascorbic acid however it is low in flavonoid content, lycopene content and reducing power (Chang, Lin, Chang, & Liu, 2006). Additionally, several studies have showed that frozen sample yield higher amount of antioxidant compared to fresh sample, this study conducted on ajwain leaves (Raza, Shukla, Fatima, & Ali, 2015). Similarly, of higher amount of antioxidant content and activity in frozen strawberries and raspberries compared to fresh sample have been reported (Turmanidze, Jgenti, Gulua, & Shaiashvili, 2017).

1.2 Problem Statement

According to World Health Organization, WHO (2014) there were around 146,000 of total death which due to NCDs, estimated around 73% from total death in Malaysia. Today, over production of free radicals has been demonstrated to be an exclusive damaging factor that cause molecular transformations and gene mutations in many types of organisms all of which leads to development of diseases such as non-communicable diseases (NCDs) and others (Barreira, Ferreira, Oliveira, & Pereira, 2008; Lai & Lim, 2011). Besides that, atherosclerosis and cancer can be due to salient free radical diseases that can cause death which is included in NCDs. According to WHO (2014), cancer account 15% from total death of NCDs in this country and there

were about 18,219 cases of cancer incidence in Malaysia (National Cancer Registry, 2007). Thus it clearly shown that free radicals can caused many chronic diseases in human body.

Dietary antioxidants appear as one of significant prevention against many diseases which can inhibit or delay the oxidation of an easy oxidized substrate. Thus, there were many studies on antioxidants as functional ingredient used in the food industry nowadays and there is also abundance of production of synthetic antioxidant in the market. Thereby, the use of natural antioxidants from plant extracts have attracted increasing interest due to consumer concern about the safety of the synthetic antioxidants in food (Sun & Ho, 2005).

In addition, citrus fruits are an important crop that are mainly used by the food industry. The by-product which is citrus peel was always being thrown or discarded as it is an inedible part of the fruits. Citrus by-product are potential sources of bioactive ingredient and valuable technological and nutritional properties which have ability to be processed as food and ingredient additives (Shea, Arendt, & Gallagher, 2012). The citrus peels are commonly viewed as primary citrus by-product which represent around 50-65% of the fruits weight which usually will be discarded and considered as a massive load toward the environment (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011). Lemon and orange peels are common waste produce from food processing and juice extraction industry worldwide. Thus, there is a need to study on potential source of cheap antioxidant. Because of that, there are limited of published data of antioxidant compound in peel part of citrus fruits in Malaysia. Therefore, the purpose of this study was to determine the total phenolic content, total flavonoid content and antioxidant activity of fresh and frozen selected citrus peels and compare with fresh peels.

1.3 Significance of research

The antioxidant is a major concern in disease prevention of non-communicable disease and other chronic diseases. The use of antioxidant compounds in foods are gaining a great deal of attention because of their potential health benefits (Datta et al., 2000).

This study provides information about antioxidant contents and antioxidant activity in frozen lemon, key lime and musk lime peels. Even though, there are studies have been conducted regarding citrus fruits in Malaysia but not much satisfactory information exists regarding the antioxidant content and activity in citrus peels. From previous study the total phenolic content, total flavonoid and antioxidant activity were based on comparison of air drying treatment and fresh citrus peels sample (Hiri, Ioannou, & Ghoul, 2015). There is also no published study comparing between frozen treated citrus peels and fresh citrus peels in Malaysia.

Previously, a study found that peel of citrus fruit contain higher amount of antioxidant compared to other components (Anagnostopoulou et al., 2006). There is study on frozen and fresh sample of orange showing that freezing treatment significantly boost the antioxidant content and amount. Therefore, in this study the frozen and fresh peels of lemon, lime and musk lime were selected to make the comparison between them.

In addition, the freezing treatment of post-harvest possesses positive affect on nutrient quality, specifically nutrient with antioxidant potential (Villa-Rodriguez, Palafox-Carlos, Yahia, Ayala-Zavala, & Gonzalez-Aguilar, 2015). However, consumer had a perception that fresh fruit and vegetable is a good source of nutrient and antioxidant compared to frozen fruit and vegetable (Antos, Sanchez, Pausal-Teresa, Cano, 2006). Thus, results of this study can reveal information on antioxidant

properties of frozen citrus peels. Although, there is report regarding effect of freezing on strawberries, blueberries, cauliflower, broccoli, green bean and sweet corn, the result are not significant for each fruits and vegetable as some of the frozen samples result higher antioxidant content while some are not. (Li, Pegg, Eitenmiller, Chun, & Kerrihard, 2017). Besides, the previous study also did not focus on the content of phenolic, flavonoid, scavenging activity (DPPH) and Ferric reducing antioxidant potential.

Besides, most of the studies on antioxidant content and activity of citrus fruits have been conducted overseas while data for local citrus fruits are still lacking even though Malaysia is one of the producer of citrus such as musk lime. Thus, it is expected that the knowledge obtained from this study will provide a baseline information on total phenolic content, total flavonoid content and antioxidant activity of frozen citrus fruits peels for research especially in Malaysia.

Next, this study will promote the potential use of citrus peel to be developed as natural functional food that high in antioxidant. Peels is a part that is always being discarded and less likely to be consumed. Thus, it will increase understanding and awareness of the significance benefits of citrus peels. Data of the study on antioxidants of the citrus peels may offer more natural materials for the food industries. Good quality of citrus peels can be used as flavouring ingredients rich in natural antioxidant. Thus, this study will determine the total phenolic contents, total flavonoid contents and antioxidant activity of frozen and fresh lemon, key lime and musk lime peels.

1.4 Objectives

1.4.1 General Objective

- To determine and compare antioxidant properties of fresh and frozen peels of lemon, key lime and musk lime.

1.4.2 Specific objectives

- To determine and compare the scavenging activity (DPPH) and ferric reducing power (FRAP assay) of fresh and frozen peel of citrus fruits.
- To determine and compare total phenolic contents and total flavonoid contents of fresh and frozen peel of citrus fruits.
- To determine and compare the total phenolic content, total flavonoid content, scavenging activity (DPPH) and ferric reducing power (FRAP) between different frozen citrus peel.
- To correlate between antioxidant content and activity of fresh and frozen peel of citrus fruits.

1.5 Null Hypothesis

- **There is no significant difference between scavenging activity and ferric reducing power of fresh and frozen peels of citrus fruits.**
- **There is no significant difference of total phenolic contents and total flavonoid content of fresh and frozen peels of citrus fruits.**
- **There is no significant difference of total phenolic content, total flavonoid content, scavenging activity and ferric reducing potential between different citrus peels.**
- **There is no significant correlation between antioxidant content and antioxidant activity of fresh and frozen peels of citrus fruits.**

CHAPTER 2

LITERATURE REVIEW

2.1 Free radicals and their biological effect

2.1.1 Reactive oxygen species (ROS) and free radicals

Any chemical species have one or more unpaired electron at outermost shell referred as free radical (Ratnam, Ankola, Bhardwaj, Sahana, & Kumar, 2006). The free radical able to cause adverse biological effect called “oxidative stress” and “nitrosative stress” (Valko et al., 2007). Most of the reactive species emit free radicals and have competency of independent existence that contains an unpaired electron in an atomic orbital which are unstable and highly reactive (Lobo, Patil, Phatak & Chandra 2010). Free radical can be generated from exogenous source and endogenous sources (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010).

Oxygen is one of the most indispensable element in every creature for life, Nevertheless, oxygen can also have several unfavorable effects on the organisms include human body under certain circumstances. Reactive oxygen species generation is essential to many important biological processes. ROS are small in size and have a

highly reactive oxygen containing molecule which may function as redox messenger however in high concentration, it may result cell damage and death (Ghosh, Das, Chaffee, Roy, K. Sen, 2018). Reactive oxygen species are unstable molecule able to react with DNA, lipid membrane and protein (Obboh & Ademosun, 2012).

Commonly, free radical attack the nearest molecule and snip the electron from other molecule to achieve its stability (Lau, Wang, & Chiu, 2008). Reaction of free radical in human as signaling molecules is important. However, excess of free radical may cause biological damage which can lead toward development of chronic and degenerative diseases such as cancer, diabetes, cardiovascular disease, autoimmune disease, aging and neurodegenerative disease (Reuter et al., 2010; Uttara, Singh, Zamboni, & Mahajan, 2009; Valko et al., 2007).

2.1.2 Effect of free radicals on human

Reactive free radical generally results in degradation of protein, lipid peroxidation and oxidation of DNA which have been considered to be linked with several chronic diseases. There are also several studies have demonstrated the potential role of fruits and vegetables in promoting health and preventing diseases (Jayathilake, Rizliya, & Liyanage, 2016). The term of oxidative stress is defined as a range of pathological conditions and reaction that together constitute a departure from a baseline homeostatic reductive state in cell or tissue (Samet & Wages, 2017). These tissues injuries can increase the production of radical generating enzymes such as xanthine oxidase, lipogenase and cyclooxygenase which cause activation of phagocytes, release of free iron, copper, iron or disruption of electron transport chains of oxidative phosphorylation and producing excess ROS (McCord, 2000). In addition, they are also highly reactive species that may cause damage of biological molecules leading to cell damage and homeostatic disruption (Young et al., 2001).

The implication of ROS can cause initiation and complication of diabetes mellitus, age-related eye diseases, and neurodegenerative disease (Rao, Bharani, & Pallavi, 2006). Furthermore, oxidative stress has been theorized in many conditions such as atherosclerosis, inflammatory condition, cancers and aging process (Lobo et al., 2010).

2.2 Antioxidant and its importance

Dietary antioxidant defined as any substances can sacrificially scavenge reactive oxygen or nitrogen species to stop the radical reactions (Huang, Boxin, & Prior, 2005). Antioxidant primarily from dietary compound have the ability to inhibit or delay the oxidation stress of the radical molecule and protect body system. Antioxidant also play role in neutralization process of excess free radical and protect from oxidative damage and disease (Wootton-Beard & Ryan, 2011). Hence, disease associate with free radical can be evaded by antioxidant therapy which gained an immense importance (Syakiroh et al., 2012; Wolfe, Wu, & Liu, 2003).

Besides, antioxidant can also add to food to prevent or delay the oxidation which normally initiated by free radical from the exposure to environmental factor such as air, light and temperature (Hraš, Hadolin, Knez, & Bauman, 2000). A strong *in-vitro* evidence exists for the antioxidants role mainly in cardiovascular disease, cancer and neurological conditions which it can perform as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents (Wootton-Beard & Ryan, 2011). Fruits and vegetable regularly contain high concentration of bio-active compound and antioxidant potential compound (Liu Rai Hui, 2013)

2.3 Classification of antioxidants

There are two type of antioxidant which are natural antioxidant and synthetic antioxidant. Natural antioxidant is the one which present in daily diet and food sources such as in fruits and vegetable. However, the synthetic antioxidant are the one which are widely use as food additives to prevent rancidity, owing to their high performance, low the cost and availability.

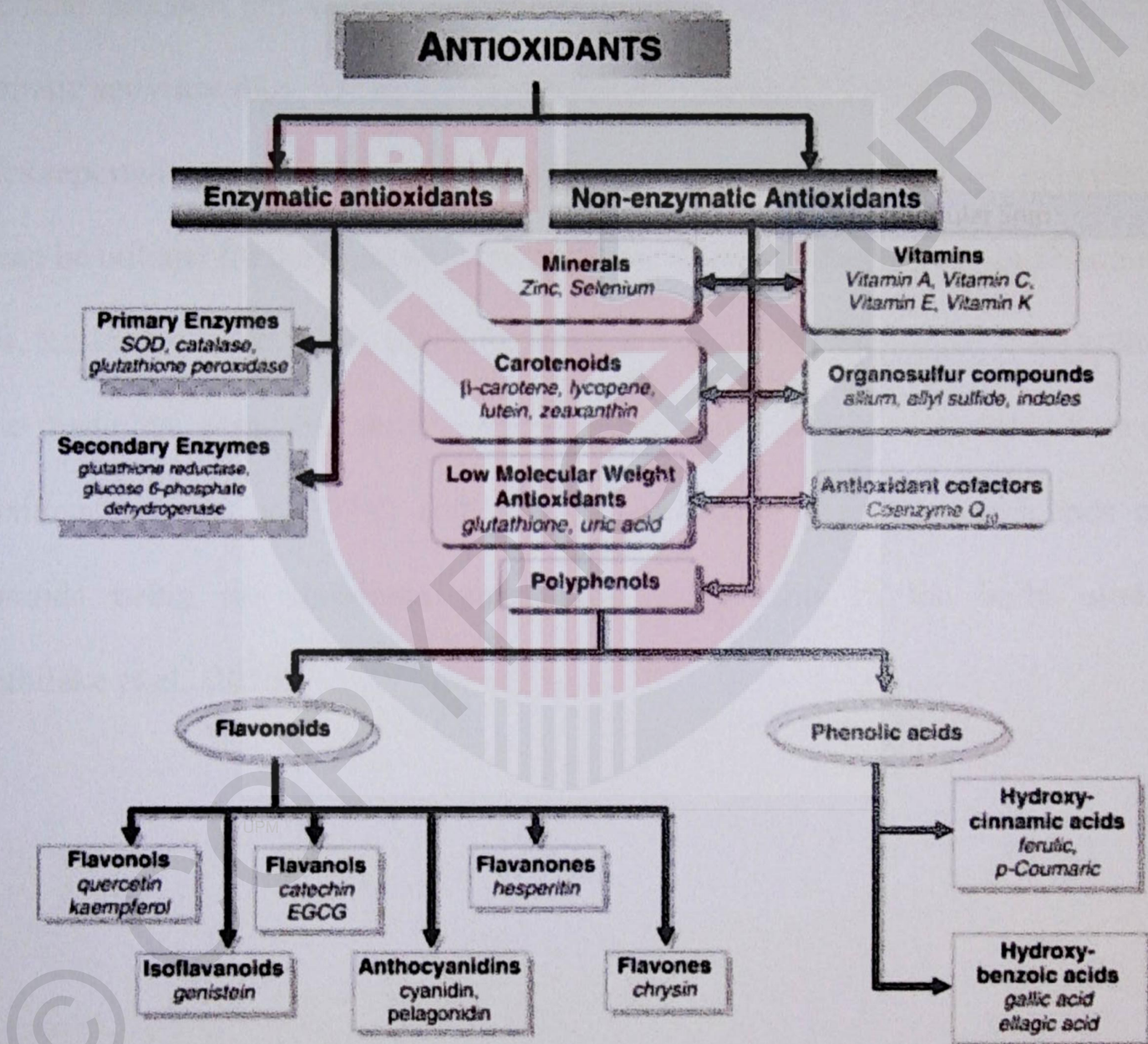


Figure 2.1: Classification of antioxidant (Ratnam et al., 2006)

2.3.1 Natural antioxidants

Natural antioxidants came in from various sources of food. One of the most natural antioxidants are from plants which contain compounds that possess antioxidant activity. One of the example is polyphenolics compound which occur in all parts of the plant which mainly come in the form of phenolic compounds, ascorbic acid and carotenoids (Zou, Xi, Hu, Nie, & Zhou, 2016). Flavonoid is one of the phenolic compound function act as free radical scavengers, have the capacity to modulate enzymatic activities thus, inhibit cell proliferation (Duthie & Crozier, 2000). There are studies reported various edible medicinal plants contain high amounts of antioxidants that can be utilised for the prevention of oxidative damage-related diseases (Katalinic, Milos, Kulisic, & Jukic, 2006; Liu, Qiu, Ding, & Yao, 2008). Moreover, there is study on the medicinal plant that show there are significantly linear relationship between antioxidant activity and TPC and TFC content with phenolic compounds and flavonoids being the dominant antioxidant components in the herbs studied (Jayathilake et al., 2016).

2.3.2 Synthetic antioxidant

Synthetic antioxidant is non-natural source antioxidant synthetically produced in the laboratory. There are many synthetic antioxidant such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) tert-butyl hydroquinone (TBHQ), and propyl, octyl and dodecyl gallates (Brewer, 2011). They have the same reaction mechanism as phenolic antioxidant compound which act as chain breaking antioxidant and involve in transfer of hydrogen atom or electron to the radical. However, their safety has been questioned due to toxicity issue (Dedoussis, Kaliora, & Andrikopoulos, 2005). The use of some synthetic antioxidants has been restricted because of their possibility of performing toxic and potential carcinogenic. A study found that BHA and BHT exhibit carcinogenic effect in experiment toward animal and the report showed that it has tumor-initiating and tumor-promoting action (Valentão et al., 2002).

2.4 Citrus fruit

Citrus genus belongs to the family of *Rutaceae* which comprise around 40 species of citrus and distributed in China, India, Malaysia, Australia and Srilanka (Ghafar, Prasad, Weng, & Ismail, 2010). This family are rich with phytochemical sources of bioactive compound that accountable for antioxidant and many other biological activities (Fejzic, 2014). Citrus fruits are well known for the fragrance which due to flavonoids and limonoids in their peels (Sawalha, Arráez-román, Segura-carretero, & Fernández-gutiérrez, 2009).

Citrus is one of the most important fruit crop in the world nowadays. Citrus has been largely grown in the tropical and subtropical countries and its annual production approximately 102 million tons (Mehl et al., 2014). In 2014, production of citrus Malaysia is around 42,212 tonnes (Yadotai, 2015). Citrus fruits are well-accepted by consumers of all over the world because of their attractive colours, pleasant flavours and aroma. Along with an increase of production, advances in storage and processing techniques, and the realization of a year-round supply (Zou et al., 2016). The citrus fruits come from the Rutaceae family which consist around 160 genera which had been grown in all. Citrus fruit is one of the important source of antioxidant such as ascorbic acid, phenolic and flavonoid compounds (Moosavy, Hassanzadeh, Mohammadzadeh, Mahmoudi, & Khatibi, 2017).

The rapid development of processing technology of frozen concentrated citrus juice after World War II has greatly expanded the international trade of citrus fruits and their other products. Citrus also had been recognized as important food and integrated as part of our daily diet which play role in supplying energy and nutrient which high in antioxidant to combat many disease (Liu, Heying, & Tanumihardjo, 2012). Among citrus parts, the peels contain the highest proportion of natural antioxidant such as natural flavonoid, phenolic compound, ascorbic acid, carotenoids and reducing sugar. It also has been reported that citrus peel extract exhibit the high antioxidant activity compared to pulp and seeds. (Guimarães et al., 2010; Sawalha et al., 2009). Besides, the citrus peel pigmentation contains abundance of carotenoid which simultaneously inverse the chlorophyll content (Ladaniya, 2008).

The phenolic compound mainly found in the citrus are flavonoid which have a different distribution of flavone glycosides. The flavone glycoside can be used along with polymethoxylated flavones to differentiate between cultivar (Peterson et al.,

2006). The citrus peel represents almost half of the total fruit mass with high flavonoids concentration (Anagnostopoulou, Kefalas, Papageorgiou, Assimopoulou, & Boskou, 2006). Citruses are from kingdom of *Plantae* in the division of *Saoindales*. It belongs to *Rutaceae* family, *Aurantioideae* subfamily, *Citrae* tribe and *Citrus L.* genus. In this study, *Citrus Microcarpa*, *Citrus Limon* and *Citrus Aurantifoliia* had been choose as samples.

Citrus by-products are potential sources of numbers bioactive ingredient and valuable technological and nutritional properties which have ability to be processed as food and ingredient additives (Shea et al., 2012). The citrus peels are commonly viewed as primary citrus by-product which represent around 50-65% of the fruits weight which usually will be discarded and considered as a massive load toward the environment (Ramful et al., 2011). Lemon and orange peels are common waste produce from food processing and juice extraction industry worldwide. The peels of lemon were normally used in the production of pectin and narirutin of flavonoid (Ghasemi, Ghasemi, & Ebrahimzadeh, 2009).

2.4.1 Medical properties of citrus

Citrus is a rich source of vitamin C, flavonoids, phenolic compound and pectin. It can provide enough daily requirement of vitamin C which is primary water soluble antioxidant that help to prevent free radical generation in the body and damage to the tissues (Milind & Dev, 2012). According to World Health Organization (WHO) reported, citrus fruits can reduce the homocysteine level which play a big role in protection from cardiovascular disease. There are also studies reported that by grating a tablespoon of the peel of citrus per day may exhibit a cholesterol-lowering benefits (Kurowska, Manthey, 2004). In addition, an in vivo study revealed that essential oil from citrus peel also exhibit the anti-carcinogenic properties which citrus have the ability to significantly lower the risk of develop of tumour (Del Toro-Arreola et al., 2005). Besides, citrus peel also shows the anti-diabetic properties where it can reduce the activity of glucose-6-phosphatase and phosphoenol pyruvate. It also stimulate the production of insulin and repair of secretory defect of pancreatic Beta-cells (Parmar & Kar, 2008). Moreover, the peel and inner white pulp of citrus exhibit an anti-inflammatory, healing and anti-arthritis properties. However this most precious part of citrus is often removed during food processing (Sandhya, Sai, Vinod, Banji, & Kumar, 2011).

2.4.2 *Citrus Limon* (Lemon)

Citrus Limon or well known as lemon is one of the important plant of the family of Rutaceae. Lemon grow on small and thorny tree which only reaches height of 3 to 6m only. The *citrus limon* leaves are dark green in colour and arranged alternately on the stem. The flower of *citrus limon* is white in colour and have a fragrant with five petals. The lemon fruits are oval shape with smooth porous skin and some have pointed tip on the bottom of the fruit. *Citrus limon* have many varieties which includes Bush lemon, Eureka, Ponderosa, Variegated Pink, Verna, Villafranca, Yen Ben and Yuzu. The colour of the lemon skin also range from greenish to bright yellow when it fully ripe (Mohanapriya, Ramaswamy, & Rajendran, 2013).

Lemon possess huge medicinal properties and benefits. There is a study on the effectiveness of dehydrated of lemon peel in promoting growth, immunity and anti-oxidant status (García Beltrán, Espinosa, Guardiola, & Esteban, 2017). In addition, studies on mice and humans had proven the lemon possess the ability to lower the uric acid value in serum and did not produce any side effect on the liver and kidney function of humans subject (Wang, Cheng, Lin, Ma, & Deng, 2017). In the other hands, lemons also provide the ability to inhibit the crystal formation by citrus bioflavonoids and lemon peels was demonstrated by the urine biochemistry which can be the crystal induced renal dysfunction (Sridharan, Mehra, Ganesh, & Viswanathan, 2016).

Besides, there were numerous of antioxidant assay to determine the bio-active compound in lemon sample such as the peels, pulp and seeds. From the previous study, lemon peel has revealed the total phenolic content was around 202.73 mg GAE/l to 224.55 mg GAE/l and the IC₅₀ value was only 0.33 mg/ml (Diankov, Karsheva, & Hinkov, 2011). The lower the IC₅₀ value exhibit the higher the antioxidant capacity to inhibit the free radical. The nutritional value of the lemon also had been discovered

which show in every 100 gram of fresh lemon possesses 29 kcal energy which contained 9.32g carbohydrates, 2.50g sugar, 2.80g dietary fibre, 0.30g fat and 1.10g protein. The micronutrient of 100g of fresh lemon are thiamine, riboflavin, niacin, pantothenic acid, vitamin B6, folate, vitamin C, iron, magnesium, phosphorus, potassium and zinc (Mohanapriya et al., 2013).



Figure 2.2: Lemon (*C. Limon*)

2.4.3 *Citrus Microcarpa* (Musk Lime)

Citrus Microcarpa is well known as musk lime or *limau kasturi* by the locals in Malaysia is a small tree with a long taproot which can grow up to 2 to 7.5 meter tall.

The musk lime native to tropical Asia and widely grown in India and throughout South East Asia countries (Shofian et al., 2011). The flowering and fruiting of the tree is all year round with peak between Augusts until October each year. *Citrus Microcarpa* fruits are round, greenish-yellow with diameters around 2 to 4.5 centimetre with smooth and shiny skin (Abdullah, Ch'ng, & Yunus, 2012). *Citrus Microcarpa* is widely used in several of food preparation and food ingredient.

For example, the pulp of the fruit is used as major ingredient in beverage, syrups concentrate and purees flavouring ingredient while the peels is made into candies, jams and marmalades. However, musk lime waste product such as seed have a potential uses that used be explored as it rich in unsaturated fatty acid and linoleic acid (Shofian et al., 2011). Moreover, the peel of the musk lime also had been explored in the development of candied musk lime peel (Hazniza et al., 2006). One of the most prominent studies found that musk lime peel and seeds are rich in oil source which account around 73% of the oil is unsaturated fatty acid which is good in preventing chronic disease such as atherosclerosis and other other heart disease (Yanty, Azizah, Oi, Kamariah, Hasanah, 2008).

The previous study found that musk lime contains lowest ascorbic acid content compared to orange, grapefruit, lemon, lime and kaffir lime. The ascorbic acid content in the musk lime obtained by using indophenol titration and high-liquid chromatography method is around 18.62 mg/100g and 10.78 mg/100g respectively (Najwa, 2017). Besides, the peel of musk lime represented higher total phenolic and flavonoid content compared to pulp of musk lime and the highest total phenolic and flavonoid are obtaining through the 70% aqueous ethanol extraction as compared to hot water (80°C, 90°C and 100°C), ethanol (50%, 60%, 80%, and 95%), methanol and ethyl acetate extraction sample (Lou, Lin, Hsu, Chiu, & Ho, 2014).



Figure 2.3: Musk lime (*C. microcarpa*)

2.4.4 *Citrus aurantiifolia* (Key Lime)

Citrus aurantiifolia or known as key lime and *limau nipis* by the locals Malaysia. Key lime is widely grown in warm tropical to sub-tropical region in the

world such as India, Mexican, United States of America, Egypt and West Indies. However, the ancestral place of its origin is still controversial as most of researchers believe that it originated from South East Asia and its native is the Indo-Malaysian region (Nicolosi et al., 2000). The lime plant is a small, shrub-like tree with approximately 5-meter height with evergreen, ever-bearing tree that have irregular branches and possess thorns. The leaves are alternate, elliptical to oblong-ovate in shape which account around 4-8 cm x 2-5cm. the flower is in yellow to white colour with purple tinge on the margins. The fruit are ovoid shape about 3 to 6 cm in diameter and sometimes have apical papilla. The fruits and flowers usually bloom during May until September in the northern hemisphere. The fruits peel is thin with dense glandular segments with yellow green pulp vesicles. The juice of *citrus aurantiifolia* is acidic and have a fragrant properties while the seeds are small, plump, ovoid, pale and smooth with a white embryo (Khan, Al-yahyai, & Al-said, 2017).

Key lime possess great medical properties with phytochemicals such as alkaloids, carotenoids, coumarins, essential flavonoids, phenolic acids and triterpenoids all of which have significant effect against carcinogenesis, stop the mobility of cancer cell, inhibit metastasis, angiogenesis and induce the tumour suppressor generating apoptosis (Narang, Jiraungkoorskul, 2016). Previous study was done to identify the antioxidant properties of fresh and processed key lime fruits found the methanol and aqueous extraction are the best extraction solvents for both fresh and processed key lime fruits. Moreover, the study found the phenolic content of pulp and peel extract of key lime ranged from 2.5 to 22.5 mg/g and 5.0 to 45.0 mg/g respectively (Divya, Jamuna, & Jyothi, 2016). Moreover, the processed key lime showed a decline in antioxidant capacity with increase in storage time (Divya et al., 2016).



Figure 2.4: Key Lime (*C. Aurantifolia*)

2.5 Principle of method of measuring the total antioxidant content

2.5.1 Total phenolic content

Phenolic acids are the secondary metabolites derived from plant which in the form of ester, amide or glycoside (Khoddami, Wilkes, & Roberts, 2013). Phenolic acid can be allocated into two subgroups which are hydroxybenzoic acids and hydroxycinnamic acid derivatives. The hydroxybenzoic acid usually found in plant which bound in the foods component of complex structures such as lignin and hydrolysable tannin or attached to the cell wall, proteins, sugar and organic acid in fruits, vegetables and whole grains. The hydrocinnamic acid in the plant primarily present in the bound form and connected to the cell wall components such as cellulose, lignin and proteins (Liu Rai Hui, 2013).

Phenolic acids are accountable for plant growth and reproduction. Furthermore, phenolic acid also plays huge part in defences against radiation and invasion by parasite, pathogens and predator as well as contribute toward plant's taste and colour properties (Dai & Mumper, 2010). Phenolic also possess huge potential in prevention of various oxidative stress associated diseases such as cancer, cardiovascular disease degenerative disease and chronic inflammatory disease (Dai & Mumper, 2010). Beside the antioxidant properties of phenolic acid, they also exhibit extensive range of biological effects such as anti-carcinogenic, anti-bacterial, anti-viral, anti-inflammatory, anti-allergic, vasodilator agent and immune-stimulating agent (Tawaha, Alali, Gharaibeh, & Mohammad, 2007).

The Folin-Ciocalteu reagent (FCR) is the main reagent that use to determine the total phenolic content of sample. Folin- Ciocalteu reagent solution containing sodium tungstate, sodium molybdate, hydrochloric acid, phosphoric acid and water (Huang et al., 2005). The yellow colour of Folin-Ciocalteu reagent is due to addition

of Lithium sulphate. The total phenolic assay implicate electron transfer pathway which translocate the electron from the antioxidant compounds such as phenolic or other reducing compounds to the molybdenum (Magalhães, Segundo, Reis, & Lima, 2008). Molybdenum reduction can cause colour changes from yellow to blue which can be monitored using spectrophotometer at 750-756 nm.

The use of Folin-Ciocalteu reagent assay not specifically measure the content of phenolic compound. Thus, there is also polyphenol oxidase method which is more precise to measure the phenolic compounds. The gallic acid is commonly used at different concentration to calculate the capacity of sample equivalent to the amount of gallic acid. Commonly, gallic acid usually used as a key standard since widely used in many studies. Thus, the result expressed as gallic acid equivalent (GAE) per amount of sample. The colour changes is correlated with the antioxidant content. The reduction of molybdenum in the complex is suggested as below:



Figure 2.5: Reaction in Folin-Ciocalteu assay

Source: Huang et al. (2005)

Despite, Folin-Ciocalteu assay also possess limitation which is highly influenced by other external factor rather than total phenolic content itself. Ascorbic acid, reducing sugar and protein have huge influence on the reading of total phenolic content of sample by the Folin-Ciocalteu assay (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013). Thus, there is a need a quantification of total phenolic content by using high performance liquid chromatography (HPLC) and gas chromatography (GC) for isolation and identification of individual phenolic

compound and other bioactive compound presented in the samples (Al-Qassabi, Weli, & Hossain, 2018).

2.5.2 Total flavonoid content

Fruits, vegetables and other plant foods are well known as a rich source of flavonoid and have ability to reduce the risk of major chronic diseases. Flavonoids are the subcategory which widely disperse in plant kingdoms with over 4000 types of flavonoid compounds (Harborne & Williams, 2000). Flavonoid are the major group of phenolic compounds that usually contain two aromatic rings (A and B rings) which connected by 3 carbons (Liu Rai Hui, 2013). Flavonoids comprehend a variety of bioactive compound which allocated into several classes. The example of flavonoid classes are hydroxybenzoic acid, anthocynins, hydroxyl cinnamic acids, proanthicynidins, flavonoids, lignans and stilbenes (Cheong et al., 2012).

The total flavonoid content usually determined by aluminium chloride (AlCl_3) colorimetric method is AlCl_3 forms acid stable complexes with C-4 ketogroup and C-3 or C-5 hydroxyl group of flavones and flavonols. Thus, it will form acid labile complexes with ortho-dihydroxy groups in the A- or B- ring of flavonoid. The catechin usually used as a standard reagent in various concentration to bild a standard calibration curve (Pal, Sannigrahi & Muzumder, 2009). Flavonoids are a group of natural compounds with variable phenolic structures and found in plants. Hydroxyl groups in flavonoid act as scavenging free radical by the chelating metal and the

capacity of flavonoids acts as antioxidant also depending on its molecular structure ions (Kumar et al., 2013).

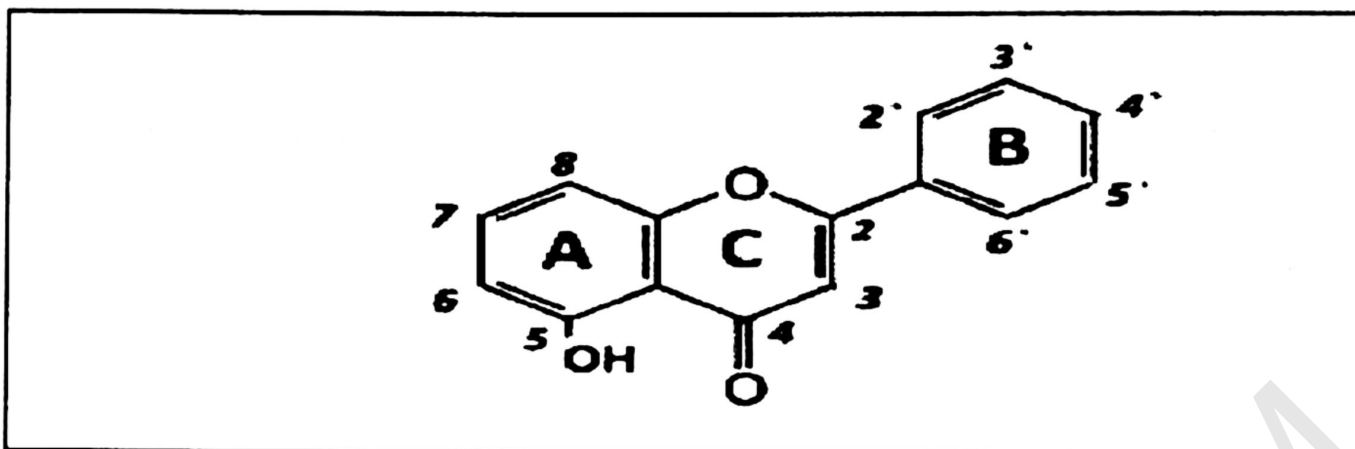


Figure 2.6: Structure of basic flavonoid

Source : Pękal & Pyrzynska (2014)

2.6 Principle of method of measuring the total antioxidant activity

There are several techniques have been developed to measure the antioxidant activity of various foods and plants. Antioxidant assays can be divided into two groups which are hydrogen atom transfer (HAT) and single electron transfer (ET). The HAT-based assay commonly consists of a synthetic free radical, an antioxidant and an oxidizable molecule probe. The HAT primary mechanism is relevant to radical chain-breaking antioxidant activity. Besides, the ET-based assay involved redox reaction to measure the reducing capacity of antioxidant and the colour changes when the process occurred (Huang et al., 2005).

The hydrogen atom transfer based assay consists of oxygen radical absorbance capacity (ORAC), crocin bleaching assay and total radical trapping antioxidant parameter (TRAP). Whereas, the electron transfer based assay consists of diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay, ferric reducing antioxidant power (FRAP) assay and Trolox equivalence antioxidant capacity (TEAC) (Huang et al., 2005).

2.6.1 Diphenyl-1- Picrylhydrazyl (DPPH) Radical Scavenging Activity

The DPPH is a stable organic nitrogen radical which contain an unpaired electron and containing purple chromogen at maximum UV- visible absorption at 515 to 520 nm (Amin, Azrina, Hock, Prasad & Kin, 2013). DPPH radical able to reduce the antioxidant and causing delocalisation of spare electron in DPPH molecule which will cause discolouration (Molyneux, 2004). DPPH is a simple method and rapid for evaluation of antioxidant activity (Huang et al., 2005). The efficiency of reducing the DPPH radical is depending on the quantity of antioxidant available in the sample. The comparison between absorbance and the negative control can be used to calculate the percentage of reduced DPPH radical. Thus, the higher amount of DPPH reduced, the higher the amount of antioxidant available (Amin et al., 2013). The DPPH radical are not reactive towards tyrosine, glucose purine and pyrimidines (Molyneux, 2004). However, it may happen a precipitation between protein and DPPH radical (Amin et al., 2013).

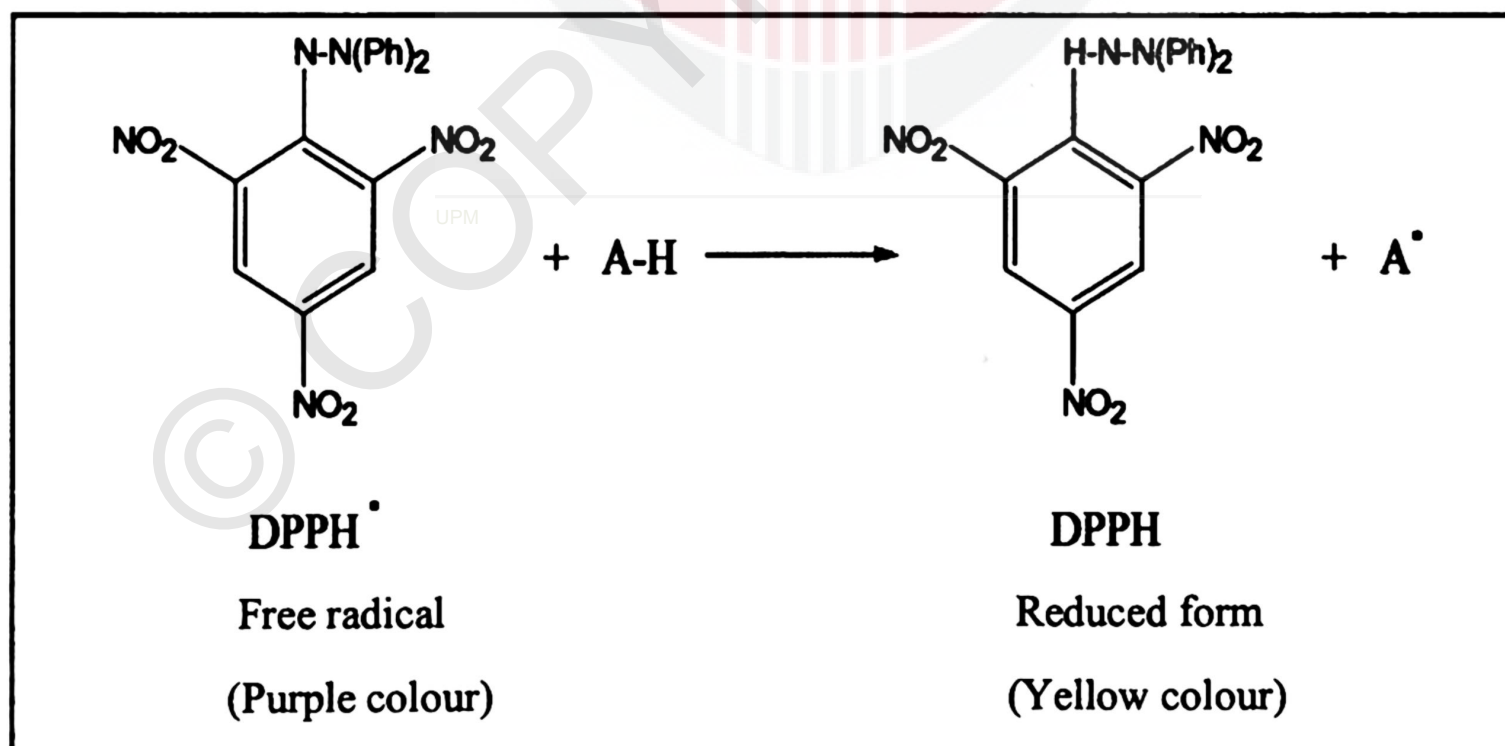


Figure 2.7: DPPH reducing activity

Source: Amin et al. (2013)

2.6.2 Ferric iron reducing antioxidant power (FRAP)

FRAP assay is one of the method used to measure the antioxidant activity in which it uses a dye of 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) act as oxidant agents. It has the ability to bind to ferrous (Fe^{2+}) ion forming navy blue colour and the absorbance measured can be used to determine the amount of iron reduced. The higher the ferric (Fe^{3+}) ion into ferrous ion by the antioxidant it will cause increase in intensity of the blue colour (Amin et al., 2013). The FRAP values obtained by comparing the absorbance change at 593 nm. The principle of FRAP assay involve reaction between electron acceptor $[\text{Fe}(\text{TPTZ})_2]^{3+}$ and electron receptor which is antioxidant.

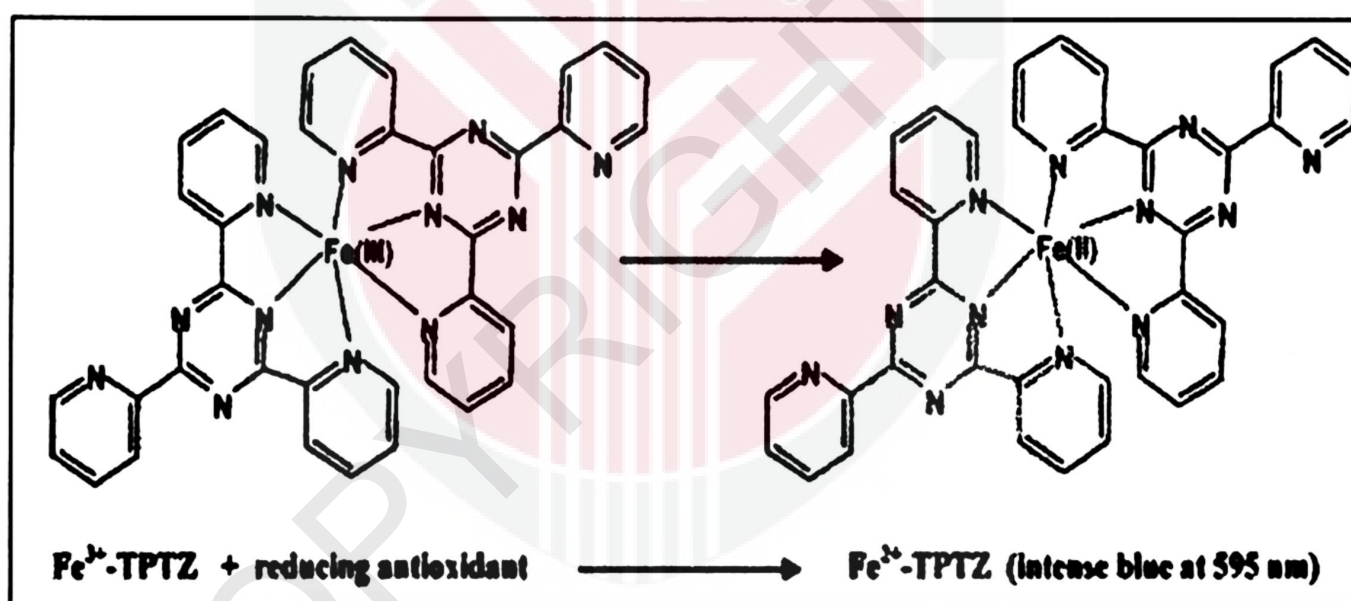


Figure 2.8: Reducing activity of Fe^{3+} to Fe^{2+}

Source: Huang et al. (2005)

2.7 Extraction methods of the sample

Phenolic compound in plant are simply classified as simple phenol or polyphenol based on the number of phenol unit in each molecules (Khoddami et al., 2013). The basic structure of polyphenol usually encompass phenolic acid and flavonoids which widely distributed in the plant tissues and exist in both free and bound form in the cell wall (Su et al., 2014). Although, the free phenolic compounds are solvent extractable, the bound phenolic compound are covalently bound toward the plant matrix and cannot be extracted by the water or aqueous solvent mixture (Pérez-Jiménez & Torres, 2011). The insoluble form of phenolic compound who had a covalent bound with the structural component of plant cell such as cellulose, polysaccharides and pectin through ester bonds which hard to hydrolyse (Acosta-estrada, Gutiérrez-uribe, & Serna-saldívar, 2014).

The insoluble bound phenolic presented a significant higher antioxidant capacity as compared to free and soluble conjugated phenolic compound (Chandrasekara & Shahidi, 2010). Therefore, there are numerous of food processing technique that will enhance the liberation and release of bound phenolic compounds such as malting, fermentation and thermomechanical process such as alkaline or acidic hydrolysis and extraction (Acosta-estrada et al., 2014).

The extraction method is depending on the physical state of the sample, type of solvent, solvent concentration, solid to liquid ratio, extraction time, temperature and pH (Prasad et al., 2012). Generally, most of the studies used aqueous mixture of alcohol such as methanol or ethanol as a solvent to extract antioxidant compound from plant sample. However, many researchers encourage the usage of ethanol than methanol due to it is greener solvent, safe and less toxic (Prasad et al., 2009). In

addition, ethanol demonstrated higher extraction yield, stronger reducing power and higher flavonoid content compared to water extraction (Kim, 2013).

Studies revealed that by using ethanol and hydroalcohol extracts with ration 4:1 resulted the highest extraction yield with maximum presence of phytoconstituents such as saponins, alkaloids, carbohydrates, flavonoids and tannins compared to other solvents such as petroleum ether, water and chloroform (Thakur & Arya, 2014). Polar solvents are more effective in extraction of biomolecule and plant. However, water demonstrated similar efficiency as ethanol except there was no trace of alkaloids in water extraction. The non-polar solvent such as petroleum ether and chloroform do not have the ability to preserve active compound and tannins extraction was very little in non-polar solvent extraction (Thakur & Arya, 2014).

Furthermore, 70% aqueous ethanol extraction had shown greater extraction yield for fruits and vegetable samples compared to distilled water extraction, however methanol extraction has higher phenolic compound compared to 70% aqueous ethanol extraction (Rababah, Banat, Rababah, Ereifej, & Yang, 2010). Moreover, 70% aqueous ethanol extraction have been widely used by the commercial product for extraction (Trusheva, Trunkova, & Bankova, 2007).

There are varies of extraction method such as soaking with aid of Soxhlet apparatus, vortex mixer, magnattic stirrer or orbital shaker for particular period (Amin et al., 2013). Beside these method, ultrasound assisted extraction or sonication extraction method as most effective methods in extraction based on high yield, less extraction time and high selectivity (Trusheva et al., 2007).

2.8 Treatment on sample

Antioxidant vulnerable toward degradation, mostly due to handling practices. Chilling injury, fungal decay, irradiation, incorrect holding temperature, relative humidity and other factors of stress (Chan & Tian, 2006; Yahia, 2010). These factors contribute to reduction of antioxidant content and nutritional value of fresh fruits and vegetables. There is established investigation on post-harvest exposure at temperature above the freezing point can contribute to negative effect on the nutrient quality of the food especially the antioxidant (Villa-Rodriguez et al., 2015).

There is study reported on the possibility of freezing process will cause the fruit cells disrupted, which activate certain enzymes, substrates and activators. Thus, this process might increase the activity of enzymes upon thawing and caused degradation of some phenolic compounds (Chang et al., 2006).

In other hand, the principle of freezing the fruits and vegetable have a benefit that could serve to lessen the problem of spoilage and degradation and provide more nutritious fruits and vegetable. There is study reported that frozen produces sample have significantly higher nutrient content than fresh stored more frequently than inverse. (L. Li et al., 2017). Thus, the finding does not support the perception of consumer who believe fresh produce is nutritionally greater to frozen produced. Freezing process also help to lower the rate of degradation and inhibit the microbiological and enzymatic activity which help to retain the nutrition of fruit and vegetable (Antos, Sanchez, Pausal-Teresa, Cano, 2006).

There are a lot of previous study successfully reported that antioxidant content and activity had significantly higher than fresh sample. This statement proved in study on Ajwain leaves where frozen ajwain have higher antioxidant content and antioxidant

activity as well as there is highly positive correlation between polyphenol concentration and freezing condition. This study conclude that freezing have an impact on the release of carotenoids from plants due to low temperature influence the obliteration of cell wall of the plant (Raza et al., 2015).

Besides, there was study on dried *H. serotina* mushroom found that freezing technique have better yield of antioxidant compared to other technique due to the freezing and vacuum condition have the ability to protect the bio-active compound maximally. This is due to it had ability to improve the thermal stability of polysaccharides, thus could effectively protect the reducing power and hydroxyl radical scavenging capacities of samples (Li, Wang, Wang, & Xiong, 2016).

Moreover, study on tomatoes found that freezing technique have higher reducing power capacity, total phenolic content and total flavonoid content as compared to fresh sample of tomatoes. Vegetables and fruits commonly have high antioxidant content at the outer part of the plant which include the total phenolic compound that usually accumulate in the vacuoles. The freezing processes have the ability to accelerate more bound phenolic compounds releasing from the cellular breakdown (Chang et al., 2006). Although, the cell wall disruption may also release oxidative and hydrolytic enzyme which have ability to destroy antioxidant in fruits, the enzyme will be inactivated after the freezing process (Pérez-Gregorio, Regueiro, González-Barreiro, Rial-Otero, & Simal-Gándara, 2011).

Furthermore, freezing have various effect depending on the type of plant sample (Wei, Chan, Tan, Chin, & Gan, 2014).. Remarkably, freezing have a positive impact on antioxidant activity of green vegetables such as beans, zucchini, and peas while have slightly negative impact or no effect on red or yellow vegetables such as

tomatoes, carrot and pepper (Danesi & Bordon, 2008). In other study proposed that, increase in amount of flavonols might attributed to release of phenolic compound from the matrix during the freezing process (Pérez-Gregorio et al., 2011).

The study of freezing effect on raspberries demonstrated freezing had a slight effect on ellagic acid which released by acid hydrolysis, vitamin C content, total phenol and antioxidant capacity (Mullen et al., 2002).



CHAPTER 3

METHODOLOGY

3.1 Chemical and reagents

The chemicals that were used in this study are ethanol solution from Fisher Scientific (Leicestershire, UK), distilled water was prepared with Favorit W4L water distillation system from Genriso (Nottingham, UK), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Sodium acetate trihydrate ($\text{C}_2\text{H}_3\text{NaO}_2 \cdot 3\text{H}_2\text{O}$), 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ), hydrochloric acid (HCl), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), sodium nitrate (NaNO_3) glacial acetic acid, ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), aluminium chloride (AlCl_3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Folin-Ciocateu reagent, sodium bicarbonate (Na_2CO_3), gallic acid and quercetin were purchased from Sigma-Aldrich (Steinheim, Germany).

3.2 Consumables and instruments

The instruments used in this research are sonicator (Power Sonic 405, Hwashin Technology.co, Seoul, Korea), freeze dryer (The VIRTIS Company, Inc Route 208, Gardiner, New York), rotary evaporator (Laborata 4000, Neidolph, Japan), Freezer (Freezer SANYO – 80°C) Weighing scale (AC Adapter Japan), Fumehood (EFH- 5A1 Singapore) UV-Vis spectrophotometer (SECOMAM CE. France) and FLOUstar Omega microplate reader (BMG labtech, Germany)

3.3 Sample collection

All the samples of lemon, key lime and musk lime were purchased from Pasar Borong Selangor. The parts of samples that had to be used in this research was only the peels of the fruits. Each type of fresh fruits samples was uniform in size, colour, maturity and shape. The fruits washed thoroughly with tap water and dried using towel tissues. Then, weighted the citrus fruits respectively according to their species. Next, peeled the fruit and removed the peel from the pulp and seed respectively. The peels were divided into two parts which was fresh sample and frozen sample. The fresh peels of the fruits grinded into smaller uniform particle then it ready to be extract. While the other half kept frozen into –80°C prior freeze dried and further analysis.

3.4 Experimental design

This study used orange, lemon, key lime and musk as sample. The part of fruits had to be used in this study is the peel. The peels of the citrus fruits were extracted by using ethanol. Each extraction samples of lemon peel, key lime peel and musk lime peel were analysed to determine total phenolic content, total flavonoid content and antioxidant activities. The experimental design of the study is shown in Figure 8:

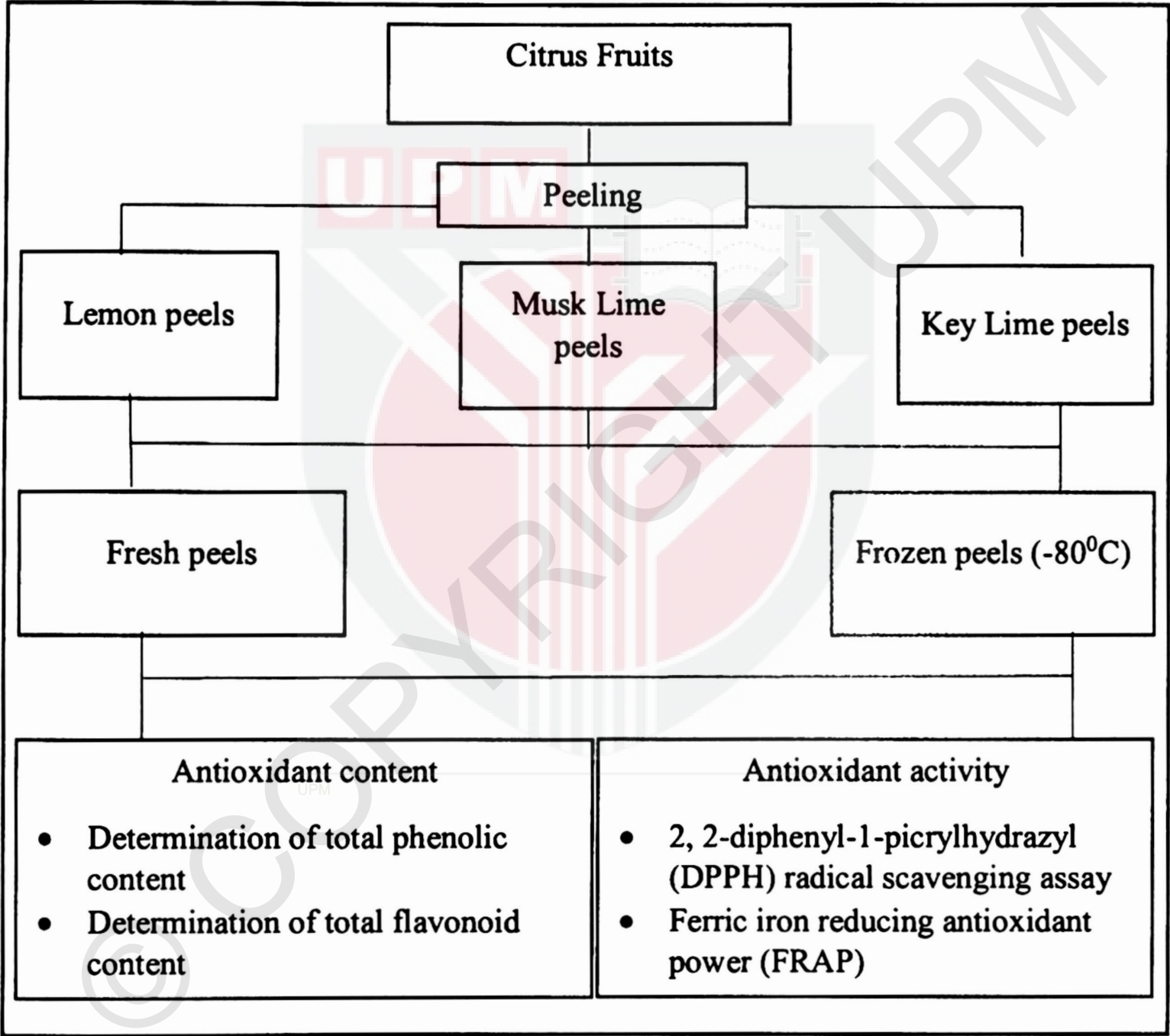


Figure 3.1: Experimental design of the study on antioxidant properties in frozen peel of citrus fruits

3.5 Preparation of sample extraction

The method used was ethanol extraction according to Ferreira, Silva, & Nunes (2018) with slightly modification. The ethanol extraction was performed between two different samples which is fresh samples and frozen samples. Both extractions were performed with similar ratio which is 1:4, sample to solvent.

For the fresh samples extraction were done by mixing 25g of each citrus peels samples into 100 ml of 70% ethanol solution and place it in a conical flask. For each sample of fresh samples have to be triplicates. The extraction performed by using sonication method for 20 minutes for each sample. Then the ethanol extraction was filtered through Whatman paper No. 1. Then, the process of extraction was repeated for three times to get the best yield of extraction which account for total 300ml of 70% ethanol solution use for each frozen or fresh samples.

For the frozen samples extraction were done by mixing 10g of each citrus peels samples into 120 ml of 70% ethanol solution and place it in conical flask for each sample of frozen samples have to be triplicates. The extraction performed by using sonication method for 60 minutes for each sample. Then, the ethanol extraction was filtered through Whatman paper No. 1.

Next, the solvent was removed from the sample by using rotary vacuum evaporator at 50°C to extract the solution. The dry extract had been freeze dried and store in -20°C for further analyse the content of antioxidant and antioxidant activity.

Yeild of extract %

$$= \frac{\text{weight (flask + extract)} - \text{weight (empty flask)}}{\text{weight of fresh sample}} \times 100\%$$

3.6 Moisture content

The moisture content determination according to Li, Pegg, Eitenmiller, Chun, & Kerrihard (2017). The moisture analyses were performed gravimetrically in triplicate for each sample. The method implemented by using AOAC Official Method (2000). Firstly, the empty aluminium dishes and its lid were dried in the oven at 105 °C for 3 hours. Then, the dishes and lids transferred into desiccator in order to let it cool. The homogenized sample were measured in 5g and placed in the dried moisture dish and spread uniformly. Then, the sample placed in the oven overnight at 105 °C.

On the next day, removed the moisture dish without its lid from the oven and put inside the desiccator at room temperature until it cool down and weighed the sample right after it reach room temperature. The weight changes were recorded. The steps repeated until the constant weight. Each of the sample were conducted in triplicates. The moisture content was determined by using the equation:

$$\text{moisture content, \% by weight} = \left(\frac{\text{loss of weight of the sample (g)}}{\text{weight of the sample taken (g)}} \right) \times 100\%$$

3.7 Determination of total phenolic content

The Folin-Ciocalteu (FC) assay was used to determine total phenolic content each of the peel of citrus fruits extract. To determine the total phenolic content according to method by Meda, Lamien, Romito, Millogo, & Nacoulma (2005) with slightly modification. The 0.2 N of FC reagent assay was diluted by added 0.15 ml of FC in 15 ml of methanol. Then, prepare 0.6 g of sodium bicarbonate (Na_2CO_3) in 10 ml of distilled water. After that, prepared the serial dilution of sample or standard concentrations (0 - 150 $\mu\text{g/ml}$). Next, added 1 ml of sample and 1ml of FC reagent. Allow standing at room temperature for 5 minutes the add 2ml of Na_2CO_3 in the cuvette. The absorbance measured at 725 nm by using spectrophotometer. Gallic acid was used as standard. All determinations are performing in triplicates. The mean of three readings are use and the content of phenolic was expressed as gallic acid equivalent (GAE) per 100 g of citrus peel sample. The total phenolic content was calculated by using the following formula:

$$\text{Total phenolic content (TPC)} = \frac{R \times D.F \times V}{DW} \times 100$$

Where R - x value obtained from standard curve

D.F. - Dilution factor

V - Total volume of sample used

DW - Dry weight of sample used for extraction

3.8 Determination of total flavonoid content

The total flavonoid contents in the peel of the citrus extract was determined by the aluminium chloride calorimetric method by Ghasemzadeh, Jaafar & Rahmat (2010). The aliquot (2ml) of approximately diluted extract were mixed with 0.2 ml of 5% sodium nitrate in volumetric flask and incubated at room temperature for 5 minutes. After 5 minutes, 0.2 ml of 10% aluminium chloride was added to the mixture then mix them thoroughly. After 6 minutes, 2 ml of 1.0 M sodium hydroxide was added. Lastly, the volume was make up to 5ml by addition of 70% ethanol and mix well. After stand for 10 minutes, the absorbance of the reaction mixture was read at 430nm using spectrophotometer against blank; 70% aqueous ethanol. All the test was performed in triplicates. The total flavonoid content value was determined from series of quercetin calibrate curve prepare from different concentration of quercetin in 70% aqueous ethanol (1- 0.001 mg/ml). Result were expressed as milligram of quercetin equivalent per gram (mg QE/g). The total flavonoid content of samples was calculated according to the formula below:

Total Flavonoid Content (TFC) =

$$\frac{R \times D.F \times V}{DW} \times 100$$

Where R - x value obtained from standard curve

D.F. - Dilution factor

V - Total volume of sample used

DW - Dry weight of sample used for extraction

3.9 Determination of antioxidant activity

3.9.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The radical scavenging activity of citrus peel extract was determined with the DPPH method. To determine the DPPH radical according to method by Thaipong et al. (2006) a modified method of Brand-Williams et al. (1995) with slightly modification. The DPPH stock solution was prepared by adding 24 mg of DPPH in 100 ml of methanol and store at -20°C. Then dilute 10.00 ml of the stock solution with 45.0 ml of methanol to obtain the working solution at absorbance of 1.1 ± 0.02 measure at 515 nm using spectrophotometer. Then add 500 μ l of sample extract to 500 μ l of DPPH solution and leave it to stand in the dark for 2 hours. After that, measure the absorbance using FLUOstar Omega microplate reader at 515 nm against a blank. All the determination was performed in triplicates. The data expressed as a percentage of scavenging activity by using following the equation:

Scavenging activity (%) =

$$\frac{\text{Absorbance sample} - \text{absorbance blank}}{\text{absorbance blank}} \times 100\%$$

3.9.2 Ferric iron reducing antioxidant power (FRAP)

The estimation of antioxidant activity was determined by using FRAP assay method. To determine the FRAP according to method by Ghasemzadeh et al. (2010) with slightly modification. Firstly, prepare working FRAP solution was prepared with a ratio of 10:1:1 by mixing 25 ml acetate buffer (300 Mm, pH 3.6 by adding 16 ml acetic acid and 3.1 g sodium acetate), 2.5 ml of TPTZ (10 mM in 40 mM HCl) and 2.5 ml of ferric chloride solution (20 Mm in distilled water). Then, mix the solution of 50µl of extract diluted with distilled water into 3.0 ml of working FRAP reagent thoroughly and incubate at 37 °C for 30 min in dark condition. Next, measure the absorbance by using spectrophotometer at 593 nm against a blank (distilled water). Result of higher absorbance value means, a higher antioxidant activity. The change of absorbance at 0 min and 30 min was calculated. Ferrous sulphate solution with various concentration (1- 1000µM) were used to plot calibration curve for quantification purpose. The antioxidant activity was expressed as millimoles of ferrous ions per gram of fresh weight (mMFe²⁺/g FW). The FRAP value of sample were calculated according to formula below:

$$FRAP\ value = \frac{R \times D.F. \times V}{DW} \times 100$$

- Where R - x value obtained from standard curve
- D.F. - Dilution factor
- V - Total volume of sample used
- DW - Dry weight of sample used for extraction

3.10 Statistical analysis

The results obtained were expressed in means $\pm$ standard deviation for further analysis. Determination of the differences between these means used, One Way analysis of variance (ANOVA) with post-hoc test (Tukey HSD). Pearson's correlation was conducted to determine the correlation between antioxidant contents and antioxidant activity in the fresh and frozen citrus peels. All p-values < 0.05 were considered to be significant. All of this test carried out by using Statistical Package for the Social Sciences (SPSS) 23.



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction yield

Solvent extraction is the most common technique used in antioxidant compound isolation as it is a widely applicable technique and efficient in extracting active compounds. Ethanol is one of the popular solvent used in extraction process as it offer higher antioxidant activity with higher phenolic content compared to water extraction (Kim, 2013; Mao, Pan, Que, & Fang, 2006). Usually, the extraction method is depending on the chemistry of active compound presence and its distribution in the plant matrix (Sultana, Anwar, & Ashraf, 2009).

The extraction yield and antioxidant activity result are highly depends on the solvent used, temperature of extraction, time, sample-to-solvent ratio, pH of extraction medium and particle size of the plant material used (Sultana et al., 2009). In addition, the structure of the active compound affects the solubility of the solvent at different polarity. Thus, type of extraction used and the isolation procedure have a significant

influence toward the yield of active compound extracted and the antioxidant activities (Rababah et al., 2010). Therefore, the selection of appropriate extraction solvent and system is very important in optimizing the recovery of phenolic, flavonoid and other antioxidant compound within the sample (Dai & Mumper, 2010).

In the recent study, 70% aqueous ethanol was chosen as the extraction solvent because ethanol has been discovered as a good solvent for the extraction of polyphenol compounds, non-toxin for environment and it is shown as safe for human consumption (Dai & Mumper, 2010; Shafekh et al., 2012). A study discovered that ethanol extraction have higher yield, reducing power and antioxidant content compared to water extraction (Kim, 2013). Water also was shown as a good solvent for polyphenol extraction from the plant matrix (Sultana et al., 2009). Thus, the combination of both organic solvent which is ethanol and water is used as the extraction solvent as it able to assist the extraction of soluble and insoluble polyphenols (Do et al., 2014). The yield of extract for fresh and frozen citrus peels are shown in Figure 4.1.

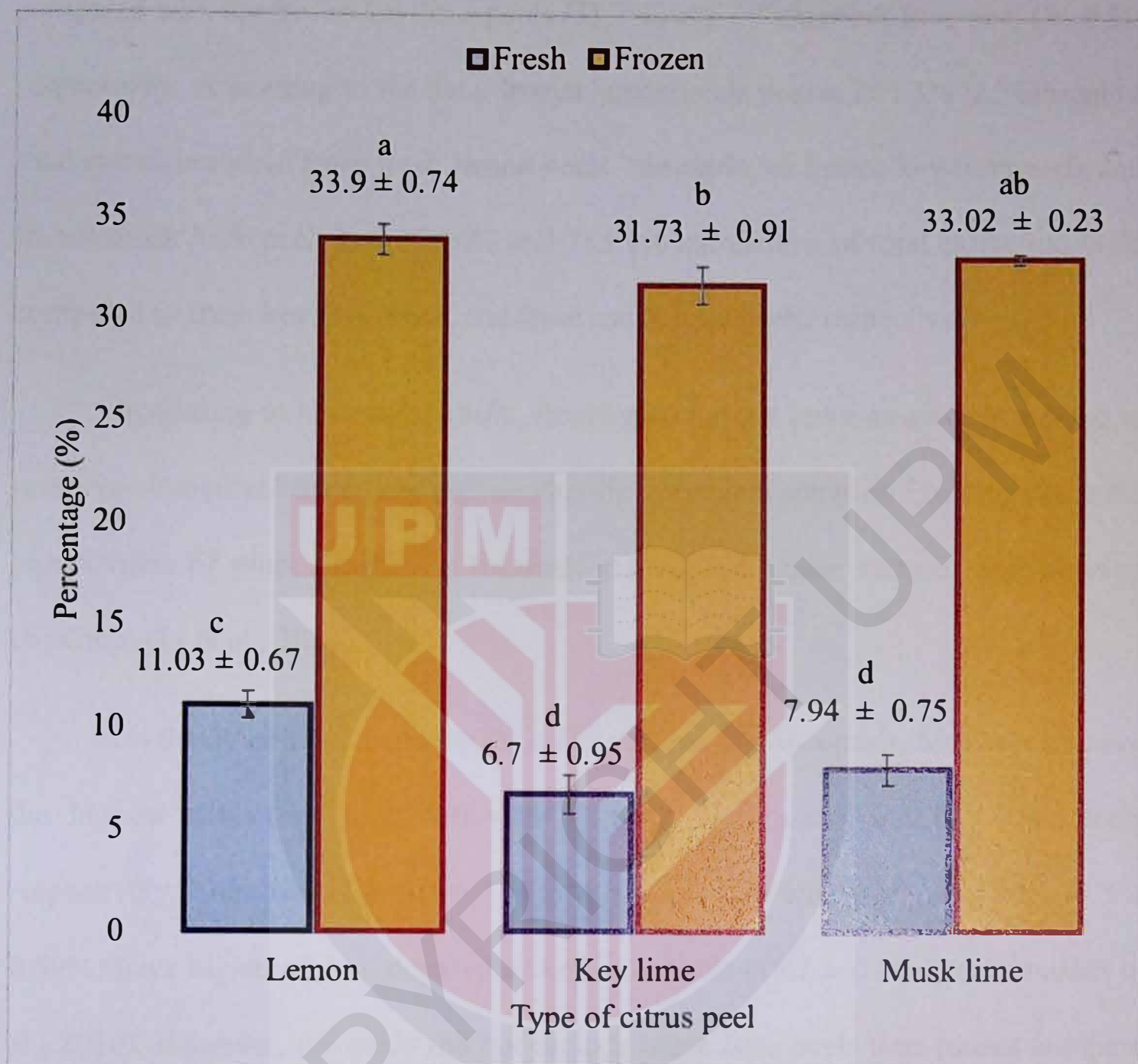


Figure 4.1 Extraction yield (%) for fresh and frozen citrus peels sample

Each of the value are expressed as mean $\pm$ standard deviation, SD (n=18). Results are expressed as percentage (%). Mean with different letters indicate there is significant difference at level $p < 0.05$.

Analysis of One Way ANOVA shows there were significant differences in species between the frozen and fresh samples of the citrus peels ($p < 0.05$) (Figure 4.1).

The extraction yield of fresh lemon peels (11.0%) was significantly low compared with the frozen lemon peels (33.9%). Similarly, the extraction yield of fresh

key lime peels (6.7%) and fresh musk lime (7.9 %) were also significantly low compared with the frozen key lime peels (31.7%) and frozen musk lime peel (33.0 %) respectively. According to the data, frozen lemon peels possess 207.3% increment of total extraction yield from fresh lemon peels. Similarly, frozen key lime peels and frozen musk lime peels had 373.6% and 315.9% increment of total extraction yield compared to fresh key lime peels and fresh musk lime peels, respectively.

According to a previous study, freezing technique serves as a better method to preserve antioxidant activity of polysaccharide from plant samples. Freezing causes the bioactivities of plant samples to be maximally protected under vacuum and freezing conditions (Li et al., 2016).

On the other hand, between the different types of citrus peels, lemon peels have the highest extraction yield followed by musk lime peels and key lime peels respectively. This data aligned well with previous studies where lemon peels ($44.7 \pm 0.59\%$) have higher yield compared to key lime peels ($15.2 \pm 0.20\%$) (Guimarães et al., 2010). However, the study did not include musk lime peels thus cannot compare the percentage of extraction yield between the lemon peels, key lime peels and musk lime peels. thus, not allowing comparison to be made.

4.2 Moisture content of samples

The moisture content of citrus peels samples (lemon, musk lime and key lime) was determined by using method established by Association of Analytical Communities (AOAC). The peels of citrus were dried using oven drying method at temperature of 105°C. The moisture content was used to calculate the antioxidant content and antioxidant activities of citrus peels samples in term of fresh weight.

Drying method is commonly used for food preservation and assist to quantify the moisture content of each food sample. The common method used in the dehydration process include oven drying, air drying and freeze drying. Water is the major constituent of food product and oven drying method is one of the simplest method available in which the sample is heated in the oven and the loss of weight is used to calculate and determine the moisture content of the sample (Bradley, 2010).

Result of ANOVA analysis showed there were significant differences in moisture content between the citrus peels sample ($p < 0.05$) (Figure 4.2). Lemon peels have the highest moisture content followed by musk lime peels then key lime peels. The results of moisture content of citrus peels are shown in Figure 4.2 where lemon peel has $83.7 \pm 0.19\%$, musk lime peel has $79.3 \pm 0.46\%$ and key lime peel has $77.300 \pm 0.36\%$. The differences of percentage of moisture content between these citrus peels might be due to different type of samples possess their own percentage of moisture content.

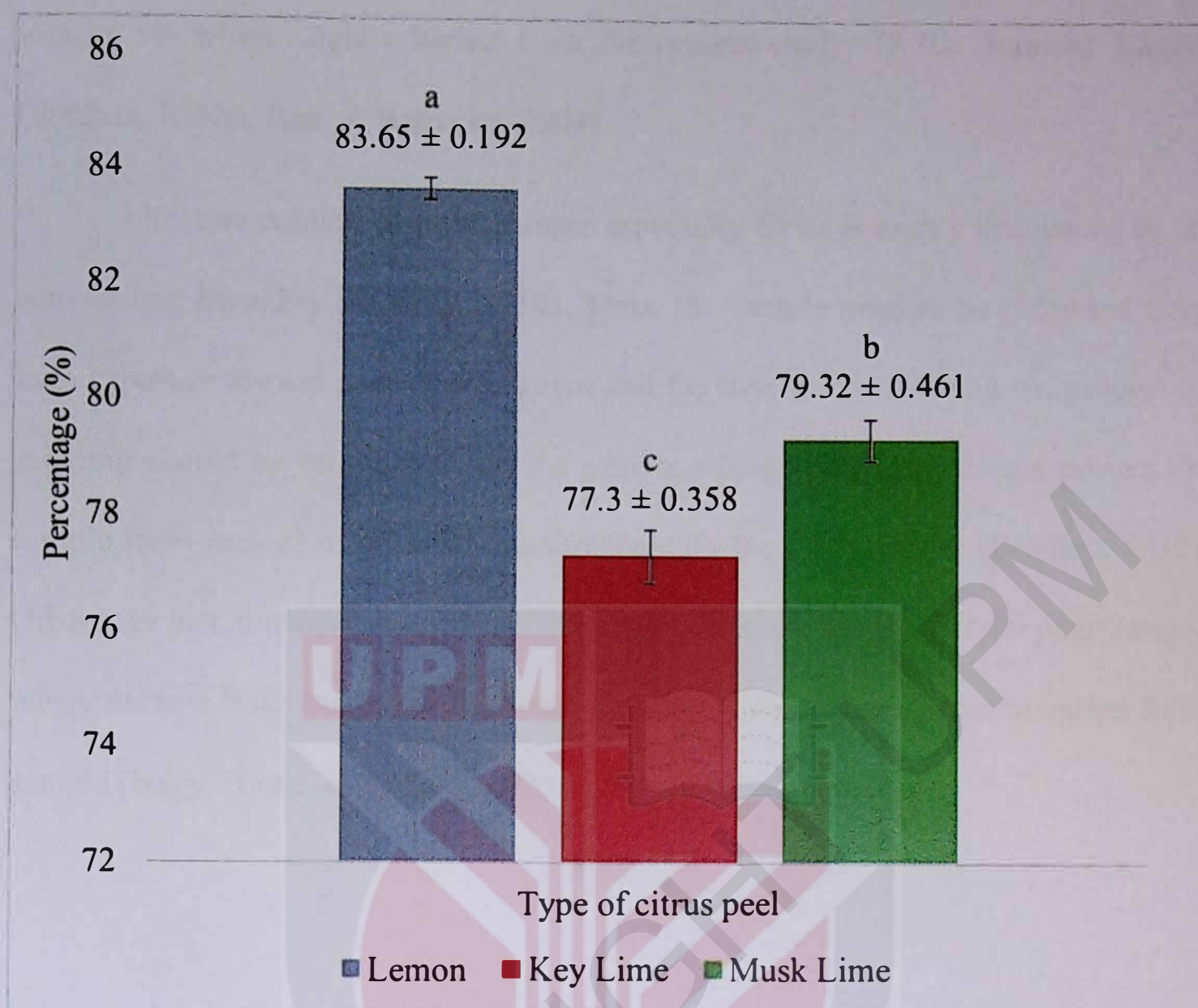


Figure 4.2 Moisture content of citrus peel

All the values are expressed as mean $\pm$ standard deviation, SD ($n = 9$). Results are expressed as percentage (%). Mean with different letters indicate there is a significant difference at level $p < 0.05$.

According to study done previously, the percentage of moisture of fresh lemon peel sample was 81.23% which is slightly lower than the present study, 83.65% (El-ghfar, Ibrahim, Hassan, Fattah, & Mahmoud, 2016). Fresh musk lime contain moisture content ranging from 84.14% to 85.95% (Abdullah et al., 2012). However, in the previous study it does not specify the specific parts of the musk lime and the moisture content represented the whole fruit including peels, pulp and seeds. Previous study by Ramesh Yadav et al., (2004) reported the moisture content of fresh key lime

was 86.5% which slightly higher from the present study, 77.3% (Ramesh Yadav, Chauhan, Rekha, Rao, & Ramteke, 2004).

Moisture content of plant sample especially fruits is highly influenced by its surrounding humidity (Bradley, 2010). Thus, the sample need to be protected from long exposure toward open environment and the heat fraction during the process of grinding should be minimized and the sample storage container should protect the sample from loss of moisture to equilibrate with the environment (Bradley, 2010). Other than that, moisture content is also influenced by the maturity of the plant sample where the ripe fruits sample contain higher moisture content compared to unripe fruits sample (Nagy, Riczu, & Tamás, 2016).

4.3 Total phenolic content

Phenolic compounds are comprising one phenolic acid or more which called polyphenol aromatic rings with attached hydroxyl groups in their structures (Minatel et al., 2017). Phenolic compound had been known to have free radical scavenging activities in various plants and have various medicinal and physiological functions in various type of plants (Sultana et al., 2009). Generally, the phenolic compounds have the ability to donate one hydrogen atom or an electron to free radical, in a reduction process of the phenolic compounds and oxidation process of the free radical compound (Dai & Mumper, 2010).

In a quite recent study, total phenolic content of citrus peels samples was measured by the Folic-Ciocalteu (FC) assay. This assay has been commonly used to measure the total phenolic compounds in various plants and natural products due to simplicity, accuracy and sensitivity. However, the FC assay also possessed some limitations and lacking in specificity and have slow reaction rate in acidic pH. It was reported, FC assay can also reacted with the amino acid, thiols, vitamin, sugar and some minerals compound (Everette et al., 2010).

For the quantification of polyphenol in the citrus peels samples, Gallic acid standard curve at concentration of 0.977 µg/ml to 250 µg/ml was used for 70% aqueous ethanol extract. The equation of standard curve for 70% aqueous ethanol extract was $y=0.0091x + 0.005$ ($r^2= 0.9892$). This equation was used to determine the concentration of total phenolic content in unit of milligram of Gallic acid equivalent per gram of sample (mg GAE/g).

The total phenolic content of the citrus peels sample of fresh and frozen are shown in Figure 4.3. The results show that the TPC values of fresh lemon peel (72.0 ± 0.67 mg GAE/g FW) and frozen lemon peel (100.0 ± 0.96 mg GAE/g FW) were significantly different. The total phenolic content between fresh musk lime peel (108.6 ± 1.34 mg GAE/g FW) and frozen musk lime peel (136.5 ± 0.58 mg GAE/g FW) were also significantly different. Moreover, for fresh key lime peel (75.7 ± 1.34 mg GAE/g FW) and frozen key lime peel (118.6 ± 0.77 mg GAE/g FW) were also show the same trend. The result of present study showed that frozen lemon peels had 38.9% increment of total phenolic content from the fresh lemon peels. Similarly, the frozen key lime peels and frozen musk lime peels possess 56.7% and 25.7% increment of total phenolic content respectively.

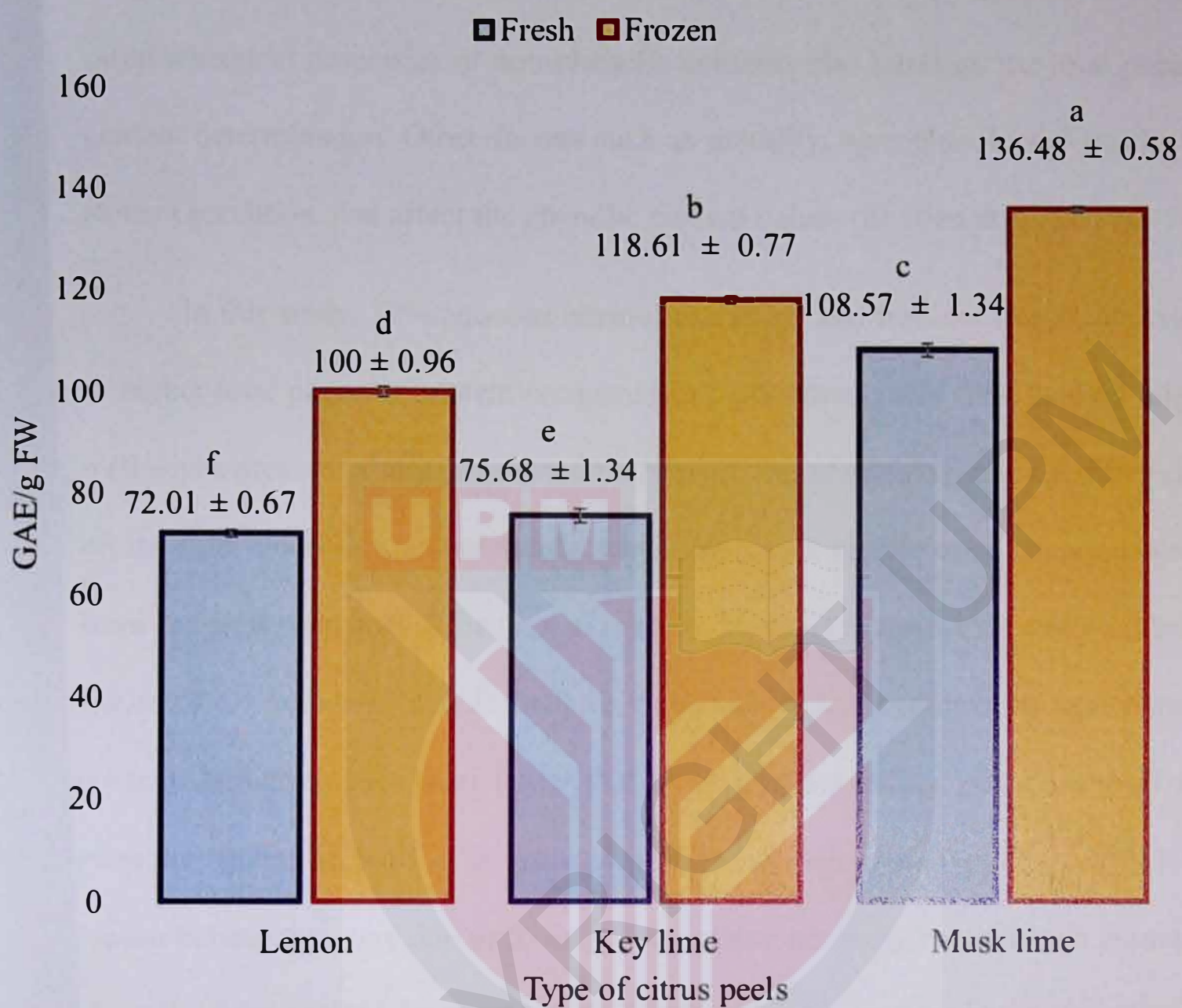


Figure 4.3 Total phenolic content of fresh and frozen citrus peels

Each value expressed as mean $\pm$ standard deviation, SD (n=18). Mean with different letter indicate there is significant difference at level $p < 0.05$.

The total phenolic contents of sample analysed in the present study are ranging from 72.01 mg GAE/g FW to 136.48 mg GAE/g FW. There is not much literature on phenolic content comparing frozen and fresh citrus peels. According to the previous study, the value of total phenolic content of Ajwain (*Trachyspermum ammi*) was 4.312 ± 0.098 mg GAE/g dry matter for frozen sample which higher than fresh sample (1.791 ± 0.040 mg GAE/g dry matter) (Raza et al., 2015). Generally, phenolic content

is strongly influenced by the type of extraction solvent, extraction time, extraction temperature and sample to solvent ratio (Khoddami et al., 2013). Besides, the physiochemical properties of non-phenolic contents also interrupt the total phenolic content determination. Other factors such as maturity, agro-climate and postharvest storage condition also affect the phenolic content values (Shofian et al., 2011).

In this study, 70% aqueous ethanol extraction and freezing treatment resulted to higher total phenolic content compared to fresh citrus peels. This finding aligned well with a previous study that shows freezing process of tomatoes had a positive effect on the total phenolic content values which increased (5.9%) total phenolic content from the fresh tomatoes (Chang et al., 2006). Moreover, a previous study on frozen leaves of *C. caudatus* and *P. betle* revealed significant increment in total phenolic content. However, they also found that there was a simultaneous decline of total phenolic content in both *A. accidentale* and *P. minus* leaves (Wei et al., 2014). The reason behind this may due to fruits and vegetable normally contain high content of phenolic compound in the outer compartment which accumulate in the vacuoles. The food processes such as freezing might accelerate more bound phenolic compound released from the breakdown of cellular part besides, the enzyme which have the ability to destroy antioxidant is deactivated (Chang et al., 2006).

In the present study, musk lime possesses the highest total phenolic content in both fresh and frozen peels followed by key lime peels and lemon peels. This parallel with a previous study which found key lime peels (124 ± 0.52 mg GAE/g extract) possesses higher phenolic content compared to lemon peels (87.77 ± 1.42 mg GAE/g extract) (Guimarães et al., 2010). Besides, other study also found the same results where key lime peels (223.3 mg GAE/g extract) have higher phenolic content compared to lemon peels (131.0 mg GAE/g extract) (Ghasemi et al., 2009). According

to another study key lime peels possess higher phenolic content compared to lemon peels (Shofinita, Feng, & Langrish, 2015). However, all of the study did not compare total phenolic content of musk lime as one of the selected citrus peels samples in their study.

There is another study comparing total phenolic content in citrus juices found that *C. hystrix* (490.74 mg GAE/100ml) possess the highest total phenolic content followed by *C. aurantifolia* (211.70 mg GAE/100ml), *C. sinensis* (135.3 mg GAE/100ml) and *C. microcarpa* (105.0 mg GAE/100ml) respectively (Ghafar, Prasad, Weng, & Ismail, 2010). This finding is slightly difference from the present result which might due to the different in the citrus fruit used, different extraction method and also can also be due to different environmental condition of cultivation. High nitrogen fertilizer exposure toward plants has negative effect toward the total phenolic content other bioactive nutrient such as vitamin C and condensed tannins (Ibrahim, Jaafar, Rahmat, & Rahman, 2011).

The reason which made the total phenolic content phenolic content in the frozen peels of citrus higher than fresh peels of citrus is due antioxidant content usually high at the outer part of plant and the phenolic compound commonly residing in the vacuoles. Freezing process have the ability to release more phenolic compound through cellular wall disruption which subsequently will release the oxidative enzyme and hydrolytic enzyme. Although these enzymes have the ability to destroy the antioxidant in fruits, however the freeze dry process inactivate them simultaneously (Chang et al., 2006; Pérez-Gregorio et al., 2011).

4.4 Total flavonoid content

Flavonoids are grouped as natural compound and usually found both edible and non-edible plants. The flavonoids include hydroxybenzoic acid, anthocynins, hydroxyl cinnamic acids, proanthicynidins, flavonoids, lignans and stilbenes (Cheong et al., 2012). Flavonoids are one of the most potent antioxidant compound due to o-diphenolic group, a 2-3 double bond conjugated with the 4-oxo function and hydroxyl groups in positions 3 and 5 structure usually presence. Flavonoids are very good in inhibiting lipid oxidation and scavenging activity of hydroxyl and peroxy radicals (Lee, Koo, & Min, 2004).

In the present study, the total flavonoid content of samples was measured by using aluminium chloride calorimetric assay. The assay has been widely used to determine the total flavonoid content as it is simple, good, repeatability and accurate method (Amin et al., 2013).

The quercetin standard curve was used for quantification of total flavonoid content at concentration of 0.977 μ g/ml to 1000 μ g/ml for 70% aqueous ethanol extract. The equation of standard curve for 70% ethanol extract was $y=0.0041x+0.0619$ ($r^2=0.987$) and this was used to determine the concentration of total flavonoid content in unit milligram of quercetin per gram of sample (mg QE/g FW).

The total flavonoid content of frozen and fresh citrus fruits sample are shown in Figure 4.4. Based on the results, the total flavonoid content values for fresh lemon peels (50.51 mg QE/g FW) and frozen lemon peels (78.15 mg QE/g FW) were significantly different. The total flavonoid content in fresh key lime peels (50.75 mg QE/g FW) also was significantly lower than the frozen key lime peels (82.22 mg QE/g FW). The present study showed that frozen lemon peels had 54.7% increment of total

flavonoid content from the fresh lemon peels. Similar to the frozen key lime peels and frozen musk lime peels possess 62.0% and 47.5% increment of total flavonoid content respectively.

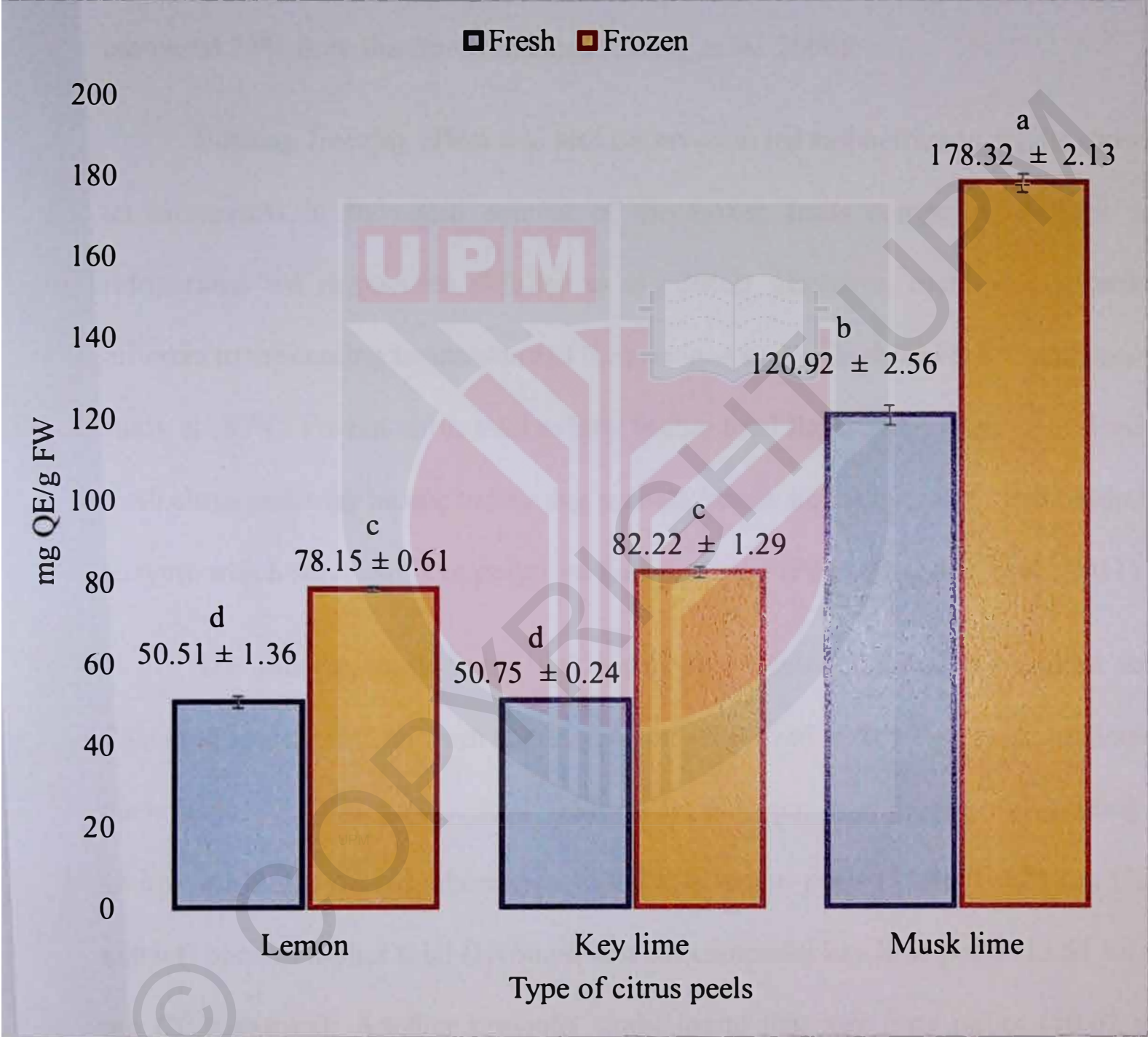


Figure 4.4 Total flavonoid content of fresh and frozen citrus peels

Each values are expressed as mean ± standard deviation, SD (n=18). Mean with different letter indicate there is significant difference at level $p < 0.05$.

The flavonoid content of sample analysed in present study range from 50.51 mg QE/g FW to 178.32 mg QE/g FW. There is a lack of literature of published data on flavonoid content of frozen and fresh citrus fruits. According to a previous study, total flavonoid content in fresh tomatoes were the lowest compared to the tomatoes which under gone the freezing processes. The flavonoid content in frozen tomatoes increased 23% from the fresh tomatoes (Chang et al., 2006).

Besides, freezing effect was also observed in red raspberries as results showed an increments in flavonoid content of the frozen fruits compared to fresh and refrigerated red raspberries (Mullen et al., 2002). However, there was a marked different in the cooling temperature of the previous study which was at 4°C and present study at -80°C. Frozen citrus peel exhibit higher total flavonoid content compared to fresh citrus peel may be due to freezing process inactivate the hydrolytic and oxidative enzyme which responsible in polyphenol degradation (Pérez-Gregorio et al., 2011)

The present study found that musk lime peels exhibited the highest total flavonoid content in both fresh and frozen peels followed by key lime peels and lemon peels respectively. However, these findings are different from the previous finding of Guimarães et al., (2010) where they found that lemon peels (15.96 ± 0.24 mg CE/g extract) possess higher total flavonoid content compared key lime peels (13.61 ± 0.64 mg CE/g extract). Another previous study found that key lime juices (10.67 mg Hesperidin equivalent/100ml) possess higher total flavonoid content compared to musk lime juices (8.70 mg Hesperidin equivalent/100ml) (Ghafar et al., 2010). These differences in flavonoid content may be due to different part of citrus were used, different extraction solvent and different extraction method being applied (Khoddami et al., 2013).

4.5 Antioxidant activity

Antioxidant have the capability to slow or prevent the oxidation process of other molecules. Fruits, vegetable, herbs and spices contain different antioxidant (Guo, Yang, Wei, Li, & Xu, 2003). Besides, method of analysis can affect the antioxidant activity of a sample, thus one single method has no ability to fully determined antioxidant activity of an extract being studied (Fu et al., 2011). Therefore, various methods have been developed to determine the antioxidant activity of various plant materials. In the current study, the antioxidant activity was determined by DPPH free radical scavenging assay and ferric reducing antioxidant power (FRAP) assay.

4.5.1 DPPH free radical scavenging assay

DPPH free radical scavenging assay is a procedure of quantifying antioxidant capacity by measuring the decrease in absorbance after oxidation processes. The activity is determined by the ability of the antioxidant to retard colour loss (Foti, Daquino, & Geraci, 2004). The application of this assay is widespread due to the quickness, highly sensitive and convenient method. The DPPH free radical scavenging assay also was introduced as one of an easy and accurate method for assessing the antioxidant activity in fruits, vegetables, medicinal plants, spices and herbs (Hibamoto, 2009). Besides, the DPPH powder are commercially available and can easily be used (Huang et al., 2005).

There are a few caution steps need to be noted while performing DPPH assay and while interpreting the data. This is because antioxidant compound reacts differently with DPPH radicals which highly depends on their structural conformation. Antioxidant may be in the form of water soluble, fat soluble or bound to the cell walls which they are not necessarily available to react with DPPH radicals. Thus, differing kinetics and the reaction will not go to accomplishment in certain assay time (Rababah et al., 2010).

In this study, quercetin standard curve at concentration of 125 µg/ml to 0.02 µg/ml was used for 70% aqueous ethanol extract. EC₅₀ was calculated as amount of antioxidant present in the sample needed to decrease the initial DPPH free radical concentration by 50%. The total DPPH radical scavenging effect of the fresh and frozen citrus peels at various concentration are shown in Figure 4.5.

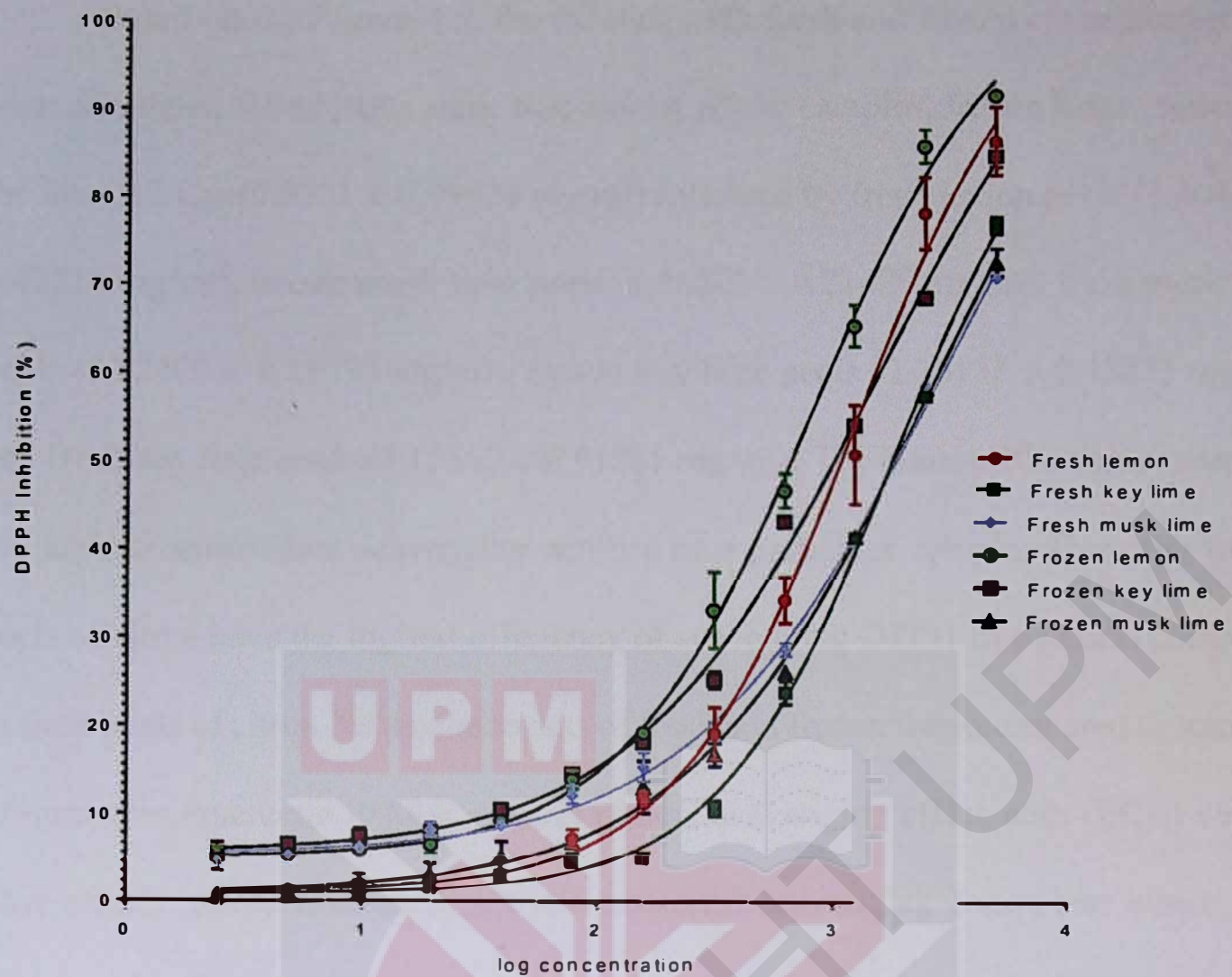


Figure 4.5 DPPH Scavenging Activity of frozen and fresh citrus peels at various concentrations.

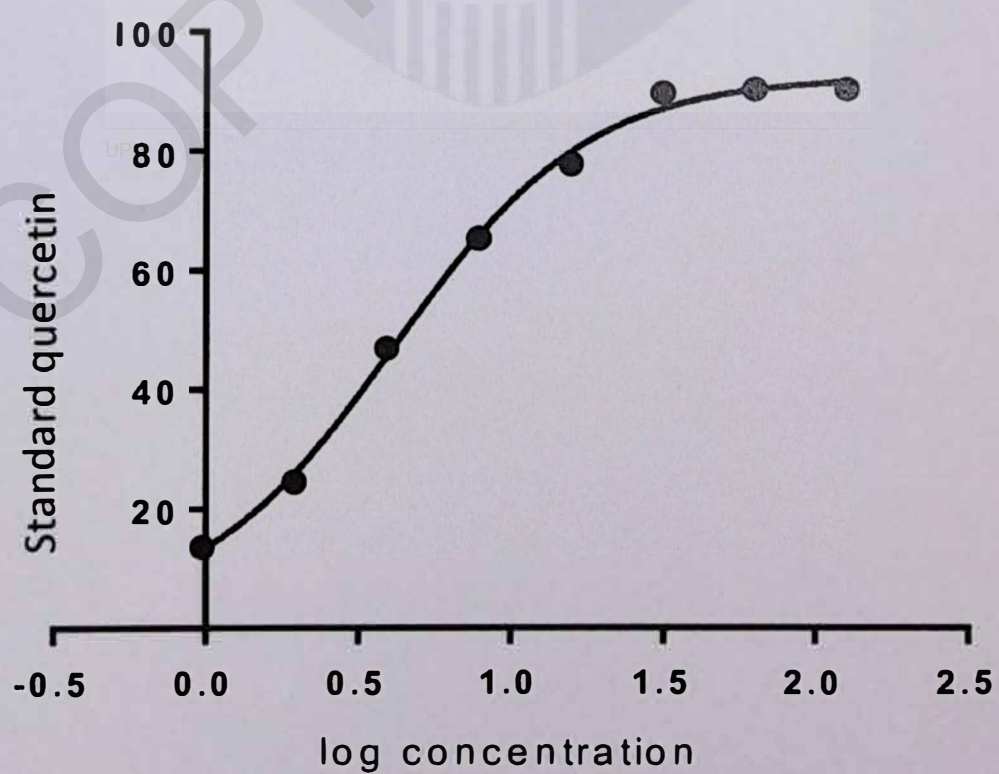


Figure 4.6 Scavenging Activity of Quercetin Standard.

Based on the Figure 4.5, the EC₅₀ of each fresh and frozen citrus sample had been calculated. The results show that among all the samples, frozen lemon peels had the lowest EC₅₀ (0.8231 ± 0.09624 mg/ml) followed by fresh lemon peels (1.30467 ± 0.42218 mg/ml), frozen musk lime peels (1.56800 ± 0.21429 mg/ml), fresh musk lime peels (1.82500 ± 0.25332 mg/ml), frozen key lime peels (2.70133 ± 0.42273 mg/ml) and fresh key lime peels (3.15567 ± 0.91865 mg/ml). The lowest EC₅₀ value represent the highest antioxidant scavenging activity of a particular sample. Thus, the frozen peels of citrus have the highest efficiency of scavenging DPPH free radical compared to fresh peels of citrus. When the results of fresh and frozen were compared to standard of quercetin, quercetin exhibit smaller radical scavenging effect with (EC₅₀) values. This clearly shows that quercetin is a powerful scavenging compound which only account for 0.0042 mg/ml to inhibit at least 50% of DPPH free radical.

The present study showed that frozen lemon peels had 36.9% decrease of EC₅₀ of total scavenging effect from the fresh lemon peels. Similarly, to the frozen key lime peels and frozen musk lime peels possess 14.2% and 14.6% decrease of EC₅₀ of total scavenging effect, respectively. Even the result show there is no significant difference between scavenging effect of fresh and frozen between fresh and frozen citrus peels, however, there is a slightly lower of the of DPPH EC₅₀ of total scavenging effect in frozen citrus peels compared to fresh citrus peels.

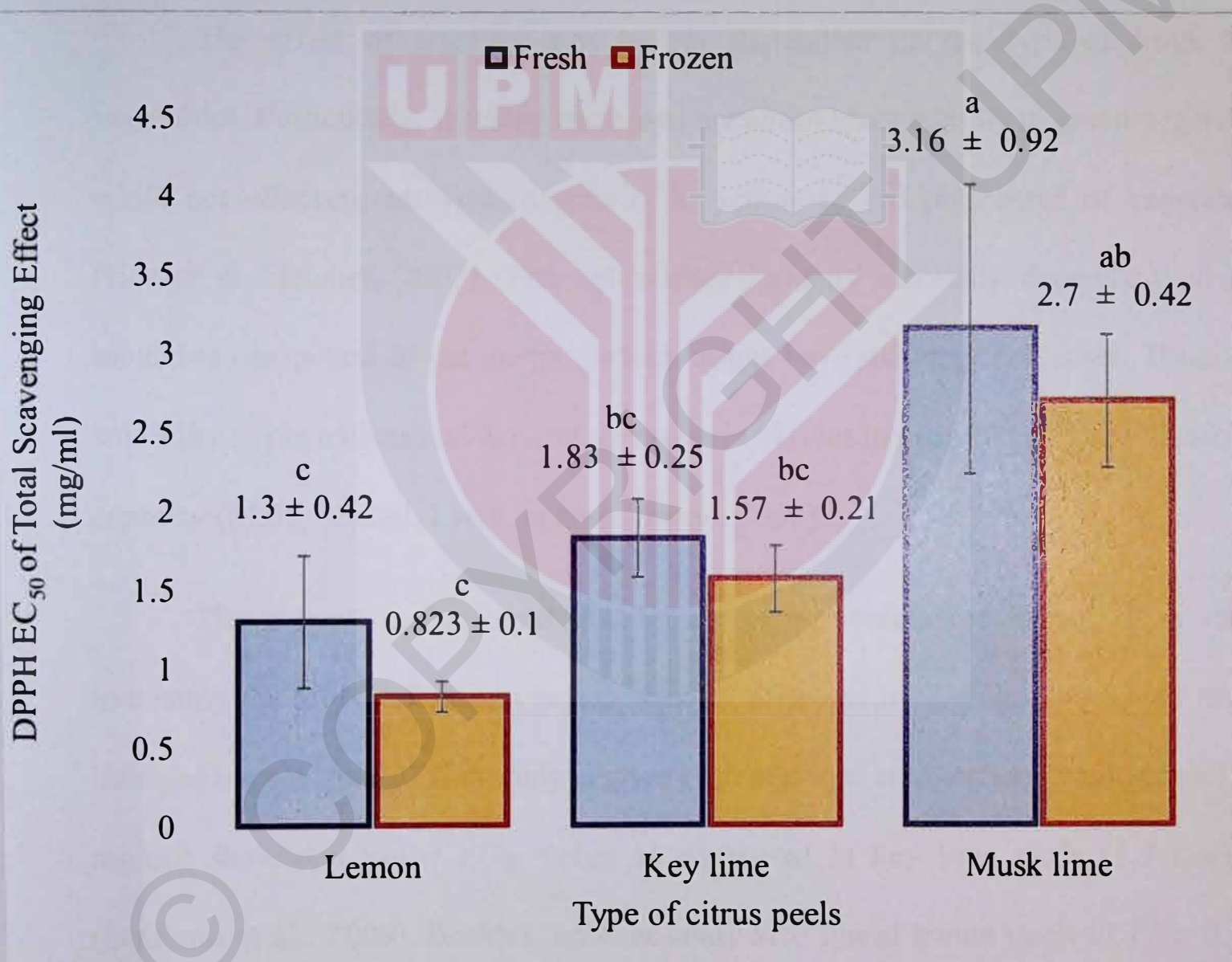


Figure 4.7 Total Scavenging effect (EC₅₀) among the fresh and frozen peel of citrus fruits.

Each of the values are expressed as mean ± standard deviation, SD (n=18). Mean with different letter indicate there is a significant difference between the total scavenging effect at level $p < 0.05$.

In this study, freezing treatment affected the phenolic and flavonoid contents as seen in DPPH scavenging effect of fresh and frozen peels of citrus fruits. This study has a similar finding with previous study done showed that frozen ajwain leaves have significantly higher scavenging effect compared to the fresh ajwain leaves (Raza et al., 2015). Furthermore, the other also study showed that frozen guava pulp also exhibit higher level of scavenging ability compared to the fresh guava pulp (Polinati, Oliveira, & Fialho, 2012).

The effect of freezing was largely dependent on the type of fruits and vegetables. Particularly, freezing increased the antioxidant activity in green vegetable while not effecting antioxidant activity in yellow or red pigmented of vegetables (Hunter & Fletcher, 2002). The antioxidant capacity is highly dependent on the bioactive compound in the sample which might have different polarities. Thus, the solubility of phytochemical in particular solvent of roles in determining the antioxidant capacity (Melo, Maciel, Lima, & Nascimento, 2008).

The present study found that lemon peels possess the lowest EC₅₀ value indicating the highest free scavenging activity followed by key lime peels and musk lime peels respectively. This study aligned with previous study where lemon peels (1.4 mg/ml) showed a lower EC₅₀ value as compared to key lime peels (1.9 mg/ml) (Ghasemi et al., 2009). Besides, another study also found lemon peels (3.77 ± 0.16) exhibited higher scavenging activity compared to key lime peels (1.72 ± 0.40) (Guimarães et al., 2010). Thus it is clearly indicating that lemon have a higher antioxidant scavenging activity compared to other citrus peels studied. However, both of the previous study did not include musk lime as one of selected citrus peel species.

Another previous study by Ghafar et al., (2010) compared the DPPH radical scavenging activities of four different citrus species found that musk lime juices possess lowest the DPPH radical scavenging activities followed by the orange, key lime and wild lime respectively. The differences in results obtain may be due to different compartment of citrus used and different extraction method.

The DPPH radical scavenging activities values in citrus peel have high possibility to be interfere with the presences of high ascorbic acid content in the peel (Divya et al., 2016). Ascorbic acid is one of powerful bioactive compound for free radical scavenging activity in the DPPH assay (Oh et al., 2010). The ascorbic acid also can be one of main antioxidant responsible for the activity in most tropical fruits such as guava and citrus fruits (Marta et al., 2005). Study by Lo Scalzo, (2008) found that ascorbic acid possess strong significant action toward the DPPH scavenging activity at a steady rate.

4.5.2 Ferric reducing antioxidant power (FRAP)

FRAP assay is another antioxidant activity determinant assay being used in the present study. This assay represents the antioxidant as a reductant in redox-linked calorimetric reaction and also used to quantify the capability of antioxidant in sample to reduce the ferric (III) ions to ferrous (II) ions in the FRAP reagents at low pH level (Halvorsen et al., 2002). Thus, this assay has the ability to measure the reducing capacity. Commonly, the reduction of ferric ion into ferrous ion by the antioxidant compound will cause increase in colour intensity from colourless to blue colour solution (Amin et al., 2013). The procedure of FRAP assay is quick, easy and simple to be perform. FRAP assay is highly suitable for antioxidant assessment of the fruit sample because there are limited amount of glutathione, this is due to FRAP assay relatively react slowly with certain antioxidant such as glutathione (Schafer & Buettner, 2001).

In the present study, ferrous sulphate standard curve was used at the concentration of $1\text{mMFe}^{2+}/\text{g FW}$ was used for 70% aqueous ethanol extract. The equation of standard curve was $y=0.0004x+0.0798$ ($r^2=0.9967$). The results were expressed as millimoles of ferrous ions per gram of fresh weight sample ($\text{Mm Fe}^{2+}/\text{g FW}$).

The results of Ferric Reducing Antioxidant Power of fresh and frozen peels of citrus are as shown in Figure 4.8. From the results, the FRAP value for the fresh and frozen citrus peels range from $0.3826\text{ mMFe}^{2+}/\text{g FW}$ to $0.5227\text{ mMFe}^{2+}/\text{g FW}$. FRAP value of frozen musk lime peels ($0.5227 \pm 0.009\text{ mMFe}^{2+}/\text{g FW}$) was significantly higher than the value of fresh musk lime peels ($0.4551 \pm 0.003\text{ mMFe}^{2+}/\text{g FW}$). Next, frozen peels of lemon ($0.5121 \pm 0.006\text{ mMFe}^{2+}/\text{g FW}$) also showed significantly higher value than fresh lemon peels ($0.3826 \pm 0.003\text{ mMFe}^{2+}/\text{g FW}$). The FRAP value

of the frozen key lime peels ($0.4626 \pm 0.006 \text{ mMFe}^{2+}/\text{g FW}$) also was significantly higher than fresh key lime peels ($0.4377 \pm 0.003 \text{ mMFe}^{2+}/\text{g FW}$).

The present study showed that frozen lemon peels had 34.2% increment of ferric reducing antioxidant power value from the fresh lemon peels. Similarly, to the frozen key lime peels and frozen musk lime peels possess 6.8% and 15.2 % increment of ferric reducing antioxidant power value, respectively. Thus, there were significant differences in reducing power of fresh and frozen of citrus peels.

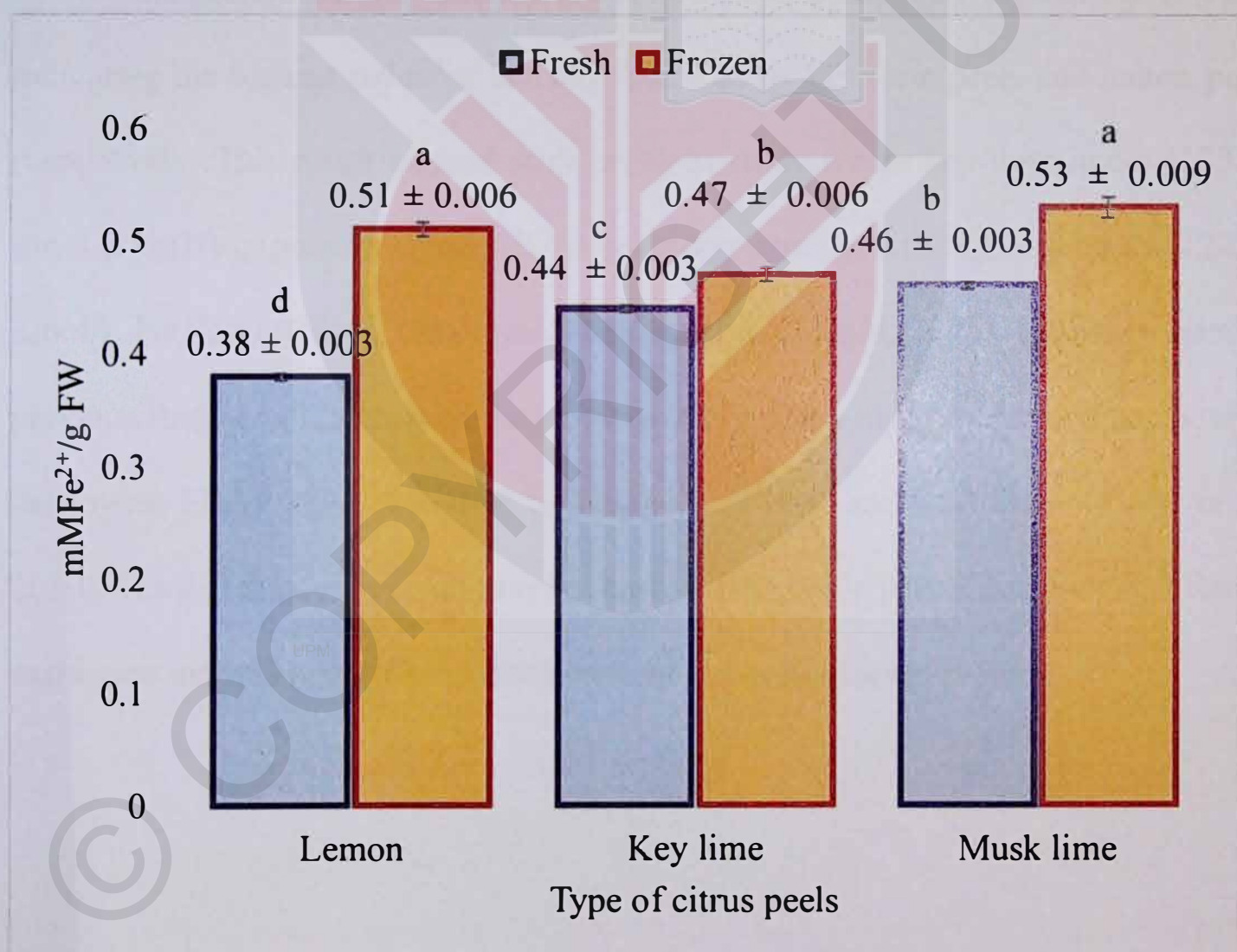


Figure 4.8 Ferric reducing antioxidant power, FRAP test on various fresh and frozen citrus peels

Value above represent mean $\pm$ standard deviation, SD (n=18). Mean with different letter indicate there is a significant difference at level $p < 0.05$.

Analysis of One Way ANOVA shows there were significant difference between the fresh and frozen citrus peels ($p < 0.05$). The results indicated that frozen peels of citrus have the highest antioxidant activity compared to the fresh peels of the citrus. There are limited published data on ferric reducing antioxidant of fresh and frozen peels of lemon, key lime and musk lime. Thus, comparison can only be made for the fresh and frozen samples using guava. Although, no significant difference between fresh and frozen sample but there is slightly higher FRAP value in the frozen treatment of guava sample compared to the fresh guava (Polinati et al., 2012).

The present study found that musk lime peels possess the highest FRAP value indicating the highest reducing activity followed by key lime peels and lemon peels respectively. This result aligned with previous study where key lime juices ($173.25 \mu\text{mol/L Fe(II)/g}$) possess higher FRAP value compared to lemon juice extract ($122.75 \mu\text{mol/L Fe(II)/g}$) (Oikeh, Omoregie, Oviasogie, & Oriakhi, 2016). However, another previous study contradicts with the previous study which found that musk lime possess the lowest FRAP value followed by orange, key lime and wild-lime (Ghafar et al., 2010). The differences in result may be due to difference in part of citrus used, different extraction method and different environment factor during cultivation.

4.6 Correlation between antioxidant content and activity

The DPPH scavenging activity (EC_{50}) of the fresh and frozen samples of citrus peels showed in table 4.1. The study showed that there is no correlation between total phenolic content and total flavonoid content. The lower DPPH scavenging activity represented the higher antioxidant effect. However, in the present study the high antioxidant capacity has not been correlated with phenolic and flavonoid compound. One of the previous study reveal that ascorbic acid is a powerful free radical scavenger in DPPH assay (Oh et al., 2010). Citrus peels are well known for its high content of ascorbic acid. According to a previous study, among three type of citrus, lemon exhibited the highest vitamin C content (43.96 ± 0.93 mg/100g) followed by the key lime (27.78 ± 0.53 mg/100g) and musk lime (18.62 ± 0.53 mg/100g) (Najwa, 2017). This aligns with result of the present study where the highest scavenging effect was presented in lemon peel followed by key lime peels and musk lime peels. An earlier study which investigated the antioxidant activity, phenolic and flavonoid content of 13 species of citrus peels and tissues found no correlation between total phenolic content and total flavonoid content with scavenging activity (Ghasemi et al., 2009)

Finding of the present study is also supported by another previous study which determined bioactive compound, vitamin C and antioxidant capacities in fresh and frozen guava. Guava is one of the fruit very high in vitamin C. Based on the study, there was no correlation between phenolic compound and antioxidant capacity of guava sample. However, there was a high correlation between vitamin C and antioxidant capacities (Polinati et al., 2012). In mulberry, (*Morus nigra*), grape (*Vitis vinifera*), acai berry (*Euterpe oletacea mart.*), guava (*Psidium guajava*), strawberry (*Fragaria vesca var.*), acerola (*Malpighia glabra linn.*), pineapple (*Ananas comosus L.*), mango (*Mangifera indica L.*), graviola (*Anona muricato L.*), cupaucu (*Theo broma*

grandiflorum) and passion fruit (*Passiflora sp.*), vitamin C is the primarily responsible compound for antioxidant activity (Marta et al., 2005). Many researchers consider vitamin C as largest contributor to antioxidant capacity in fruits samples (Thaipong, Boonprakob, Crosby, Cisneros-zevallos, & Hawkins, 2006). In a previous study done by Thaipong et al (2006), it was confirmed that there was a strong correlation exist between antioxidant capacity and ascorbic acid contents in fruit samples such as orange and guava.

Table 4.1 shows the FRAP value of the fresh and frozen samples of citrus peels in the present study showed positive correlations with the total phenolic content ($r=0.783$) and total flavonoid content ($r=0.681$). Therefore, the higher the FRAP value of the frozen and fresh citrus peel samples, the higher the total phenolic and total flavonoid contents. The total phenolic and flavonoids are highly correlated with the ferric reducing power. It suggests that phenolic and flavonoids may contribute to the reducing power of the extracts. Preceding study reported that peel of *Citrus sinensis L.* and *Citrus aurantium L.* showed strong correlation between phenolic content and reducing power (Lagha-Benamrouche & Madani, 2013).

**Table 4.1 Summary of the correlation between antioxidant content and activity
among the fresh and frozen of citrus peels**

Antioxidant Content Antioxidant Activity	Total Phenolic Content	Total Flavonoid Content
DPPH Scavenging Activity (EC ₅₀)	NC	NC
Ferric Reducing Antioxidant Power	r= 0.783	r= 0.681

NC= no correlation; r= Pearson coefficient; significant at p<0.01;

CHAPTER 5

CONCLUSION

5.1 Conclusion

The fresh and frozen peel of citrus fruits (lemon, musk lime and key lime) were selected in the present study and analysed for its antioxidant content using the Folin-Ciocalteu (FC) Reagent and aluminium chloride calorimetric assays to determine total phenolic content (TPC) and total flavonoid content (TFC) of the fresh and frozen citrus peels samples, respectively. Besides, DPPH free radical scavenging assay (DPPH) and ferric reducing antioxidant power (FRAP) were used to determine the antioxidant capacity for the samples. For DPPH free radical scavenging assay, frozen peels of citrus samples presented a higher antioxidant capacity as compared to fresh peels of citrus. Furthermore, all samples of frozen citrus peels exhibited higher ferric reducing antioxidant power values as compared to fresh citrus peels. The frozen samples of citrus peels also showed greater values of total phenolic content and total flavonoid content as compared to the fresh citrus peels. Thus, there was a significant higher in antioxidant capacity and antioxidant content in the frozen citrus peels compared to fresh sample.

Overall, it can be concluded that frozen peels of the citrus possess higher antioxidant properties as compared to fresh citrus peels. Therefore, food industry are encouraged to utilise the by-product of citrus which is the peel as it is rich in antioxidant. Furthermore, citrus products are widely available in the market as they are well-known with various of health benefits. Thus, the peels of citrus can also be promoted so that more people can benefit from this cheap natural antioxidant. All in all, the utilisation of the citrus peel would also help to lessen the wastes and environmental pollution.

5.2 Limitations and Strengths

One of the study limitation was that finding may not representative as the fruits only purchased from the Pasar Borong Selangor. Different planting area and different way of cultivation may affect the antioxidant. Besides, the amount of fertilizer, pesticide, fruit's maturity and type of soil are not known. Each of the factors may result in different phytochemical and nutrient composition in the samples.

Besides, other limitation is this study was unable to detect and quantify the amount and type of antioxidant presented in the citrus peels. The phenolic content and scavenging activity may influence by other factor such as vitamin C and other non-polyphenol antioxidant. Thus, the quantification of antioxidant present in the samples are crucial to determine the actual bioactive compound present.

Moreover, there is a need for more antioxidant test as Folin-Ciocalteu assay, aluminium chloride calorimetric assay, DPPH free radical scavenging activity and ferric ion reducing antioxidant power (FRAP) were not enough to determine the antioxidant content and antioxidant activity of the fresh and frozen citrus peels. Other assay such as oxygen radical absorbance capacity (ORAC), ABTS, phosphomolybdenum assay, hydroxyl radical scavenging activity assay and others.

Despite the limitations, present study had added the knowledge and information of antioxidant content and activity in the fresh and frozen citrus peels by using scientific methods. Since there is limited study on the effect of freezing on the citrus peels in Malaysia.

Furthermore, the present study had increased the value of citrus peels which treated as waste in the environment. This study found that citrus peels contain high bio-active compound which had benefit to human health. Thus, the citrus peels should be fully utilized as it can be developed into pharmaceutical products or nutraceutical products.

5.3 Recommendations

The present study only determined the total phenolic content, total flavonoid content, DPPH scavenging activity and ferric reducing antioxidant power of selected fresh and frozen citrus peels (lemon, key lime and musk lime) extract by using 70% aqueous ethanol solvent. Future research may consider the usage of other solvent such as methanol, hexane and chloroform for extraction to extract the bioactive compound in the sample of fresh and frozen citrus peels. Moreover, other factor can be modified such as extraction time, extraction temperature, concentration ratio of solvent to sample and solvent concentration which have big influence on phenolic profile for future research.

Besides, researcher should further investigate the effect of freezing on the amount of ascorbic acid in the citrus peel. The citrus are high in the vitamin C and it also possess a scavenging power. Thus, research on vitamin C in the frozen citrus peel is needed to see whether there is degradation of vitamin C during the freezing duration. According to previous study, vitamin C in citrus do degrade with increase in temperature (Najwa, 2017). Moreover, vitamin C also do influence the Folin-Ciocalteu assay and DPPH scavenging free radical assay. In future study, researcher should investigate on the isolation and purification of bioactive compound by using high performance liquid chromatography (HPLC) to confirm each bio-active compound present. This is because other compound such as sugar and protein also can influence the antioxidant activity of the plant sample. Therefore, HPLC is important to identify, purify and quantify the component of the citrus peels.

Apart from that, in future study researcher can also determine the effect of freezing in different period of freezing of citrus peel. There was a study investigated the effect of freezing in different period, however it not being done on the citrus peels

yet. Thus, researcher can know the optimal freezing period for the citrus in in order to maximize the antioxidant content and antioxidant capacity of citrus peels. Thus, the optimal freezing time could be a standardized for the use of food industry in the future.

Furthermore, future research can be done to identify the full potential of vitamin C and other potent antioxidant in the frozen citrus peels as it has huge beneficial effect towards human health. Last but not least, citrus peel samples possess high antioxidant activities. Therefore, further in-vivo study can be conducted to better appraise the potential health benefit and profit of citrus peels samples. Nevertheless, future researches can also further extend the study into nutraceuticals and pharmaceuticals purpose in the same time widespread the profiling the nutritional value of various citrus peels.

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APPENDICES

Appendix 1: Standard Curve for total phenolic content (TPC)

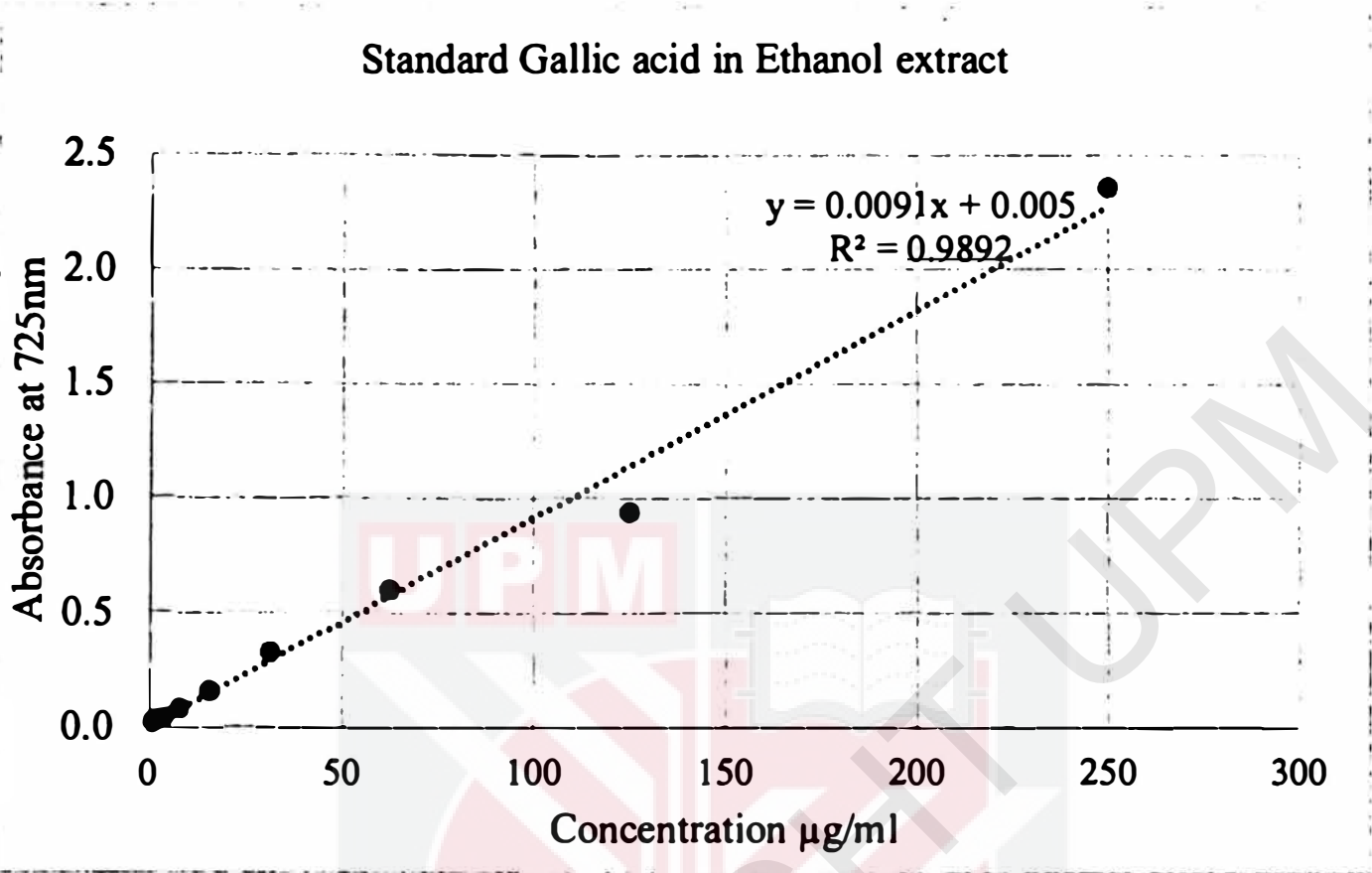


Figure 1: Gallic acid standard for ethanol extract

Appendix 2: Standard for total flavonoid content (TFC)

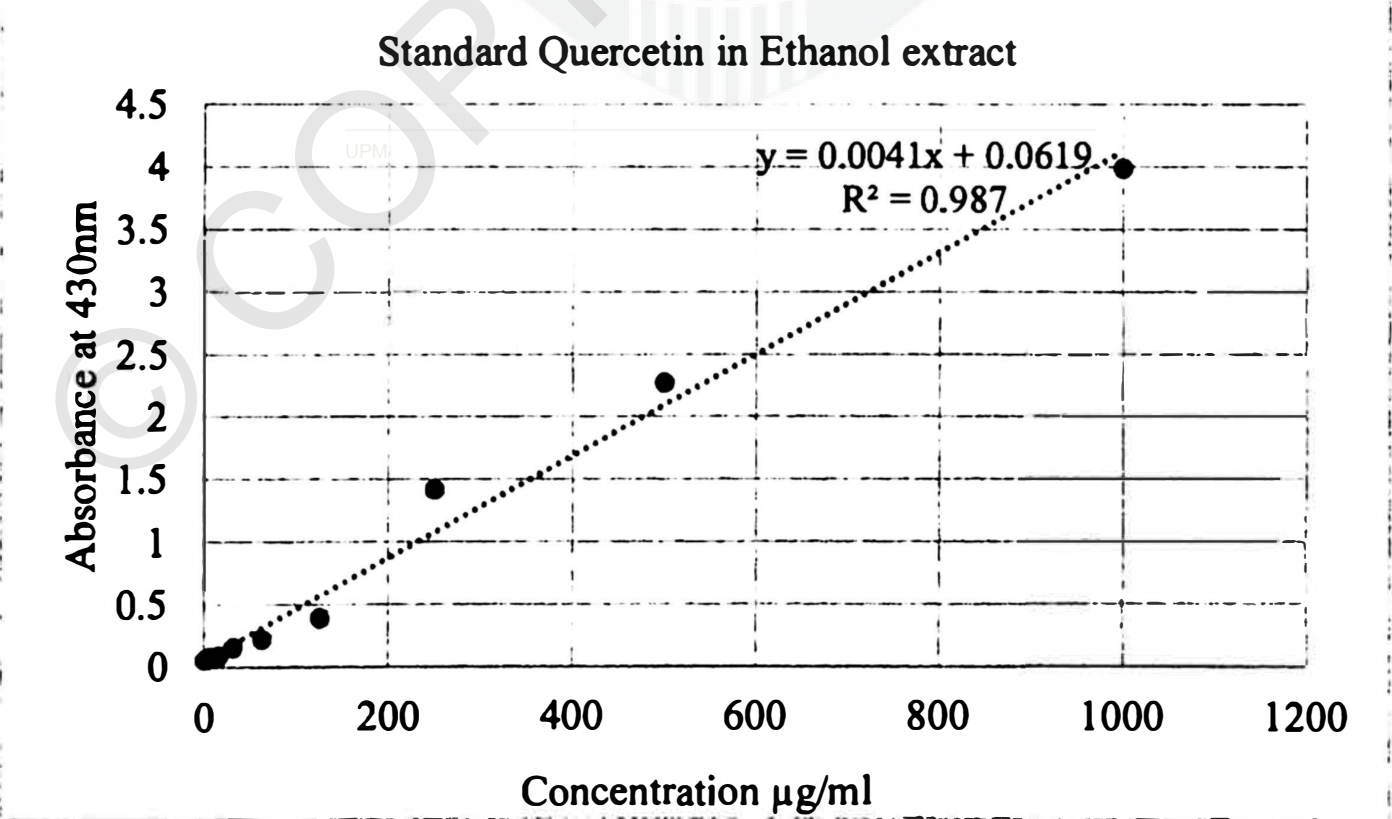


Figure 2: Quercetin standard for ethanol extract

Appendix 3: Standard for DPPH Scavenging Activity assay (DPPH)

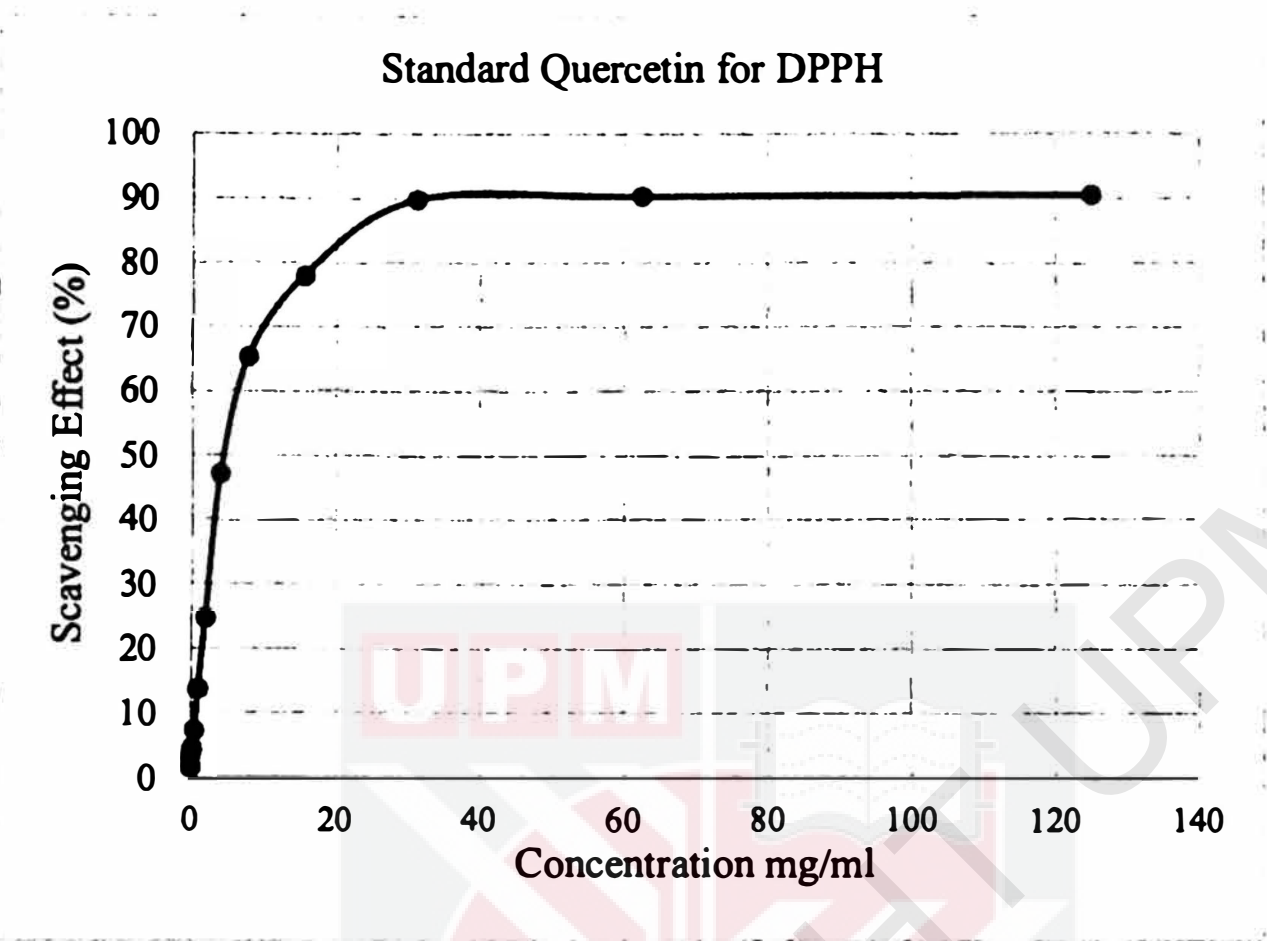


Figure 3: Quercetin standard

Appendix 4: Standard for ferric ion reducing antioxidant power (FRAP)

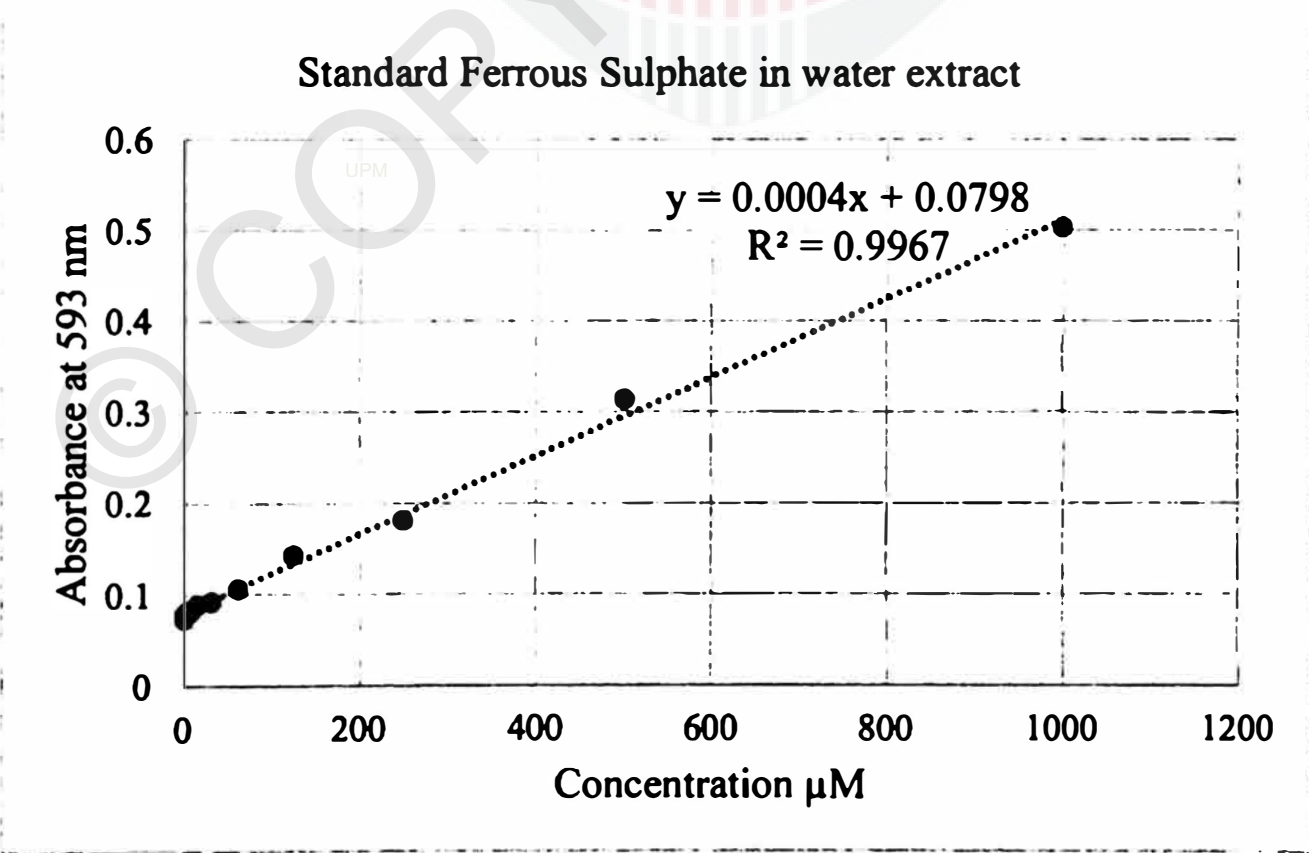


Figure 4: Ferrous sulphate standard in water

Appendix 5: Sample preparation

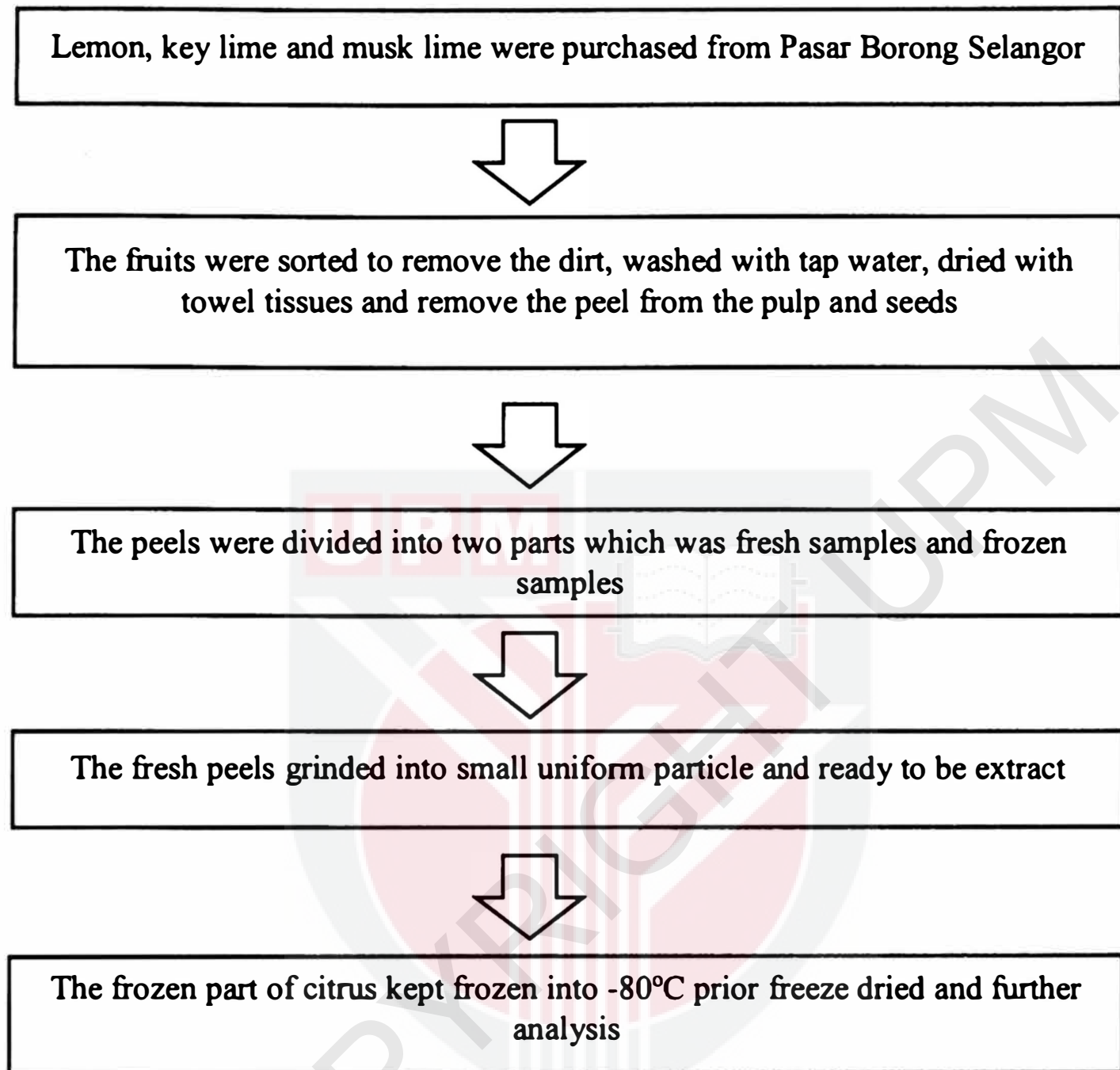


Figure 5: PRM samples preparation

Appendix 6: Sample extraction

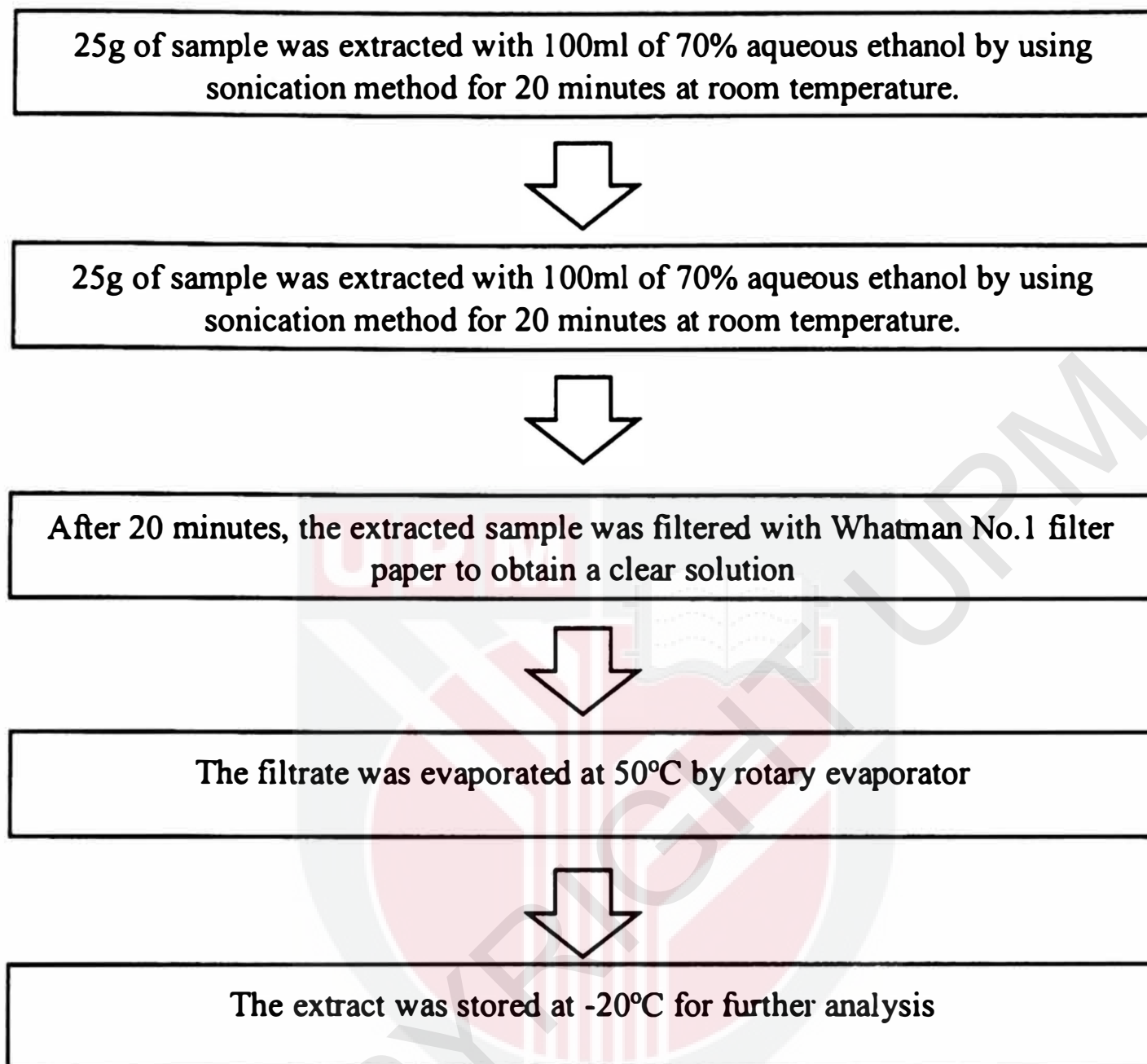


Figure 6: Sample extraction with 70% ethanol

Appendix 7: Determination of moisture content

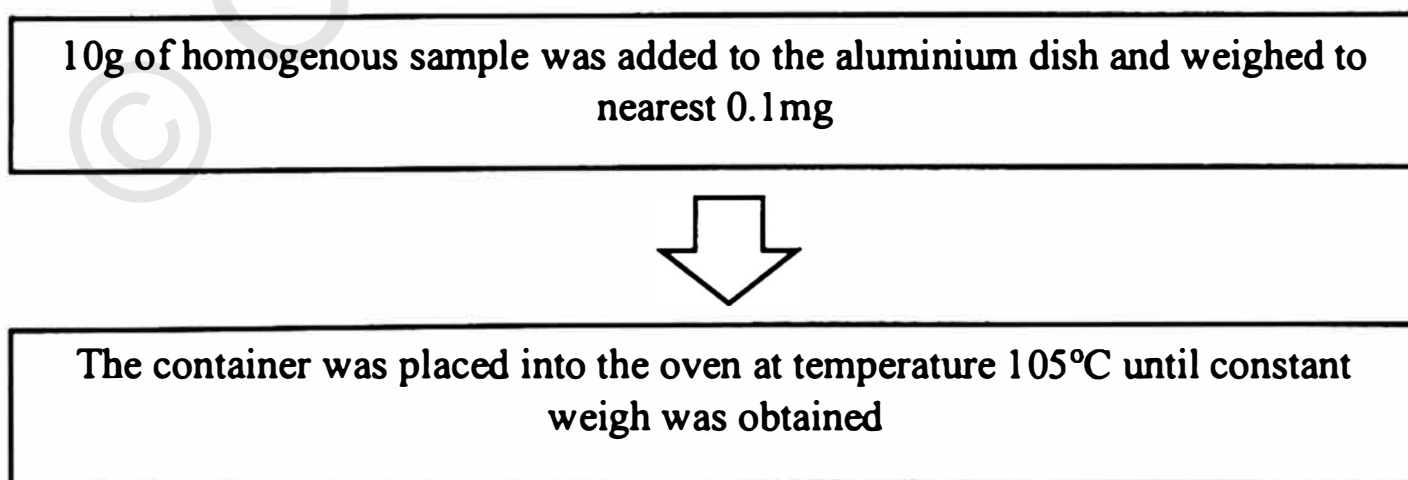


Figure 7: Determination of moisture content

Appendix 8: Folin-Ciocalteu (FC) Reagent assay

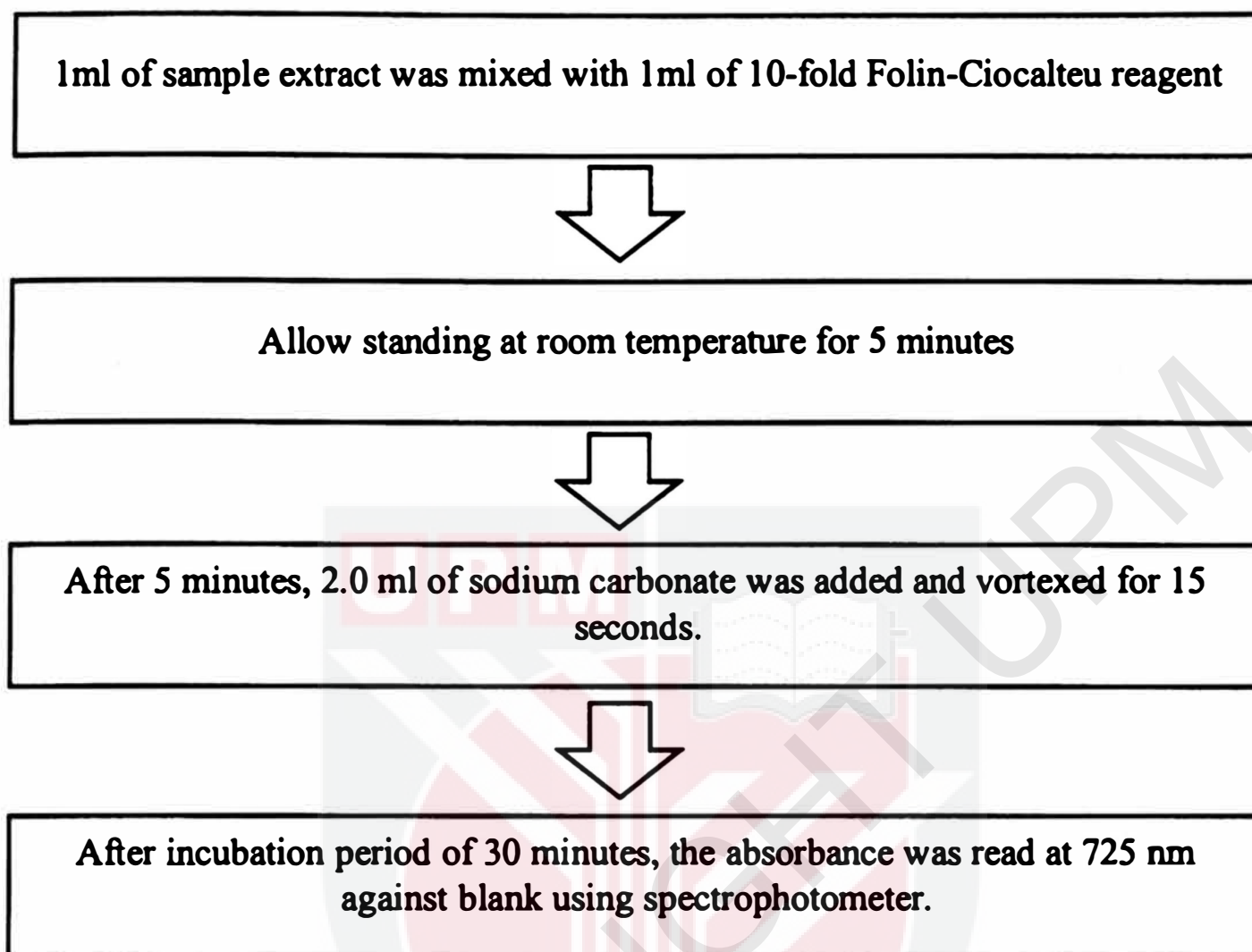


Figure 8: Determination of total phenolic content with Folin-Ciocalteu reagent

Appendix 9: Aluminium chloride calorimetric assay

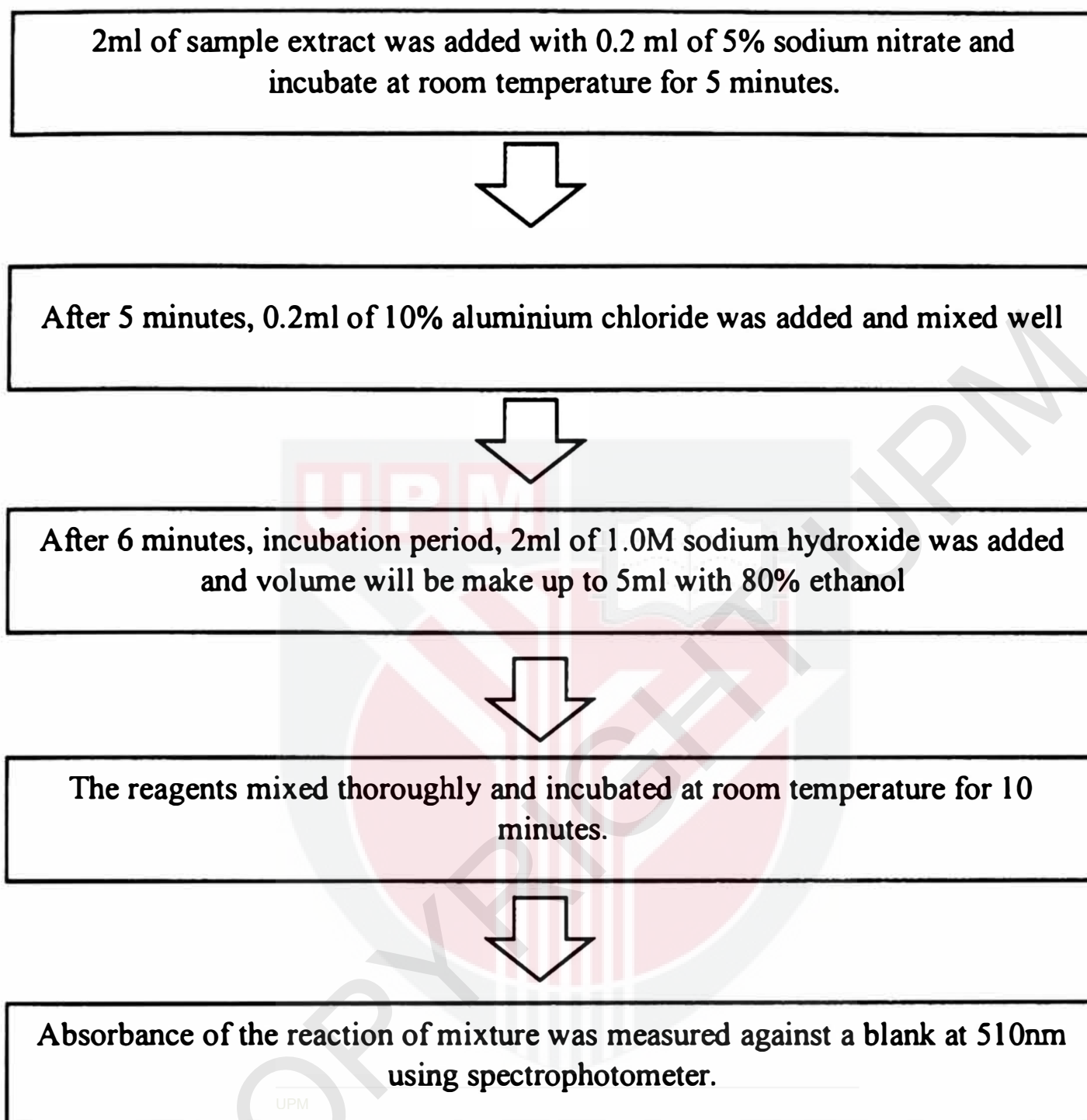


Figure 9: Determination of total flavonoid content with aluminium chloride colorimetric assay

Appendix 10: DPPH free radical scavenging capacity assay

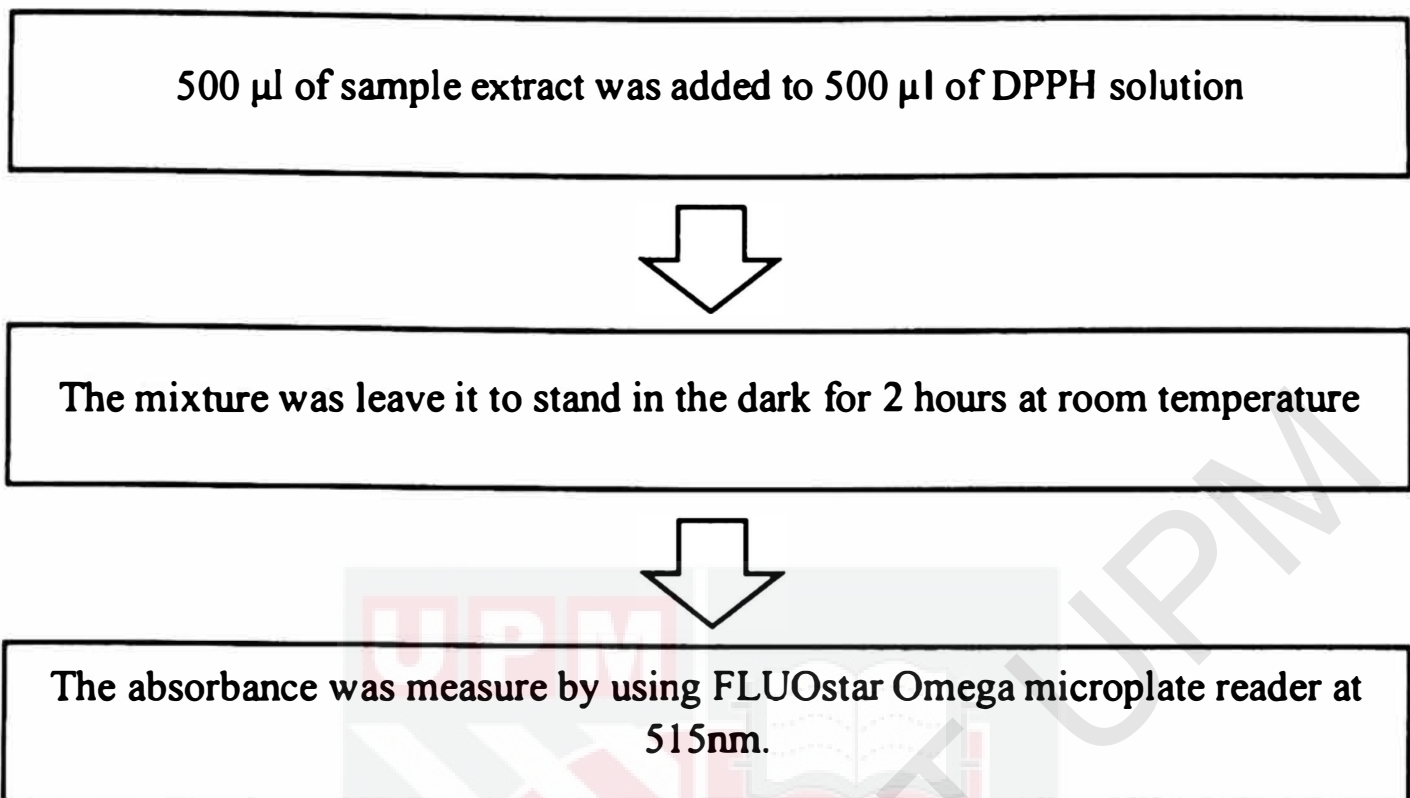


Figure 10: Determination of DPPH free radical scavenging capacity of PRM samples

Appendix 11: Ferric Reducing Antioxidant Power (FRAP)

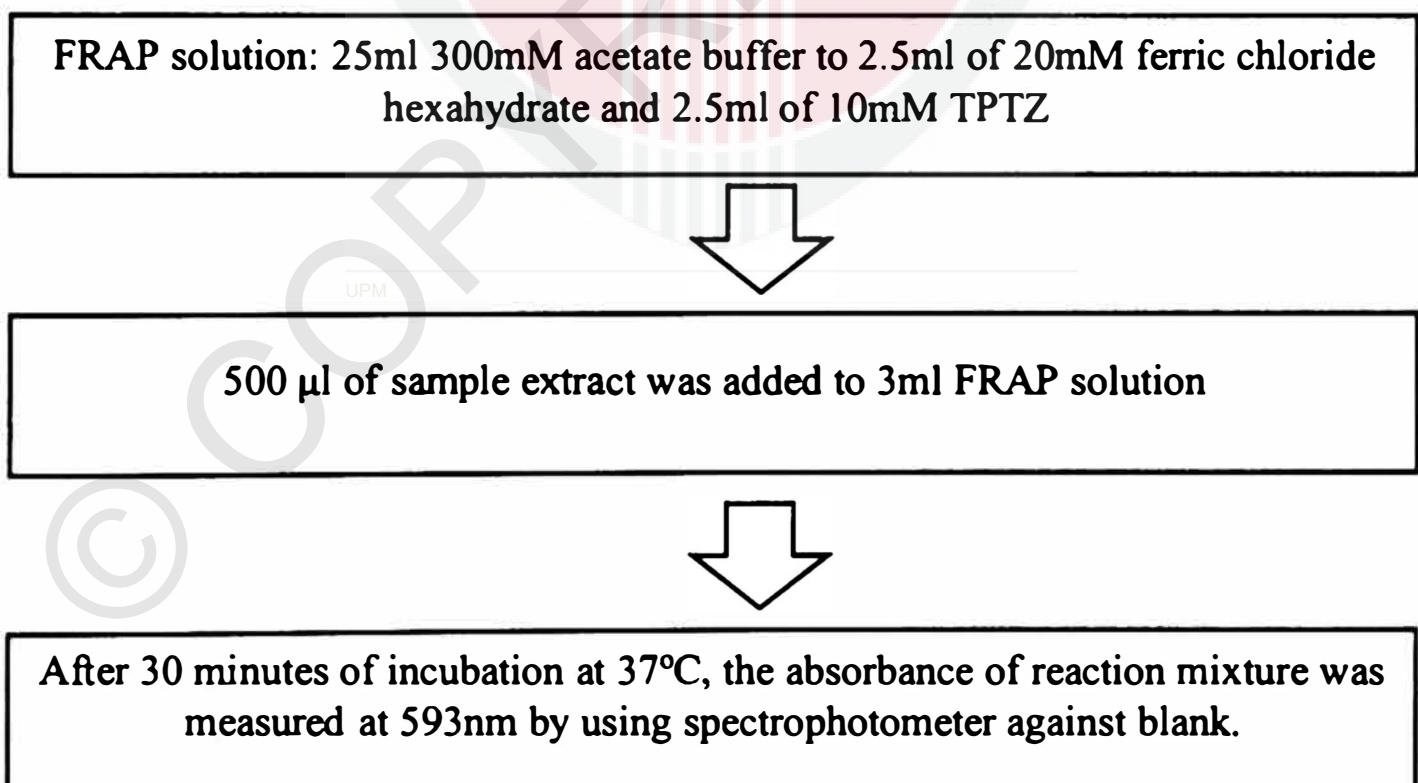


Figure 11: Determination of ferric reducing antioxidant power (FRAP) of citus peel sample

Appendix 12.1: SPSS output (Yield of extraction)

Descriptive

Yield

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	11.0253	.66737	.38531	9.3675	12.6832	10.29	11.59
ML	3	7.9427	.75372	.43516	6.0703	9.8150	7.12	8.60
KL	3	6.6987	.95143	.54931	4.3352	9.0622	6.10	7.80
FL	3	33.9033	.73569	.42475	32.0758	35.7309	33.42	34.75
FML	3	33.0233	.23007	.13283	32.4518	33.5949	32.79	33.25
FKL	3	31.7333	.90970	.52521	29.4735	33.9932	30.94	32.73
Total	18	20.7211	12.62027	2.97463	14.4452	26.9970	6.10	34.75

Yield

Tukey HSD^a

Sample	N	Subset for alpha = 0.05			
		1d	2c	3b	4a
KL	3	6.6987	11.0253	31.7333	33.0233
ML	3	7.9427			
L	3				
FKL	3			31.7333	33.0233
FML	3			33.0233	
FL	3				
Sig.		.375	1.000	.340	.702

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ANOVA

Yield

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2700.931	5	540.186	970.272	.000
Within Groups	6.681	12	.557		
Total	2707.612	17			

Appendix 12.2: SPSS output (Moisture content)

Descriptive

Moisture								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	83.6547	.19205	.11088	83.1777	84.1318	83.43	83.78
ML	3	79.3214	.46113	.26623	78.1759	80.4669	78.98	79.85
KL	3	77.3001	.35787	.20662	76.4111	78.1891	76.92	77.64
Total	9	80.0921	2.82843	.94281	77.9180	82.2662	76.92	83.78

Test of Homogeneity of Variances

Moisture			
Levene Statistic	df1	df2	Sig.
1.401	2	6	.317

ANOVA

Moisture					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	63.245	2	31.622	251.242	.000
Within Groups	.755	6	.126		
Total	64.000	8			

Moisture

Tukey B ^a				
Sample	N	Subset for alpha = 0.05		
		1c	2b	3a
KL	3	77.3001		
ML	3		79.3214	
L	3			83.6547

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 12.3: SPSS Output (Total phenolic content)

Descriptive

TPC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	72.0147	.67144	.38766	70.3467	73.6826	71.43	72.75
ML	3	108.5714	1.33687	.77184	105.2505	111.8924	107.03	109.45
KL	3	75.6777	1.34288	.77531	72.3418	79.0136	74.51	77.14
FL	3	100.0000	.95800	.55310	97.6202	102.3798	98.90	100.66
FML	3	136.4835	.58148	.33572	135.0390	137.9280	135.82	136.92
FKL	3	118.6081	.77184	.44562	116.6907	120.5254	117.80	119.34
Total	18	101.8926	23.41934	5.51999	90.2464	113.5387	71.43	136.92

Test of Homogeneity of Variances

TPC

Levene Statistic	df1	df2	Sig.
1.164	5	12	.381

ANOVA

TPC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9312.125	5	1862.425	1896.239	.000
Within Groups	11.786	12	.982		
Total	9323.911	17			

TPC

Tukey B^a

Sample	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
L	3	72.0147	75.6777	100.0000	108.5714	118.6081	136.4835
KL	3						
FL	3						
ML	3						
FKL	3						
FML	3						

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 12.4: SPSS output (Total flavonoid content)

Descriptive

TFC

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	50.5122	1.35799	.78404	47.1388	53.8856	49.05	51.73
ML	3	120.9187	2.56194	1.47914	114.5545	127.2829	118.32	123.44
KL	3	50.7561	.24390	.14082	50.1502	51.3620	50.51	51.00
FL	3	78.1545	.61381	.35438	76.6297	79.6793	77.59	78.80
FML	3	178.3171	2.12629	1.22762	173.0351	183.5991	175.88	179.78
FKL	3	82.2195	1.29061	.74513	79.0135	85.4256	81.24	83.68
Total	18	93.4797	46.00537	10.84357	70.6017	116.3576	49.05	179.78

Test of Homogeneity of Variances

TFC

Levene Statistic	df1	df2	Sig.
2.083	5	12	.138

ANOVA

TFC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35950.337	5	7190.067	2870.146	.000
Within Groups	30.061	12	2.505		
Total	35980.399	17			

TFC

Tukey HSD^a

Sample	N	Subset for alpha = 0.05			
		1	2	3	4
L	3	50.5122			
KL	3	50.7561			
FL	3		78.1545		
FKL	3		82.2195		
ML	3			120.9187	
FML	3				178.3171
Sig.		1.000	.071	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 12.5: SPSS output (DPPH scavenging activity)

Descriptive

DPPH EC50

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	1304.6667	422.17690	243.74395	255.9211	2353.4122	1008.00	1788.00
ML	3	3155.6667	918.65463	530.38550	873.6021	5437.7313	2145.00	3940.00
KL	3	1825.0000	253.31996	146.25434	1195.7183	2454.2817	1556.00	2059.00
FL	3	823.1000	96.23518	55.56141	584.0386	1062.1614	737.20	927.10
FML	3	2701.3333	422.72962	244.06306	1651.2147	3751.4519	2265.00	3109.00
FKL	3	1568.0000	214.29186	123.72146	1035.6695	2100.3305	1329.00	1743.00
Total	18	1896.2944	913.51403	215.31732	1442.0146	2350.5743	737.20	3940.00

Test of Homogeneity of Variances

DPPH EC50

Levene Statistic	df1	df2	Sig.
3.093	5	12	.051

ANOVA

DPPH EC50

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	11546207.669	5	2309241.534	10.495	.000
Within Groups	2640426.420	12	220035.535		
Total	14186634.089	17			

Tukey HSDa

Sample	N	Subset for alpha = 0.05		
		1	2	3
FL	3	823.1000		
L	3	1304.6667		
FKL	3	1568.0000	1568.0000	
KL	3	1825.0000	1825.0000	
FML	3		2701.3333	2701.3333
ML	3			3155.6667
Sig.		.167	.097	.835

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 12.6: SPSS output (Ferric reducing antioxidant power)

Descriptive

FRAP

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
L	3	347.1667	59.91313	34.59086	198.3342	495.9991	278.00	383.00
ML	3	476.3333	37.44440	21.61854	383.3163	569.3504	445.50	518.00
KL	3	444.6667	28.97556	16.72905	372.6874	516.6460	425.50	478.00
FL	3	464.6667	38.18813	22.04793	369.8021	559.5312	423.00	498.00
FML	3	497.1667	12.58306	7.26483	465.9086	528.4247	485.50	510.50
FKL	3	398.8333	33.29164	19.22094	316.1323	481.5344	370.50	435.50
Total	18	438.1389	61.18602	14.42168	407.7118	468.5660	278.00	518.00

Test of Homogeneity of Variances

FRAP

Levene Statistic	df1	df2	Sig.
1.999	5	12	.151

ANOVA

FRAP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	46530.903	5	9306.181	6.526	.004
Within Groups	17112.500	12	1426.042		
Total	63643.403	17			

FRAP

Tukey B^a

Sample	N	Subset for alpha = 0.05	
		1	2
L	3	347.1667	
FKL	3	398.8333	398.8333
KL	3		444.6667
FL	3		464.6667
ML	3		476.3333
FML	3		497.1667

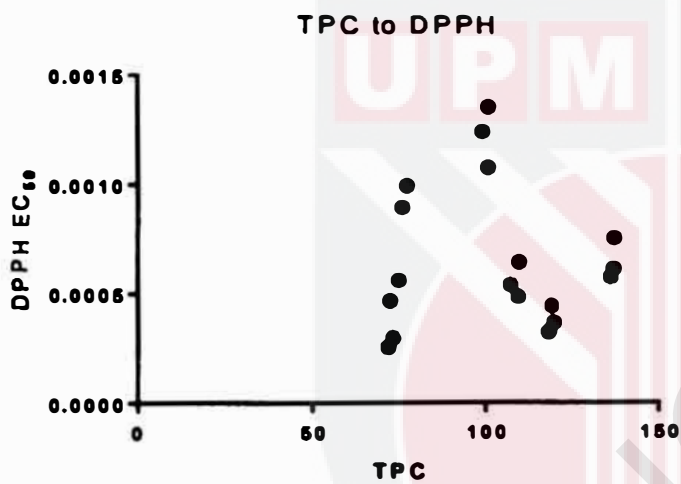
Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 12.7: SPSS output (correlation between antioxidant content and antioxidant activity)

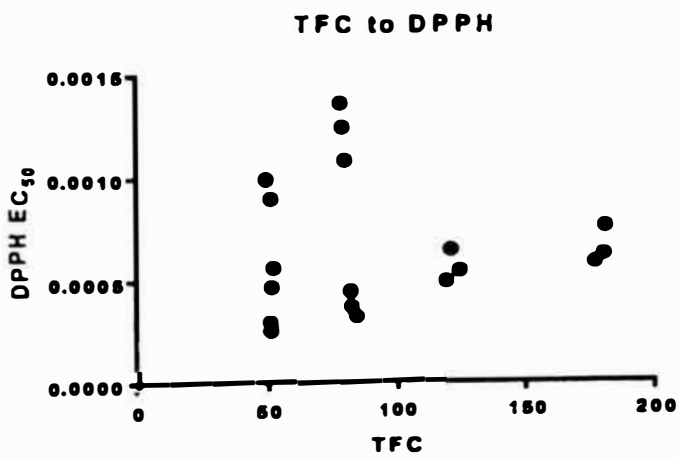
a) DPPH and TPC

Correlations		DPPH EC50	TPC
DPPH_EC50_	Pearson Correlation	1	-.068
	Sig. (2-tailed)		.787
	N	18	18
TPC	Pearson Correlation	-.068	1
	Sig. (2-tailed)	.787	
	N	18	18



b) DPPH and TFC

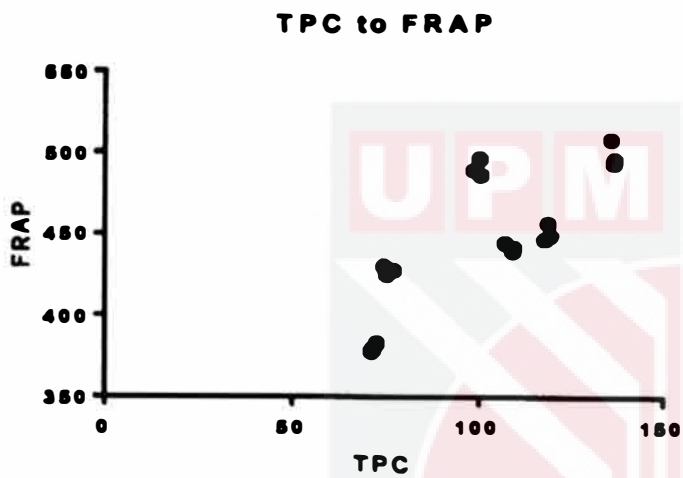
Correlations		TFC	DPPH EC50
TFC	Pearson Correlation	1	-.030
	Sig. (2-tailed)		.905
	N	18	18
DPPH_EC50_	Pearson Correlation	-.030	1
	Sig. (2-tailed)	.905	
	N	18	18



c) FRAP and TPC

Correlations		
		FRAP
		TPC
FRAP	Pearson Correlation	1
	Sig. (2-tailed)	.783**
	N	.000
TPC	Pearson Correlation	.783**
	Sig. (2-tailed)	.000
	N	1

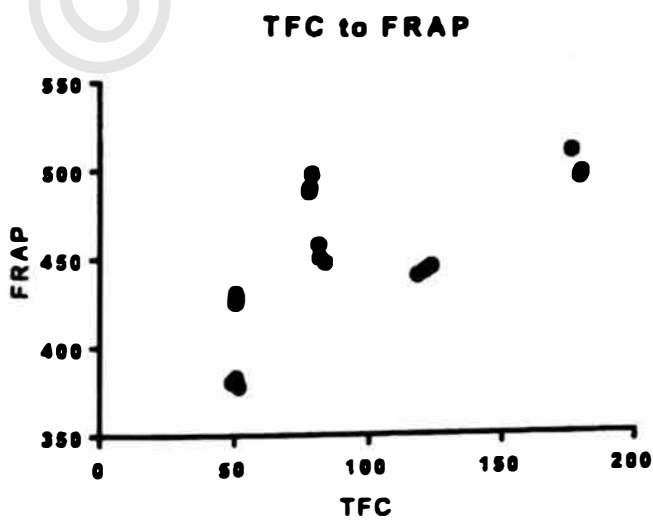
** . Correlation is significant at the 0.01 level (2-tailed).



d) FRAP and TFC

Correlations		
		FRAP
		TFC
FRAP	Pearson Correlation	1
	Sig. (2-tailed)	.681**
	N	.002
TFC	Pearson Correlation	.681**
	Sig. (2-tailed)	.002
	N	1

** . Correlation is significant at the 0.01 level (2-tailed).



Appendix 13: Gantt chart

Years	2017				2018					
Month	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun
Project introduction & objective										
Review literature work										
Methodology & work simulation										
Proposal presentation										
Laboratory work										
Analyse and discussion										
Presentation of final report/ seminar										
Submission report to second reader										
Submission full report										