



UNIVERSITI PUTRA MALAYSIA

***ANTIOXIDANT CAPACITY AND TOTAL PHENOLIC
CONTENT OF PEPPEROMIA PELLUCIDA LEAVES, STEM AND
FRUITING SPIKES***

NURUL MARSHITAH BINTI ABDUL MANAN

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FRUITING SPIKES**

BY

NURUL MARSHITAH BINTI ABDUL MANAN

**A project submitted as a partial fulfilment of the requirement for the degree of
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Abstract

ANTIOXIDANT CAPACITY AND TOTAL PHENOLIC CONTENT OF *PEPPEROMIA PELLUCIDA* LEAVES, STEM AND FRUITING SPIKES

NURUL MARSHITAH BINTI ABDUL MANAN

Globally, chronic diseases such as heart disease, stroke, cancer and diabetes, are by far the leading causes of morbidity even with advances in technology, mortality from them still remains high. Diets containing antioxidants have received more attention because of their potential health benefits and the ability to lower the risk of chronic diseases. Nowadays, used of medicinal herbs gain more attention due to their natural antioxidants properties. *Pepperomia pellucida* which is a tree belonging to the Piperaceae family that commonly found among Asian and South American countries have been identified as one of the nutritional sources of antioxidants. In this study the antioxidant capacity and total phenolic content of *Pepperomia pellucida* leaves, stem and fruiting spikes were investigated. Three different parts of *P. pellucida* (leaves, stem and fruiting spikes) were extracted by using 70% ethanol. The antioxidant capacity DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, FRAP (Ferric Reducing Antioxidant Power) assay, ORAC (Oxygen Radical Absorbance Capacity) assay and total phenolic content (Folin- Ciocalteu assay) was used in this study. The results indicated that the *P. pellucida* leaves had the greatest antioxidant capacity and total phenolic content followed by fruiting spikes while the stem had the lowest. *P. pellucida* leaves had the highest scavenging effect which was represented by the value $96.30 \pm 2.00\%$ followed by fruiting spikes which was $95.53 \pm 2.29\%$. *P. pellucida* stems showed the lowest scavenging effect among the other parts which was $54.43 \pm 0.60\%$. For FRAP assay, *P. pellucida* leaves shows outstanding ferric reducing ability which represented by the value $141.57 \pm 24.24 \mu\text{mol Fe}^{2+}/100 \text{ g}$ followed by the fruiting spikes which was $72.97 \pm 10.17 \mu\text{mol Fe}^{2+}/100 \text{ g}$ while the stems exhibited the lowest FRAP values which was $17.46 \pm 4.07 \mu\text{mol Fe}^{2+}/100 \text{ g}$. In ORAC assay, it is clearly showed that ORAC value of the leaves attained the highest value which was $764.83 \pm 22.28 \mu\text{mol TE/g DW}$ followed by the fruiting spikes which was $592.77 \pm 31.54 \mu\text{mol TE/g DW}$ while the stems attained the lowest ORAC values which was $85.13 \pm 5.33.54 \mu\text{mol TE/g DW}$. *P. pellucida* leaves had total phenolic of $492.29 \pm 6.14 \text{ mg GAE}/100 \text{ g DW}$ which was the highest followed by fruiting spikes which was $284.15 \pm 14.80 \text{ mg GAE}/100 \text{ g DW}$ while the stems had the lowest phenolic content which was $71.16 \text{ mg GAE}/100 \text{ g DW}$. A negative high correlation was found between total phenolic content and IC_{50} of DPPH value. While, a positive and very high correlation were found between total phenolic content with both FRAP and ORAC. The correlations indicate that the phenolic compounds could be the main contribution of antioxidant potential for *P. pellucida* plants. In summary, *P. pellucida* leaves is the best source of dietary antioxidants among the other parts (fruiting spikes and stem). *P. pellucida* leaves seems contain high amounts of phenolic compounds with antioxidant activities as well as a good natural source of antioxidants which may potentially reduce the oxidative stress (triggered by chronic diseases). Thus, *P. pellucida* leaves could be considered as an alternative and potential source of natural antioxidant.

Abstrak

KAPASITI ANTIOKSIDA DAN KANDUNGAN FENOLIK *PEPPEROMIA PELLUCIDA* DAUN, BATANG DAN PANCANG BERBUAH

NURUL MARSHITAH BINTI ABDUL MANAN

Di seluruh dunia, penyakit kronik seperti penyakit jantung, strok, kanser dan kencing manis, adalah penyebab utama morbiditi walaupun dengan kemajuan teknologi, kematian dari penyakit kronik ini masih tinggi. Diet yang mengandungi antioksidan telah mendapat lebih perhatian kerana manfaatnya untuk kesihatan serta berpotensi untuk mengurangkan risiko penyakit kronik. Pada masa kini, penggunaan ubat-ubatan herba mendapat lebih banyak perhatian kerana sifat-sifat antioksidan semulajadi yang terdapat pada tumbuhan herba. *Pepperomia pellucida* yang merupakan pokok dari keluarga Piperaceae biasanya ditemui di negara Asia dan Amerika Selatan telah dikenalpasti sebagai salah satu sumber pemakanan antioksidan. Dalam kajian ini, kapasiti antioksidan dan jumlah kandungan fenolik *P. pellucida* (daun, batang dan pancang berbuah) telah dikaji. Tiga bahagian *P. pellucida* (daun, batang dan pancang berbuah) telah diekstrak dengan menggunakan 70% etanol. Kaedah DPPH (1,1-difenil-2-pikrilhidrazyl), kaedah FRAP (penurunan ferik/kapasiti antioksidan), ORAC (Kapasiti Penyerapan Radikal Oksigen) dan jumlah kandungan fenolik (Folin-Ciocalteu assay) telah digunakan dalam kajian ini untuk menentukan kapasiti antioksida. Keputusan menunjukkan bahawa daun *P. pellucida* mempunyai kapasiti antioksidan yang paling tinggi dan jumlah kandungan fenolik diikuti oleh pancang berbuah manakala bahagian batang yang paling rendah. Daun *P. pellucida* kesan penghapusan radikal tertinggi yang diwakili oleh nilai $96.30 \pm 2.00\%$ diikuti oleh pancang berbuah yang menunjukkan nilai $95.53 \pm 2.29\%$. Batang *P. pellucida* menunjukkan kesan penghapusan radikal yang paling rendah di kalangan bahagian lain iaitu $54.43 \pm 0.60\%$. Untuk kaedah FRAP, daun *P. pellucida* menunjukkan keupayaan mengurangkan ferrik yang luar biasa iaitu $141.57 \pm 24.24 \mu\text{mol Fe}^{2+} / 100 \text{ g}$ diikuti dengan pancang berbuah iaitu $72.97 \pm 10.17 \mu\text{mol Fe}^{2+} / 100 \text{ g}$ manakala batang menunjukkan nilai FRAP terendah iaitu $17.46 \pm 4.07 \mu\text{mol Fe}^{2+} / 100 \text{ g}$. Dalam ujian ORAC, jelas menunjukkan bahawa nilai ORAC daun mencapai nilai tertinggi iaitu $764.83 \pm 22.28 \mu\text{mol TE} / \text{g DW}$ diikuti dengan pancang berbuah yang $592.77 \pm 31.54 \mu\text{mol TE} / \text{g DW}$ manakala batang mencapai nilai ORAC terendah iaitu $85.13 \pm 5.33.54 \mu\text{mol TE} / \text{g DW}$. *P. pellucida* mempunyai jumlah fenolik $492.29 \pm 6.14 \text{ mg GAE} / 100 \text{ g DW}$ yang paling tinggi diikuti dengan pancang berbuah iaitu $284.15 \pm 14.80 \text{ mg GAE} / 100 \text{ g DW}$ manakala batang mempunyai kandungan fenolik terendah iaitu $71.16 \text{ mg GAE} / 100 \text{ g DW}$. Korelasi tinggi negatif didapati antara jumlah kandungan fenolik dan nilai IC₅₀ pada DPPH. Walau bagaimanapun, korelasi yang positif dan sangat tinggi didapati di antara jumlah kandungan fenolik dengan FRAP dan ORAC. Hubungannya menunjukkan bahawa sebatian fenolik boleh menjadi sumbangan utama kepada potensi antioksidan dalam tumbuhan *P. pellucida*. Kesimpulannya, *P. pellucida* merupakan sumber terbaik antioksidan dalam pemakanan di antara bahagian lain (batang dan pancang berbuah). Daun *P. pellucida* mengandungi sejumlah besar sebatian fenolik dan aktiviti antioksidan serta sumber antioksidan semulajadi yang berpotensi mengurangkan tekanan oksidatif (yang disebabkan oleh penyakit kronik). Oleh itu, daun *P. pellucida* dapat dianggap sebagai sumber alternatif antioksidan semula jadi.

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CHAPTER 1

INTRODUCTION

1.1 Background

Globally, chronic diseases such as heart disease, stroke, cancer and diabetes, are by far the leading cause of morbidity even with advances in technology, mortality from them still remains high. According to World Health Organisation 2017, cardiovascular disease, cancers, respiratory diseases and diabetes are the leading global cause of death and are responsible for 70% of death worldwide. They are the leading causes of death in developed countries and even in developing countries. Based on National Health and Morbidity Status 2015, the prevalence of diabetes, hypertension and hypercholesterolemia in Malaysia is worrying as about 3.5 million or 17.5 percent of its citizen aged 18 years and above have diabetes while 6.1 million or 30.3 percent have hypertension and 9.6 million or 47.7 percent have hypercholesterolemia. They continue to be an important public health challenge in all countries, including low- and middle-income countries where more than three quarters of non-communicable disease deaths occur. Now, there is an increasing realization that what is eaten as diet plays a significant role in the pathogenesis of chronic diseases and therefore dietary components can be used in the prevention of chronic diseases. Recently, diets containing antioxidants have received more attention because of their potential health benefits and the ability to lower the risk of chronic diseases.

Antioxidant are part of the components present in the diet that has the ability to scavenge the free radicals. Free radicals are toxic by-products produced through oxidation process can cause significant damage to living cells and tissues and contributed to various type of disease. The attraction of consuming food rich in antioxidants has become more popular recently and the importance of this food components has expanded and develop huge market for antioxidant rich products and ingredients (Wang, Chen, & Yin, 2010) particularly in those intended to prevent presumed deleterious effects of free radicals in the human body. Previous studies have shown a negative correlation between the intake of various antioxidant components and degenerative disease caused by free radical (Lobo, Patil, Phatak, & Chandra, 2010). A wide variety of antioxidant components contains in food such as flavonoids, carotenoids, phenolic compounds and certain vitamins.

Pepperomia pellucida is a tree belonging to the Piperaceae family and commonly found among Asian and South American countries. In Malaysia, it is known as “pokok ketumpang air”(Wei, Wee, Yong, Siong, & Syamsumir, 2011) and “sireh cina” which grows in clumps, loose and humid soils. *P. pellucida* is an annual, shallow-rooted herb, usually growing to a height of about 15 to 45 cm. It consists of three compartments which is the leaves characterize as shiny, fleshy and heart-shaped, the stems which is succulent and high water content, and the fruiting spikes opposite to the leaves which is attached by dot-like seeds.

Medicinal uses of this plant vary depending on the region. Local community claimed that decoction of the plant was helpful to treat bone aches and pains. *P. pellucida* also popularly known in Nigeria as shiny bush or riri and is used locally for hypertension, diabetes and generally as tonic for healthy well-being (Ganiyat, Patricia A., & Bamidele B., 2016). In some parts in Cameroon the seeds

and the whole plant are used to treat fracture (Aziba, Adedeji, Ekor, & Adeyemi, 2001). It is also used in the treatment of various ailments such as abdominal pain, headache and hypertension (Ragasa, Dumato, & Rideout, 1998).

1.2 Problem Statements

As the chronic diseases remains the leading cause of morbidity and mortality, further researcher on alternative treatments need to be studied. Due to the popularity of herbaceous plant intake for maintaining good health and treating various disease, there is a need to study the antioxidant properties of Malaysian medicinal plants.

In folk medicine, *P. pellucida* species have been studied that it had some biological properties such as antibacterial, anti-inflammatory, analgesic, antifungal and anticancer (Arrigoni-blank et al., 2004). To date, limited studies have been showed that which part of *P. pellucida* plant(stem, fruiting spikes and leaf) has the highest antioxidant capacity.

1.3 Significance of the Study

It has been shown that intake of antioxidants in diet is related to the maintenance of health, protection and treatment for various type of disease. This study would give new and useful information to nutritionists, food technologists, and consumers and increase knowledge among society regarding the issue of nutritional benefits of *P. pellucida* as an antioxidant-containing food from natural sources. This study would investigate and compare the antioxidant properties of *P. pellucida* from different compartment of the plants (stem, fruiting spikes and leaf).

1.4 Objectives

General Objective

To investigate the antioxidant capacity in leaves, stem and fruiting spikes of *P. pellucida*.

Specific Objectives

1. To determine and compare the antioxidant capacity of *P. pellucida* leaves, stem and fruiting spikes in 70% ethanolic extract using DPPH radical scavenging and FRAP, and ORAC methods.
2. To measure and compare the total phenolic contents of *P. pellucida* leaves, stem and fruiting spikes in 70% ethanolic extract.
3. To measure the relationship between antioxidant capacity and total phenolic contents of *P. pellucida* leaves, stem and fruiting spikes.

1.5 Hypothesis

1. There is significant difference in the antioxidant capacity of *P. pellucida* leaves, stem and fruiting spikes in aqueous and 70% ethanolic extract using DPPH radical scavenging and FRAP methods.
2. There is significant difference in the total phenolic contents of *P. pellucida* leaves, stem and fruiting spikes.
3. There is relationship between antioxidant capacity and total phenolic *P. pellucida* leaves, stem and fruiting spikes.

CHAPTER 2

LITERATURE REVIEW

2.1 *Peperomia Pellucida*

Peperomia Pellucida plants belongs to the family Piperaceae, which the division is Magnoliophyta and class is Magnoliopsida (Wei, Wee, Yong, Siong, & Syamsumir, 2011). This plant commonly found among Asian and South American countries. In Malaysia, it is known as “pokok ketumpang air” and “sireh cina” while in Philippines this plant known as “pansit-pasitan”, shiny bush or riri in Nigeria, little heart in Brazil, “suna kosho” in Japan, and “pak krasang” in Thailand (Khan & Omoloso, 2002).



Fig 2.1 Picture of *Peperomia pellucida*

2.1.1 Plant Description

P. pellucida is an annual, shallow-rooted herb, which characterized by succulent and high water content stem usually growing to a height of about 15 to 45 cm. The leaves are shiny, fleshy, alternate oval shaped with 2 to 5 cm long and 2 to 4 cm wide. The opposite part of the leaves are the fruiting spikes which inflorescences and attached by dot-like seeds.



Fig 2.1.1(a) Size of the leaves *Peperomia pellucida*

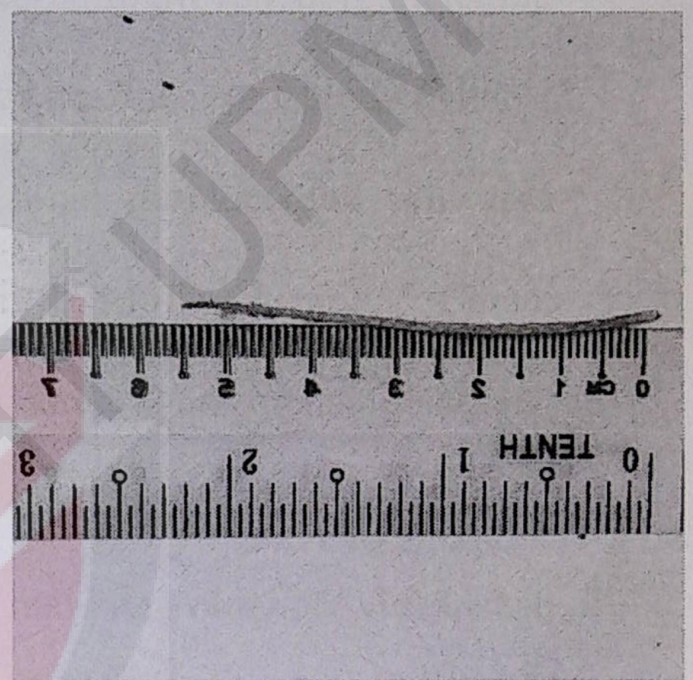


Fig 2.1.1(b) Size of the fruiting spikes *Peperomia pellucida*

2.1.2 Uses of *P. pellucida* and biological activity

The medicinal properties of *P. pellucida* vary depending on region. People believed that decoction of the plant was useful to treat bone aches and pains (Ong & Nordiana, 1999). The leaf was also utilized in treating headache, fever, eczema, abdominal pains and convulsions (Yusuf, Wahab, Yousuf, Chowdhury, & Begum, 2007). Its roots are used to treat fevers and aerial parts used for dressing wounds (Muñoz et al., 2000). Elsewhere, this plant served multi-function, including mental disorder treatment in Bangladesh, haemorrhages treatment in Bolivia (Muñoz et al., 2000), cholesterol reduction in Brazil, and renal problem and uric acid reduction in Guyana and Philipines (De C. Bayma, Arruda, Müller, Arruda, & Canto, 2000). Other literature data indicates its use for treating gout, renal disorders, rheumatic joint pain and to stop bleeding (Majumder, Abraham, & V, 2015). A study also confirmed the antimicrobial and analgesic effects including other activities, such as anti-inflammatory effect of this plant (Wei et al., 2011).

2.1.3 Biological activity of *P. pellucida*

Several biological activities were reported on the crude extracts and pure compounds isolated from *P. pellucida*. Wei et al. (2011) had suggested that *P. pellucida* has anti-cancer and anti-microbial properties. Colorimetric MTT (tetrazolium) assay was used to determine the anticancer activity of *P. pellucida* leaf extract against human breast adenocarcinoma (MCF-7) cell line and the outcomes of the study showed that the leaf extract had anti-cancer activity with half maximal inhibitory concentration (IC₅₀) of 10.4±0.06 µg/ml. The anti-microbial properties

were revealed by using two-fold broth micro-dilution method against 10 bacterial isolates and the result indicated that the plant extract has the ability to inhibit the growth of *Edwardsiella tarda*, *Escherichia coli*, *Flavobacterium sp.*, *Pseudomonas aeruginosa* and *Vibrio cholerae* at 31.25 mg/l; *Klebsiella sp.*, *Aeromonas hydrophila* and *Vibrio alginolyticus* at 62.5 mg/l. The plant was able to control the growth of *Salmonella sp.* and *Vibrio parahaemolyticus* at 125 mg/l. This study also concluded that potent antioxidant activity was exhibited by their ability to scavenge DPPH (1, 1-diphenyl-2-picryl hydrazyl) by 30% at the concentration of 0.625 ppt of the plant extract.

Arrigoni-blank et al., (2004) stated that the aqueous extract of the aerial part of *P. pellucida* exhibit an analgesic and anti-inflammatory activity. It was tested paw edema induced by carrageenin and arachidonic acid for anti-inflammatory activity and abdominal writhes and hot plate for analgesic activity in rats and mice, respectively. Oral administration of 200 and 400 mg/kg of the aqueous extract exhibited an anti-inflammatory activity in the carrageenin test, which was based on interference with prostaglandin synthesis, as confirmed by the arachidonic acid test. In the abdominal writhing test induced by acetic acid, 400 mg/kg of the plant extract had the highest analgesic activity, whereas in the hot-plate test the best dose was 100 mg/kg. Thus, this study showed that the aqueous extract has an anti-inflammatory action interfering with prostaglandin synthesis and analgesic activity by the models used.

2.2 Antioxidants

Antioxidant can be defined as “any substance that when presence at low concentration compared to that of an oxidizable substrate (lipid, protein, carbohydrate or DNA), significantly delays or prevents oxidation”(B. Halliwell, Aeschbach, Löliger, & Aruoma, 1995). Antioxidants delays or prevents oxidation mechanism can be divided by two ways: antioxidant react as a free radical scavenger by donating electron to unpaired electron of free radical, hence breaking the radical chain process. Vitamin E (tocopherol) and phenolic compounds are the examples of free radical scavenger. Whereas, the other way does not involve direct scavenging of free radicals but by interrupting events that lead to the oxidation process. For examples, by binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen (Pokorný, Yanishlieva, & Gordon, 2001).

Antioxidants consist of two types which are synthetic and natural antioxidants. Typical non-dietary antioxidants are synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) whereas natural antioxidants are the dietary bioactive compounds that have the ability to inhibit or prevent oxidation. Natural antioxidants could be phenolic compounds (Caillet et al., 2007), nitrogen compounds or carotenoids as well as ascorbic acid. Compounds consist in synthetic antioxidants were restricted to consumed and studies shown that they suspected as promoters of carcinogenicity (Gazzani, Papetti, Massolini, & Daglia, 1998).

Antioxidants range from the hydrophilic or water soluble antioxidants such as ascorbic acids, betacyanins, phenolic acids to lipophilic or lipid soluble

compounds such as tocopherols and carotenoids. The hydrophilic nature of the antioxidant compounds might restrict them to get into contact with oxidation occurring in the lipid phase (Laguerre, Lecomte, & Villeneuve, 2007). Hence, the hydrophilic and lipophilic antioxidants might act synergistically in inhibiting the oxidation process.

Autoxidation of lipids and the generation of free radicals are actually natural phenomena in biological food systems. Autoxidation is a reaction of organic compounds with oxygen. In biological systems, there are variety biochemical defence mechanisms including enzymes, trace minerals and antioxidant vitamin that protect cellular components against oxidative damage while in food systems, they impart protection from oxidation.

Antioxidants are the first line of defense against free radical damage, and are critical for maintaining optimum health and well-being. The need for antioxidants even becomes more important with increased exposure to free radicals such as pollution, cigarette smoke, drugs, toxic, stress, and even exercise (Sies, Stahl, & Sundquist, 1992). Internal antioxidative systems in humans consist of enzyme such as catalase, peroxidase and superoxide dismutase and also uric acid, serum albumin and glutathione (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002) but those defenders as antioxidant are inadequate to give full protection to human body. Thus, antioxidants naturally occurring in food like in plants play an important role to aid and secure the body against harmful effect of free radical (Stratil, Klejdus, & Kuban, 2006).

Natural antioxidants mostly derived from plants species are intense scavengers against free radicals and helpful in the prevention and reduction the risk

of numerous chronic disease such as cancer, arteriosclerosis, diabetes, arthritis and others. They play an important role in different systems: i) in plants, they act as protective agents against radiation or microbial infections, ii) in foods, they delay or inhibit the formation of toxic lipid oxidation products, maintaining nutritional quality and increasing shelf life, and iii) in biological systems, along with endogenous defenses (enzymes, vitamins, proteins, and others), dietary antioxidants may help prevent or slow the oxidative stress caused by free radicals (Willcox, Ash, & Catignani, 2004).

Evidence showed that high intake of antioxidant vitamins can lower the risk of coronary heart disease in humans (Ness & Powles, 1997). The evidence is strongest for vitamin E, limited promising for β -carotene and vitamin C (Pokorný, 2007). Oxidation of LDL-cholesterol causing the coronary heart disease to occur and damaging the vascular system and the heart (Heinecke, 2006). A study conducted by Stampfer et al., 1993 reported and successfully proved that vitamin E can inhibits oxidation of LDL-cholesterol in plasma and β -carotene might give protection from endothelial damage by diminishing uptake into cells. However, a study reported that, protective effects of vitamin E might be effective only in high dose (Niki, Noguchi, Tsuchihashi, & Gotoh, 1995). Flavonoids are more potent antioxidants than carotenoids and vitamin E (Aviram et al., 2004) and it shown to be more related to reduce the risk of morbidity and mortality of coronary heart disease (Peterson & Kim, 2011). Plant flavonoids can reduce the metal's capacity from generating free radicals by hydrogen atom-donating antioxidants via their metal chelatin properties (Brown, 2001).

Dietary antioxidants such as β -carotene, vitamin E, vitamin C, and polyphenols have a good ability to scavenge free radicals. They can be found

naturally in vegetables, fruits, and beverages such as cocoa, dark chocolate, tea, tomato, seaweeds and fish (Ginter, Simko, & Panakova, 2014; Middleton, Kandaswami, & Theoharides, 2000; Pokorný, Yanishlieva, & Gordon, 2001). Flavonoids is one of the powerful natural antioxidants which exhibit a wide range of biological effects including antibacterial, anti-inflammatory, anti-allergic, anti-thrombotic and vasodilatory actions (Pietta, 2000).

2.3 Phenolic Compound

Phenolic compounds are one of the major categories of phytochemicals that are an integral part of the human diet and most widely found in plants and have attracted considerable interest in the past fifteen years (Aviram, 2002). Phenolic are one group of essential dietary components that have been associated with inhibition of atherosclerosis and cancer. Plant phenolics are compounds that have one or more aromatic ring, and can be found in many foods and drinks from plant origin such as fruits, vegetables, and coffee (Parr and Bolwell, 2000). This compound is one of the most active phytochemical in the plants (Akowuah et al., 2005). All phenolic compounds have one or more aromatic ring which contains various attached substituent groups.

Phenolic compounds are known as antioxidant due to its capability in scavenging the free radicals (Seyoum et al., 2006). These compounds act as antioxidants by chelating metal ions, preventing radical formation and improving the antioxidant endogenous system, inhibit lipoxygenase (Mallavadhani et al., 2006; Al-Azzawie and Alhamdani, 2006). Phenolic are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are stable radical intermediates. Besides that, it also functions as antimutagenic, anti-

inflammatory and antitumour. In plants, phenolic serve as effective defence against herbivores (Gurib-Fakim, 2006).

Phenolic compounds include different subclasses (flavonoids, phenolic acids, stilbenes, lignans, tannins and oxidised polyphenols) displaying a large diversity of structures, some of which may escape the usual methodologies of analysis, commonly carried out by high performance liquid chromatography (HPLC) coupled to distinct detection devices. Various reasons for this exist, such as the existence of isomers, difficulty for chromatographic separation of some compounds, lack of commercial standards, or the structure has not yet been discovered (Georgé et al., 2005). Huda-Faujan et al. (2007) mentioned that different levels of phenolic content can be contributed from different plants, procedures and standards used to express the results: the colour measurement of Folin-Ciocalteu reagent was non-specific on phenol, and there were other components that can react with Folin-Ciocalteu reagent such as ascorbic acid. This is in agreement with findings of Sun and Ho (2005) who found that extracting solvent will significantly affect the yield of phenolic content of buckwheat extract.

2.4 Antioxidant activity

There are various methods to measure antioxidant activity in foods such as Trolox Equivalent Antioxidant Capacity (TEAC), Total Radical Absorption Potentials (TRAP), Ferric Reducing Antioxidant Power (FRAP), 1,1-Diphenyl-2-Pecrylhydrazyl (DPPH), Oxygen Radical Absorption Capacity (ORAC), and 2,2-Azino-bis-(3-ethyl Benzoethiozoline-6-Sulfonate) (ABTS) assays. Basically, the principle of these methods are based on either single electron transfer (SET) reaction or a hydrogen atom transfer (HAT) reaction between an oxidant and free radical. The end of this reaction is the same, despite of mechanisms, kinetics and potential for side reaction is different (Prior, Wu, & Schaich, 2005). Although there are various methods of measuring antioxidant activity in food, but there is no single antioxidant assay that can fully evaluate the antioxidant capacity in food.

DPPH radicals scavenging assay

DPPH is a stable free radical in a methanolic solution containing an odd electron in its orbit. When DPPH reacts with a hydrogen donor, the color turns from purple to yellow the reduced form (DPPH) is generated. The effect of antioxidants on DPPH radical scavenging is thought to be due to DPPH hydrogen-donating ability. DPPH has a violet color which can be monitored spectrophotometer at 517 nm. This method was originally introduced nearly 50 years ago by Marsden Blois (Blois, 1958).

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) effect of the stable free radical by the hydrogen atom or electron was transferred to the odd electron in DPPH decolorization of the spare electron over the molecule as a whole, so that the molecules not dimerise, as would be the case with most other free radicals showing an absorbance at 517 nm which disappears upon reduction by an antioxidant and violet color (Bondet, Brand-Williams, & Berset, 1997). When the substance is mixed with a solution of DPPH can donate a hydrogen atom and reduces the violet color to a yellow color from the picryl group (Molyneux, 2004).

Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was originally developed by Benzie and Strain in 1996 to measure the reducing power in plasma. Later, this assay has been adapted and used hugely for measuring antioxidant activity in plants (Pellegrini et al., 2003). FRAP assay depends on the reduction of the ferric tripyridyltriazine complex to ferrous tripyridyltriazine by a reductant at low pH (3.6). Ferrous tripyridyltriazine has an intensive blue color and can be monitored spectrophotometer at 593 nm. In contrast to other tests the total antioxidant power of the FRAP assay is simple, speedy, inexpensive, highly reproducible and does not require specialized equipment (Xu and Chang, 2007). This method can be carried using automated, semiautomatic or manual methods (Prior et al., 2005). However, the possible disadvantage with FRAP assay is the fact that it does not react fast with some antioxidants such as glutathione (Guo et al., 2003). The final results expressed as micromole Trolox equivalents (TE) per gram on dried basis ($\mu\text{mol TE/g, db}$) or microgram extract/mL ($\mu\text{g/mL}$). The ferric reducing antioxidant potency mechanism is based on the reaction between an oxidant and a free radical, when antioxidants are oxidized by oxidants.

The single electron is transferred from the antioxidant molecule to the oxidant. The change of absorbance of either antioxidant or oxidant is measured by an ultraviolet-visible spectrometer and the absorbance value is used as the quantifying the reducing capability of the antioxidant (Ou et al., 2002).

Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC assay was developed in the early 1990s by Dr. Alexander N. Glazer in order to determine reactive oxygen species in biological systems. The ORAC assay is a kinetic assay measuring fluorescein decay and antioxidant protection over time. This assay measures antioxidant inhibition of peroxy radical induced oxidation, and thus reflects classical radical chain breaking antioxidant activity by hydrogen atom transfer (Ou et al., 2001). This assay also measures the protection provided by antioxidants against either hydroxyl or peroxy radicals.

The ORAC assay works upon the principle of inhibition of peroxy radical when formed from the breakdown of AAPH (2,2'-azobis-2-methylpropanimidamide, dihydrochloride) at 37 °C (Cao & Prior, 1999). The peroxy radical can oxidize fluorescein to generate a product without fluorescence. Antioxidants suppress this reaction by a hydrogen atom transfer mechanism, inhibiting the oxidative degradation of the fluorescein signal. The fluorescence signal is measured over 30 minutes by excitation at 485 nm, emission at 538 nm, and cut off at 530 nm. The concentration of antioxidant in the test sample is proportional to the fluorescence intensity through the course of the assay and is assessed by comparing the net area under the curve to that of a known antioxidant, trolox (Huang et al., 2001).

The antioxidant activity in biological fluids, cells, tissues, and natural extracts can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present. ORAC assay is unique as it measures both inhibition time and inhibition degree of free radical action by antioxidants using an area under curve technique and for quantification, ORAC assay can be performed in both hydrophilic and lipophilic solution (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006)

2.5 Free radicals

Free radicals can be defined as molecules that exist under certain condition which is unstable, highly reactive and contain an unpaired electron in an atomic orbital (Lobo, Patil, Phatak, & Chandra, 2010). They act as oxidant or reductants which they can either donate or accept an electron from other molecules (Cheeseman & Slater, 1993). Free radicals and other reactive oxygen/nitrogen/chlorine species are strongly believed to contribute to the development of several age-related diseases, and even to the aging process itself by causing oxidative stress and oxidative damage.

Many free radicals are unstable such as hydroxyl, alkoxyl, superoxide, but there are also free radicals which are not reactive but stable enough to survive longer such as nitric oxide, tocopheroxyl and dehydroascorbate (Barry Halliwell & Whiteman, 2004). These free radicals have the ability to react in the nucleus, and in the membranes of cells and damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids (Ginter, Simko, & Panakova, 2014). Free radicals attack important macromolecules leading to cell damage and homeostatic disruption. Oxygen-containing free radicals such as the superoxide and hydroxyl radicals are

associated with damage to cell structures and, consequently, human diseases, including those affecting immune functions. Targets of free radicals include all kinds of molecules in the body compromise health. Among them, lipids, nucleic acids, and proteins are the major targets.

Free radicals derived either from normal essential metabolic processes in the human body or from external sources such as exposure to X-rays, ozone, cigarette smoking, air pollutants, and industrial chemicals. Free radical formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Enzymatic reactions, which serve as source of free radicals, include those involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis, and in the cytochrome P-450 system (Liu, Stern, Roberts, & Morrow, 1999). Free radicals can also be formed in nonenzymatic reactions of oxygen with organic compounds as well as those initiated by ionizing reactions.

The term is used to describe the condition of oxidative damage resulting when the critical balance between free radical generation and antioxidant defenses is unfavourable (Young & Lowe, 2001). Oxidative stress, arising as a result of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acids (McCord, 2000). Short-term oxidative stress may occur in tissues injured by trauma, infection, heat injury, hypertoxia, toxins, and excessive exercise. These injured tissues produce increased radical generating enzymes such as xanthine oxidase, lipogenase, cyclooxygenase and activate phagocytes, release of free iron, copper ions, or a disruption of the electron transport chains of oxidative phosphorylation, producing excess ROS. The initiation, promotion, and progression of cancer, as well as the side-effects of radiation and chemotherapy, have been linked

to the imbalance between ROS and the antioxidant defense system. ROS have been implicated in the induction and complications of diabetes mellitus, age-related eye disease, and neurodegenerative disease.



CHAPTER 3

METHODOLOGY

3.1 Materials and Instruments

3.1.1 Chemicals

Folin-ciocalteu reagent, ferric chloride ion III, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium chloride, sodium carbonate, gallic acid, ascorbic acid, acetate buffer, Tris HCl Buffer and ethanol.

3.1.2 Instruments

Orbital shaker (719, protech electronic), freeze dryer (the VIRTIS Company, Inc. Route 208, Gardiner, New York, 12525), blender (Torrington CT, USA), rotary evaporator (Laborata 4000, Heidolph, Japan), centrifuge machine (Universal 32R, Hettich Zentrifugen, England), water bath (Mettmert, Germany), UV/VIS spectrophotometer (UV-1601, Kyoto, Japan), high-pressure liquid chromatography (HPLC), weighing scale (AC Adapter, Japan), vortex (EVM-V1000, Erla, jayu, Korea) and magnetic stirrer (Stuart scientific, UK).

3.2 Methods

3.2.1 Preparation of samples and extracts

Three kilogram of *Peperomia Pellucida* plants were collected from home garden in Sungai Merab, Selangor. Only young and fresh plants were chosen and used for the sample preparation. The plants were separated for three different compartment of leaves, stem and fruiting spikes. The *P. pellucida* leaves, stem and fruiting spikes were stored at -80°C for 3 days. After these process, the samples were dried using freeze dryer and ground into powder using a grinder (Sharp, Malaysia). Lastly, the samples were kept at -20°C prior to analysis.

P. pellucida leaves, stem and fruiting spikes powder were extracted with 70% ethanol at ratio 1:4. The process of the extraction was performed by shaking the mixture continuously at 200rpm for 120 min at 50°C using a temperature controlled orbital shaker. The mixture was filtered through a Whatman No.1 filter paper using a Buchner funnel. The filtrate was used for determination of antioxidant capacity and total phenolic content.

3.2.3 Determination of antioxidant capacity

3.2.3.1 DPPH radicals scavenging assay

The antioxidant activity or the capacity to scavenge the “stable” free radical DPPH was determined by using DPPH radicals scavenging method. Ascorbic acid, α -tocopherol and polyphenols are the antioxidants which able to act as reducing agents to DPPH free radical and remove the spare electron in DPPH (Molyneux,2004) by changing the radical’s initially purple colour to pale yellow.

A 3.94 mg of DPPH was dissolved in 100ml methanol to give a 100 μ m solution and then 3.0ml of the solutions was added with 0.5ml of the sample extract with doses ranging from 1.0 mg/ml to 0.0625 mg/ml (Gülçin, Oktay, Küfrevioğlu, & Aslan, 2002). The mixture was shaken vigorously and left to stand for 20min at room temperature in a dark room. The absorbance was read using a spectrophotometer at 517 nm. Triplicate measurements were carried out and the radical scavenging activity was calculated based on the percentage of scavenged DPPH as follows:

$$\text{Scavenging activity (\%)} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample of the extract.

3.2.3.2 Ferric reducing/antioxidant power (FRAP) assay

FRAP assay involves electron transfer reaction which uses ferric salt as an oxidant at low pH. This method was carried out according to Benzie and Strain, (1996) with some modifications. The FRAP reagent was prepared by mixing 300mM acetate buffer at pH3.6, 10mM TPTZ solution and 20Mm $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a ratio of 10:1:1, and heated to 37°C . 3ml FRAP reagent was pipetted into test tube followed by a total of 100 μL of sample extracts and 300 μL of distilled water. The extraction was mixed thoroughly and incubated at 37°C for 4 min and measure the absorbance at 593 nm using a UV-spectrophotometer. Each sample was conducted in triplicates. The change of absorbance of either antioxidant or oxidant was measured and the absorbance value were used as the quantifying the FRAP value (Ou *et al.*, 2002) and were calculated based on the following equation:

$$\text{FRAP value} = A_{\text{final}} - A_{\text{initial}}$$

A_{final} - Final absorbance at 593 nm (4 min)

A_{initial} - Initial absorbance at 593 nm (0 min)

3.2.3.3 Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC value of different parts of the *P. pellucida* samples was determined using method as described by Cao and Prior (1999). 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) was used as the generator of peroxy radical ($\text{ROO}^\cdot$), fluorescein as fluorescent probe and 6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox) as standard. Sample extracts and different dilutions of Trolox (12.5- 800 μM) were prepared in 75mM phosphate buffer (pH 7.4). All solutions were prepared fresh. The assay was performed at 37°C in 75mM phosphate buffer (pH 7.4) and the volume of the final solution for each well was 200 μl . In each well of the 96-well plate, 150 μL of 10nM fluorescein was added followed by 25 μl of the sample extracts, phosphate buffer (blank) or Trolox. Prior to the initiation of the reaction, the mixture was incubated at 37°C for 10 minutes. After the incubation, fluorescence measurements (excitation wavelength: 485nm, emission wavelength: 520nm) were taken every 90 seconds in order to determine the background signal. Twenty-five microliters of 240mM AAPH was injected into the mixture using on-board injector after 4 cycles. The test was continued and fluorescence intensity measurements were taken up to 120 minutes. ORAC value were calculated based on the difference in the area under the fluorescence curve between blank and sample. The ORAC value was calculated as follows:

$$\text{ORAC value} = [(AUC_{\text{sample}} - AUC_{\text{blank}}) / (AUC_{\text{Trolox}} - AUC_{\text{blank}})] \times [\text{Trolox}] \text{ DF}$$

Where: AUC = Area under the curve

DF= Dilution factor

Final ORAC values were expressed as μmol Trolox equivalent (TE)/g dried weight (DW)

3.2.4 Determination of Total Phenolic Content

Total phenolic content was determined using Folin-Ciocalteu method according to Singleton and Rossi (1965) with slight modifications. 200 μl of the sample extracts were added to 1.5ml of Folin-Ciocalteu reagent and incubate for 5 min at room temperature. Then, 1.5ml of sodium bicarbonate (Na_2CO_3) solution (0.6M) was added to the mixture. The absorbance was measured at 765 nm after 90 min by using spectrophotometer. The total phenolics were quantified by using a standard calibration curve of gallic acid at the concentrations of 0.025-0.1mg/mL and was expressed as gallic acid equivalent (GAE) per 100 gram of extract. All the measurements were conducted in triplicates and the mean values was calculated.

3.3 Statistical Analysis

The experiments were repeated three times. The results were expressed as mean values $\pm$ standard deviations. Statistical analyses were performed using IBM SPSS Statistics 23. One-way ANOVA and Pearson correlation coefficient were used to determine whether there were correlations between total phenolic content and antioxidant activity in *P. pellucida* leaves, stem and fruiting spikes. The level of significance was used in this study based on $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 DPPH radicals scavenging activity

DPPH is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, its purple colour will fade and turn into pale yellow (Miliauskas et al., 2004).

Figure 4.1 shows the scavenging effect of the plant extract tested on DPPH radical. The results shown an increasing trend of DPPH free radical scavenging effects as concentration of the 70% ethanol extract of *P. pellucida* leaves, stems, fruiting spikes and ascorbic acid increases. IC_{50} value was determined from the plotted graph of scavenging activity against the concentration of the extract of *P. pellucida* leaves, stems and fruiting spikes, which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical by 50% based on their scavenging activity. A lower EC_{50} value corresponds to a higher antioxidant power of the sample extracts.

By referring to Table 4.1, the tested samples showed very good scavenging effect when compared to the standard (ascorbic acid) used. *P. pellucida* leaves had the highest scavenging effect which was represented by the value $96.30 \pm 2.00\%$ followed by fruiting spikes which was $95.53 \pm 2.29\%$. This might be due to the higher content of the total polyphenolic compounds in the leaves and fruiting spikes. However, *P. pellucida* stems showed the lowest scavenging effect among the other parts which was $54.43 \pm 0.60\%$. A study by Malar et al., 2014 also reported that the percentage yields of free radical scavenging activity obtained for different *L. sativum* stem in ethanol extract are the lowest which was $2.69 \pm 05\%$ compared to the other parts which were the leaf ($10.21 \pm 09\%$) and seed ($11.63 \pm 03\%$). The results showed significant differences ($p < 0.05$) in means scavenging effect among the stems with the ascorbic acid, leaves and fruiting spikes.

Figure 4.1 Radical scavenging effects (%) of the sample extract

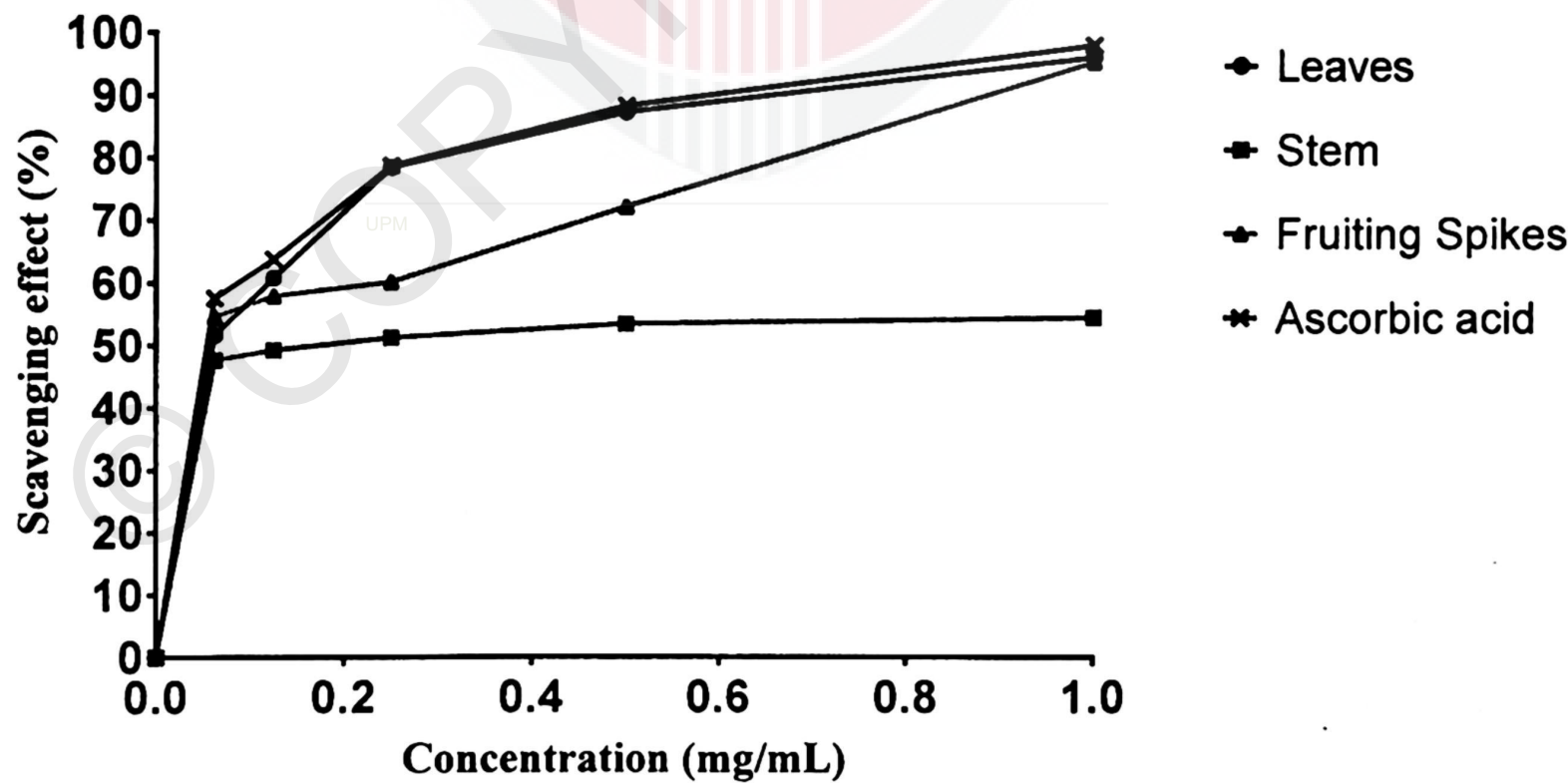


Table 4.1 Radical scavenging effect at highest concentration 1 mg/ml of sample extract in DPPH radical scavenging assay

Sample	DPPH (%)
Leaves	96.30 ± 2.00 _a
Stems	54.43 ± 0.60 _b
Fruiting Spikes	95.53 ± 2.29 _a
Ascorbic Acid	98.40 ± 0.92 _a

Values are expressed as mean ± standard deviation, SD (*n*=3). Means in the column with different letter are significantly different at the level of *p*< 0.05

Table 4.2 IC₅₀ of sample extract

Sample	IC ₅₀ (mg/ml)
Leaves	0.06 ± 0.01 _a
Stems	0.19 ± 0.01 _b
Fruiting Spikes	0.06 ± 0.01 _a
Ascorbic Acid	0.04± 0.01 _a

Values are expressed as mean ± standard deviation, SD (*n*=3). Means in the column with different letter are significantly different at the level of *p*< 0.05

Table 4.2 shows IC₅₀ values of *P. pellucida* leaves and fruiting spikes had the same and lowest value which was 0.06 ± 0.01_a. This can be concluded that *P. pellucida* leaves and fruiting spikes had the greatest antioxidant power. A study reported that low concentration of plant which had high antioxidant power can be used for the treatment of ailments resulting from oxidative stress such as Parkinson’s disease, Alzheimer’s disease, cancer, cardiovascular disorders, bacterial and viral infections and inflammation, coronary heart disease and stroke (Ganiyat, Patricia A.,

& Bamidele B., 2016). On the other hand, *P. pellucida* stems had the highest IC₅₀ value which was $0.19 \pm 0.01_b$. The results showed significant differences ($p < 0.05$) in means scavenging effect among the stems with the ascorbic acid, leaves and fruiting spikes.

Previous reports have stated the antioxidant activity of *P. pellucida* using various methods of extractions. Ethanol extracts of the leaves of *P. pellucida* showed antioxidant activity. The compounds present in the extract are hexadecanoic acid, methyl ester and, octadecadienoic acid. These compounds may have contributed to the antioxidant property of *P. pellucida* (Kumar, Kumaravel, & Lalitha, 2010).

Mutee et al. (2010) investigated the petroleum ether, chloroform and methanol extracts of *P. pellucida* using DPPH. The same study reported that the methanol extracts have the strongest free radical scavenging activity compared to the low antioxidant activity of the petroleum ether and chloroform extracts. The test showed that as the concentration of the extract increased, the antioxidant activity increased (Mutee et al., 2010).

4.2 Ferric reducing/antioxidant power (FRAP) activity

FRAP assay is commonly used to determine antioxidant capacity in fruits, vegetables, and medicinal herbs. In this assay, the antioxidant capacity of the *P. pellucida* leaves, stems and fruiting spikes by 70% ethanol extracts were observed based on the plotted standard curve of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ at the concentration range between 0.025 – 0.2 mg/ml. By referring to the Figure 4.2, calibration curve exhibited a high positive linear ($R^2 = 0.9834$) relation with FRAP value and concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (standard). The standard curve therefore was used to estimate the antioxidant capacity of the *P. pellucida* leaves, stems and fruiting spikes extracts.

Figure 4.2 A concentration curve of ferrous sulphate, FRAP assay (n=3). Values are expressed as mean $\pm$ standard deviation, SD (n=3).

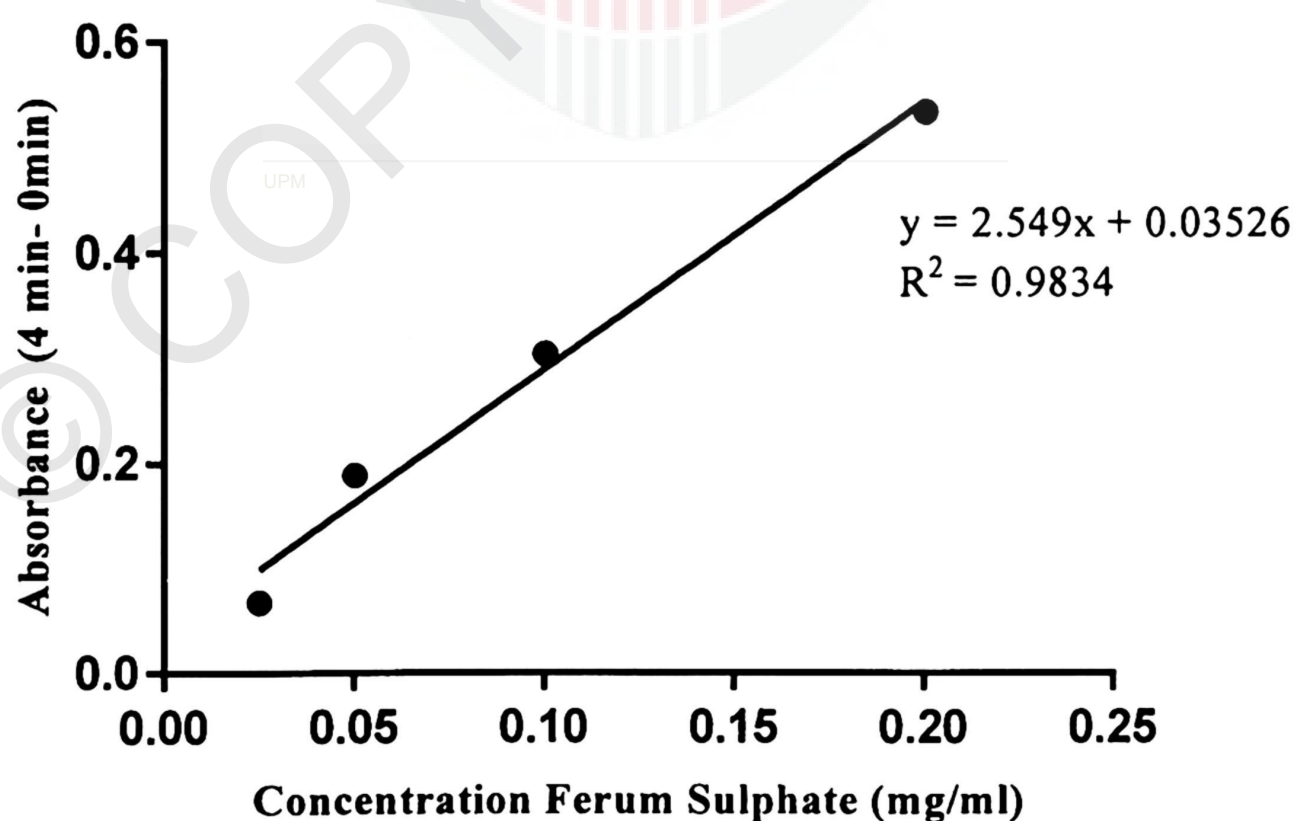


Table 4.3 Ferric reducing antioxidant power of the sample extract

Sample	FRAP
Leaves	141.57 ± 24.24 ^a
Stems	17.46 ± 4.07 ^b
Fruiting Spikes	72.97 ± 10.17 ^c

Values are expressed as mean ± standard deviation, SD (*n*=3). Results are expressed as ferum sulphate µmol Fe²⁺/100 g and values with different letters are significantly different at the level of *p* < 0.05

Table 4.3 shows the FRAP values of *P. pellucida* leaves, stems and fruiting spikes by 70% ethanol extracts. The *P. pellucida* leaves shows outstanding ferric reducing ability which represented by the value 141.57 ± 24.24 µmol Fe²⁺/100 g followed by the fruiting spikes which was 72.97 ± 10.17 µmol Fe²⁺/100 g while the stems exhibited the lowest FRAP values which was 17.46 ± 4.07 µmol Fe²⁺/100 g. Total antioxidant capacity from both DPPH and FRAP assays does not show similar trend as showed by the IC₅₀ of DPPH which the value of the leaves and the fruiting spikes were the same but in FRAP assay it showed different and huge gap between the both part. This could be due to different mechanism of assay method, structure of different phenolic compounds, the antioxidant protection mechanism exhibited by compounds, and also the synergistic effects of different compounds (Malar et al., 2014).

FRAP assays measures the reducing potential of the antioxidant to react with a ferric tripyridyltriazine (Fe³⁺ -TPTZ) compex to produce a coloured Fe²⁺ -TPTZ form at 593 nm at low PH (Benzie & Strain, 1996). The reducing properties are generally associated with the presence of reducing agents (Duh, Tu, & Yen,

1999), which have been shown to exert antioxidant action by breaking the free radical chain through donating a hydrogen atom (Blois, 1958). Examples of reducing agents are ascorbyl palmitate, sulphites, ascorbic acid, glucose oxidase and erythorbic acid (Kaur & Kapoor, 2001). However, some antioxidants such as glutathione (GSH) are not able to reduce Fe^{3+} . Therefore, FRAP assay does not account for SH—group of antioxidants which is involved in quinone tanning process (Prior & Cao, 2000). Tannin is a group of polyphenolic phytochemicals, which is believed to be responsible for dietary astringency. Tannin is widely distributed in fruits, vegetables, cocoa, red wine and certain food grains. ANOVA analysis showed that there were significant differences ($p < 0.05$) in the means of FRAP values among the *P. pellucida* leaves, stems and fruiting spikes where the leaves had the stronger reducing power than the other parts. This could be due to the high amount of reducing agents in the leaves extract compared to the fruiting spikes and stems.

4.3 Oxygen Radical Absorbance Capacity (ORAC) activity

Other than the antioxidant assays mentioned before, ORAC assay also was used in this study. This assay measures the loss of fluorescein fluorescence over time due to peroxy-radical formation by the breakdown of AAPH (2,2'-azobis-2-methyl-propanimidamide, dihydrochloride) and this allows the determination of antioxidant activity. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] was used as standard and serves as a positive control inhibiting fluorescein decay in a dose dependent manner.

ORAC values of *P. pellucida* leaves, stems and fruiting spikes were determined by using Trolox calibration curve with the concentration ranging from 0µM to 800µM. From that, a linear calibration curve was plotted with equation $y = 96.66 x + 11166.74$ ($R^2= 0.9837$) as shown in Appendix 1. ORAC values of *P. pellucida* leaves, stems and fruiting spikes were expressed as µmol Trolox equivalent (TE)/g dried weight (DW) in Table 4.3.

Table 4.4 ORAC value of the sample extract

Sample	ORAC value
Leaves	764.83 ± 22.28 ^a
Stems	85.13 ± 5.33.54 ^b
Fruiting Spikes	592.77 ± 31.54 ^c

Results are expressed as mean ± standard deviations, SD (n=3). Results are expressed as µmol Trolox equivalent (TE)/g dried weight (DW) and values with different letter are significantly different at the level of $p< 0.05$

By referring to Table 4.4, it is clearly shows that ORAC value of the leaves attained the highest value which was $764.83 \pm 22.28 \mu\text{mol TE/g DW}$ followed by the fruiting spikes which was $592.77 \pm 31.54 \mu\text{mol TE/g DW}$ while the stems attained the lowest ORAC values which was $85.13 \pm 5.33.54 \mu\text{mol TE/g DW}$. This result suggested that leaves parts of *P. pellucida* had strong antioxidant activity. This result can be important both for human diet and food safety.

Since the ORAC method is preferred for the measurement of the antioxidant activity of foods and biological samples, it is surprising that in the literature there are ORAC data just for several of the medicinal plants investigated in the current study. Previous study performed a comprehensive evaluation of different foods and spices using the ORAC method (Ninfali, Mea, Giorgini, Rocchi, & Bacchiocca, 2005). On the basis of fresh weight, they reported ORAC values for thyme ($274.26 \mu\text{mol TE/g}$), sage ($320.04 \mu\text{mol TE/g}$) and common balm ($59.97 \mu\text{mol TE/g}$). Zheng and Wang (2001), determined ORAC of five herbs from the current study, but again the results were expressed on the basis of fresh weight. Moreover, they used Rphycoerythrin as a fluorescent agent, which could significantly alter the ORAC results in their study (Ou et al., 2001). The results showed *P. pellucida* leaves significantly ($p < 0.05$) higher in the means of ORAC values followed by the stems while fruiting spikes showed significantly lowest ORAC value. Other study had found this herb to contain very potent antioxidant compounds, six of which reduced menadione-induced DNA damage in cultured breast cells (Burdette et al., 2002).

4.4 Total Phenolic Content

The total phenolic contents (TPC) are considered as a major group of chemicals that contribute to the antioxidant potential of medicinal plants. So far as plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was reasonable to determine their total amount in the selected plant extracts (Cook & Samman, 1996). Phenolics have also played an important role against chronic diseases (Simopoulos, 2004). The Folin-Ciocalteu procedure is nonspecific because it detects all phenolics (phenolic acids, flavonoids, and tannins) (Niklová, Schmidt, Habalová, & Sekretár, 2001) , so it does not give details of the quantity and quality of the phenolic constituents of the extracts. Nevertheless, this widely used method provides a rapid and useful overall evaluation of the phenolic content of extracts (Luximon-Ramma, Bahorun, & Crozier, 2003).

Table 4.5 Total phenolic content (TPC) of the sample extract

Sample	TPC (mg GAE/100g extract)
Leaves	492.29 ± 6.14 ^a
Stems	71.16 ± 0.00 ^b
Fruiting Spikes	284.15 ± 14.80 ^c

Results are expressed as mean ± standard deviations, SD (n=3). Results are expressed as gallic acid mg GAE/100 g DW and values with different letter are significantly different at the level of $p < 0.05$

The content of total phenolic content in ethanolic extracts expressed in gallic acid equivalents (GAE) are shown in Table 4.5. The *P. pellucida* leaves had total phenolic of 492.29 ± 6.14 mg GAE/100 g DW which was the highest. The *P. pellucida* fruiting spikes had the second highest phenolic content of 284.15 ± 14.80 mg GAE/100 g DW while the *P. pellucida* stems had the lowest phenolic content which was 71.16 mg GAE/100 g DW. The phenolics contain hydroxyls that are responsible for the radical scavenging effect mainly due to redox properties (Rice-Evans et al., 1997). Based on the result, the high phenolic content in *P. pellucida* leaves explain its high free radical scavenging activity. The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes (Shahidi et al., 1992).

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). A study reported that total phenolic contents of *Rumex japonicus* Houtt grown in Japan was 200 mgGAE/g extract (Elzaawely, Xuan, & Tawata, 2005). The variability could be due to plant species used, environmental factors, and collection period (Coruh, Gormez, Ercisli, & Sengul, 2008). The results showed significant differences ($p < 0.05$) in total phenolic contents of *P. pellucida* leaves was the highest followed by *P. pellucida* fruiting spikes and the lowest phenolic content was *P. pellucida* stems.

4.5 Correlation between total phenolic content and antioxidant capacity

A number of studies have shown that the antioxidant capacity of fruits and vegetables is strongly correlated to the phenolic content. For example, previous study had reported that there is very high correlation between antioxidant potential of various fruit (by using FRAP) and total phenolic which the R value range from 0.97 to 0.99 (Gardner, White, McPhail, & Duthie, 2000). A study by Prior et al. (1998) also found a high correlation (R=0.84) antioxidant activity (ORAC) and total phenolic content of different cultivars of *Vaccinium* species. However, several studies had reported that there was no such correlation between total phenolic and antioxidant capacity, but other compounds have been responsible for antioxidant activity (Kähkönen et al., 1999).

Table 4.6 Correlation between total phenolic content and antioxidant capacity

	Total phenolic content	
	r	P
IC ₅₀ of DPPH	-0.859**	0.003
FRAP	0.969**	0.000
ORAC	0.963**	0.000

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

Based on the results shown in Table 4.6, total phenolic content is highly correlated and showed a negative correlation with the IC₅₀ of DPPH value. Negative correlation demonstrated that the higher the total phenolic content, the lower the IC₅₀ of DPPH. While, total phenolic content was very highly correlated and significant with the FRAP value and ORAC value. It also showed that antioxidant capacity based on FRAP and ORAC had a positive and very high correlated with total phenolic content. This implies that the higher the total phenolic content in the sample extract, the higher the ORAC and FRAP value. The correlations indicate that the phenolic compounds could be the main contribution of antioxidant potential for *P. pellucida* plants. This is in agreement with the previous findings where many phenolic compounds in plants are good sources of natural antioxidants (Othman, Ismail, Abdul Ghani, & Adenan, 2007). This result was supported by previous study who found a strong correlation between total phenolic content and FRAP assays (Huda-Faujan, Noriham, Norrakiah, & Babji, 2007).

Findings by Zheng and Wang (2001) also reported positive linear correlation between total phenolic content and antioxidant activity of selected medicinal herb extracts such as *Aloe Vera*, *Thymus vulgaris* and *Ginkgo Biloba*. Phenolic compounds also considered to be the most important antioxidative plant components, and antioxidant activity of plant materials, was well correlated with the content of their phenolic compounds (Elzaawely et al., 2005).

Generally, there have been divergent views with regards to correlation between amount of total phenol and antioxidant capacity, and interestingly Konyalioglu *et al.*, (2005) reported positive correlations according to this. Koffi *et al.* (2010) also reported that in recent years, many research has focused on antioxidant activity of phenolic compounds in traditional medicinal plants, showing positive

correlation between high phenolic content and strong antioxidant activity (Djeridane *et al.*, 2006). Safaa *et al.* (2010) also reported similar findings of strong correlation between free radical scavenging activity and total phenolics in plant samples. Caliskan *et al.* (2011) who found a strong significant correlation between the FRAP of fig fruits and total phenolics ($r = 0.74$). In addition, the relationship between total phenolic and antioxidant activity from other could be due to high content of reducing agents and environmental influences (Jagdish *et al.*, 2007)



CHAPTER 5

CONCLUSION

The results from all the three different antioxidant assays and total phenolic content showed that among the ethanol extracts of the different parts of *P. pellucida*, the leaves extracts obtained the highest value in DPPH, FRAP, ORAC and TPC value followed by the fruiting spikes while the stems had the lowest value for all the test. Hence this can be concluded that the leaves extracts of *P. pellucida* signified the best antioxidant activities and can be recommended and selected as a prophylactic agent against oxidative stress and infectious diseases.

According to the results obtained in this study, it can be suggested that the consumption of *P. pellucida* leaves could possibly offer some dietary benefits as it contains highest antioxidant constituents, which are able to protect against lipid peroxidation and to scavenge free radicals. Data obtained from this study can be further be extended to community nutrition by means encouraging the public to consume more vegetables. It is therefore recommended that people can eat more *P. pellucida* as part of their meal.

A simple linear regression test was used to analyse the relationship between total phenolic and antioxidant capacity of *P. pellucida* extracts. Our present results showed, that total phenolic content have strong positive correlation with FRAP and ORAC assay which indicate that as total phenolic content value increases,

FRAP and ORAC also increases. Meanwhile, total phenolic content had strong negative correlation with IC_{50} where that as the TPC value increase, IC_{50} decreases which indicates stronger antioxidant activity.

However, findings obtained from this study cannot be generalized to all types of *P. pellucida* plant as convenience sampling was applied in this study. The samples were collected from home grown garden and in one place only. The samples were chosen in such a way that those plants are available during the period sample collection were being selected. Therefore, the findings from this study might not necessary representative to other samples.

Besides, the limitation of this study is that it only involved one extraction solvent which is ethanol and only three methods were used to determine the antioxidant activities. Therefore, we suggested future studies should use more different extractions solvents and variety method to compare the antioxidant activity of *P. pellucida* by it different parts which are leaves, stems and fruiting spikes.

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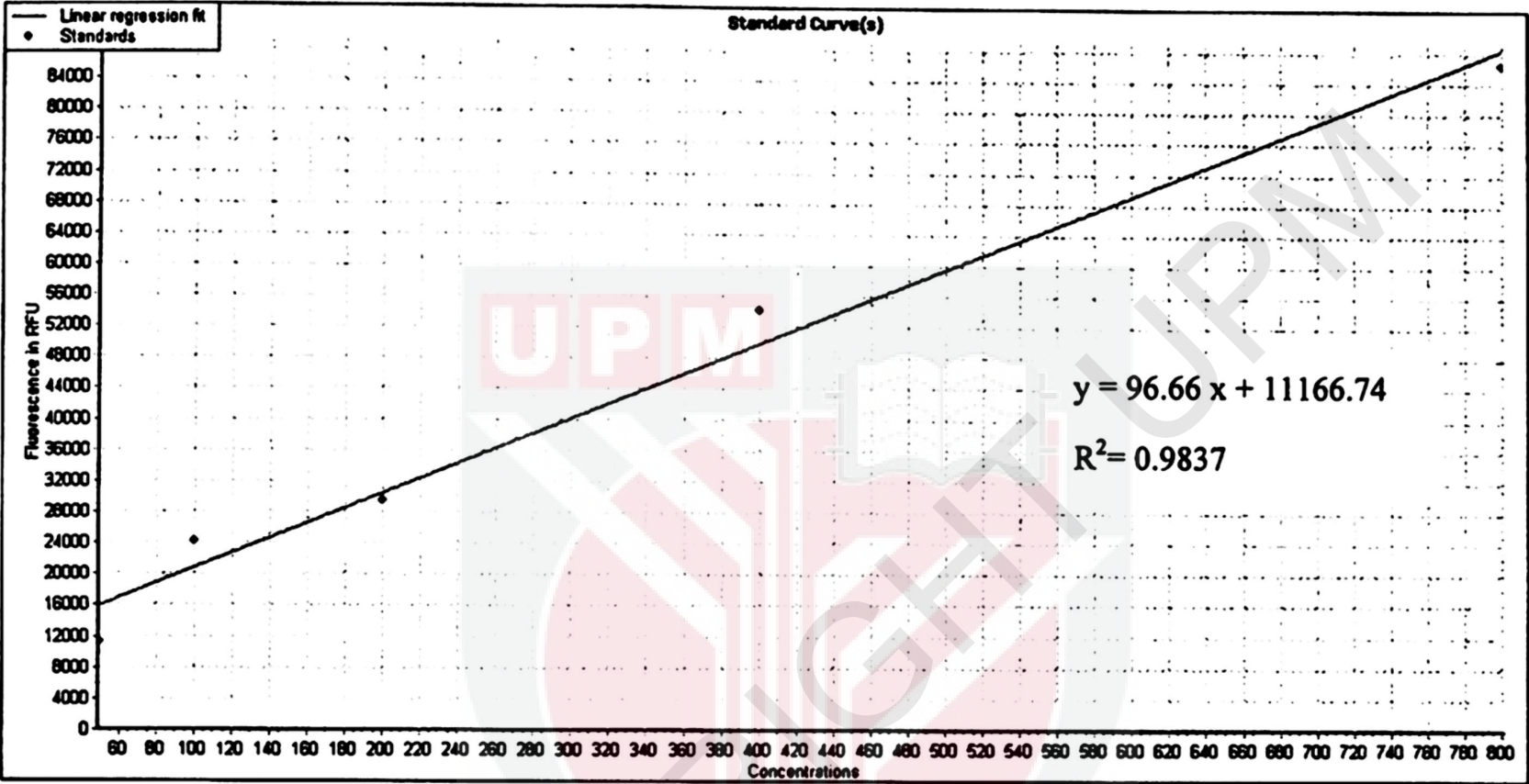
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APPENDICES

Appendix 1

Standard curve of Trolox for ORAC



Appendix 2

Standard curve of gallic acid for total phenolic content

