MONASH University

THE EFFECT OF LIGHT-EMITTING DIODES ON THE GROWTH, ANTIOXIDANT AND ANTI-MICROBIAL ACTIVITY OF MISAI KUCING *(ORTHOSIPHON STAMINEUS* BENTH*)*

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Date: 21/5/2021

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I had been in Monash for over 4 years before I decided to fully embrace Monash's motto: "Ancora Imparo" (Still, I am learning). I believe that learning is a life-long journey, and here I am, 3 years later, writing the acknowledgement for my Master's degree, trying to remember everyone who has helped me along the way. I am truly sorry if I missed out any names.

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ABSTRACT

Misai kucing, or scientifically known as *Orthosiphon stamineus* is a plant believed to be native to the Southeast Asia region. The leaves of this plant have been used extensively as tea in Southeast Asia countries as well as Europe. They are processed and known as the "Misai Kucing tea" or "Java tea". *O. stamineus* is believed to have several medicinal properties such as hyperdiuretic and hypouricemia, anti-pyretic, anti-inflammatory, analgesic, antioxidant, antimicrobial, bilirubin-reducing, and plasma glucose reducing properties. Of all the concentrations, 80% methanol was identified as the best extraction solvent in this study. The main objective of this study was to study the effect of LED lights on the secondary metabolites production and growth of *O. stamineus*. The plants were subjected to 3 growing conditions of varying percentages of blue, red, far-red and green LED (35B40R, 25B50R, and 15B60R); and 3 control groups namely sunlight (SUN), shaded rooftop (SRT), and fluorescence light (FLUO) for the duration of 11 weeks. Standard antioxidant assays were employed: Total Phenolic Content (TPC), Free Radical Scavenging (FRS), and Ferrous Iron Chelating (FIC). Additional tests such as growth, number of leaves, total carbohydrate content, chlorophyll content, and antimicrobial tests (disc diffusion and broth dilution assay) were also conducted on five microorganisms: *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Streptococcus pyogenes,* and *Enterococcus faecalis*. Reverse Phase High Performance Liquid Chromatography (RP-HPLC) was used to identify and quantify the bioactive compounds. Statistical tests of one-way ANOVA with Tukey HSD post-hoc were used to compare the various parameters while Pearson correlation was used to determine the correlation. This study revealed that 25B50R (Red: 50%, Blue: 25%, Green: 15%, Far-red: 10%) was the best condition for growing *O. stamineus* as demonstrated by high antioxidant content and activity, rosmarinic acid content, antimicrobial's zone of inhibition and low MIC and MBC values. However, these gains were accompanied by the reduction of carbohydrate and total chlorophyll content, height, and the number of leaves. Strong correlations between TPC, IC50, AEAC, and rosmarinic acid content were identified, while TPC was negatively correlated with total chlorophyll and carbohydrate content. In conclusion, growing *O. stamineus* under varying percentages of LED lights is capable to have beneficial effects as demonstrated by the increase of secondary metabolites production in this study.

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LIST OF ABBREVIATION

AEAC	Ascorbic acid equivalent antioxidant capacity
AIP	Aminoindan-2-phosphonic acid
ANOVA	Analysis of variance
CC_{50}	Chelating concentration (where half is chelated)
CO_2	Carbon dioxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
FIC	Ferrous iron chelating
FRS	Free radical scavenging
GAE	Gallic acid equivalent
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration (where half is inhibited)
LED	Light emitting diode
MBC	Minimum bactericidal concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibition concentration
MTHF	Methyltetrahydrofolic acid
NPK	Nitrogen, phosphorus, Potassium
PAL	Phenylalanine Ammonia-Lyase
PAR	Photosynthetically active radiation
PPFD	Photosynthetic photon flux density
RP-HPLC	Reverse phase high performance liquid chromatography
TPC	Total phenolic content
UV	Ultraviolet

CHAPTER 1
INTRODUCTION

1.1 Introduction of *Orthosiphon stamineus* (Misai Kucing)

Misai kucing, or scientifically known as *Orthosiphon stamineus* is a plant believed to be native to the Southeast Asian region. The plant is common in tropical climate, such as found in Malaysia, Indonesia, Brunei, and Philippines; and can be found growing on the roadside or recently can be found sold in the nursery due to beliefs that it has a lot of medicinal effects. In general, the plant has four-angled stem, with leaves in the shape of lanceolate-like, rhomboid or elliptical. There are two distinct varieties of *O. stamineus*, the purple variety (Figure 1.1), which bears pale, lilac-coloured flowers and white variety (Figure 1.2), which bears white-coloured flowers (Ameer et al., 2012).

Figure 1.1: Purple variety of *O. stamineus*

Figure 1.2: White variety of *O. stamineus*

Morphologically, both varieties have similar square-shaped stem structure, except the stem color. The purple variety has greenish maroon stem, while the white variety has green stem. Both varieties have petiolated green leaves with two leaves grew on opposite side of each other to form a pair. The purple variety's leaves have ovate shape with acute apex and truncated base. Purple veins also can be observed on the leaves together with light yellowish spots appearing unevenly on both sides of the leaves. On the other hand, the white variety has rhomboid shaped leaves and acuminate apex and obtuse base. The yellowish spots that exist on the purple variety is absent on the white variety. The vein of the leaves is also light green, compared to purple vein on the purple variety. The two varieties of *O. stamineus* could be differentiated merely by the color of the flowers and stems, and the leaf characteristics. (Keng

& Siong, 2006). The purple variety of *O. stamineus* was found to have significantly higher rosmarinic acid and bioactive compounds compared to the white variety based on a previous study (Lee, 2004).

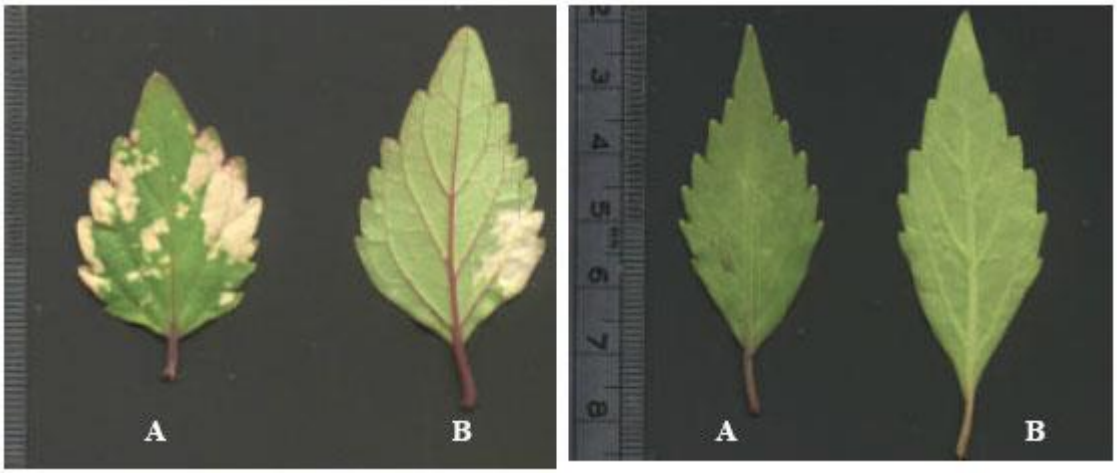

Figure 1.3: Leaf morphology of the white variety (right) with rhomboid shaped and acute apex and light green veins; and purple variety (left) with ovate shape and yellowish spots and purple veins (Keng & Siong, 2006)

The leaves of this plant have been used extensively as tea in Southeast Asia countries as well as Europe. They are processed and known as the "Java tea" in Indonesia and "Misai Kucing Tea" in Malaysia. *O. stamineus* is believed to have several medicinal properties such as hyperdiuretic and hypouricemia (Arafat et al., 2008), anti-pyretic (Yam et al., 2009), anti-inflammatory and analgesic (Yamet al., 2008), antioxidant and antimicrobial (Chun-Hoong et al., 2010), and plasma glucose reducing properties (Sriplang et al., 2007).

CHAPTER 2
LITERATURE REVIEW

2.1 Light Emitting Diodes (LED)

Light Emitting Diodes or also known as LED was first patented in the United States by Biard and Pittman in 1966 (Biard & Pittman, 1966). By referring to the principle of electroluminescence, LED is a solid-state semiconductor. The flow of electricity in the semiconductor excites the electrons and causing them to move from higher to lower energy orbital (Gupta & Agarwal, 2017) leading to the emission of light. The world's first working LED was produced by Nick Holonyak Jr. using gallium arsenide phosphide diode which produced red light; however, it was not deemed useful for agriculture due to the lack of blue color LED. In the year 2000, Nakamura et al. (2000) produced the first working blue light LED, which emits light at wavelength of 450 nm. A Nobel Prize was awarded for the creation of efficient blue LED in the year 2014; and later through placing a phosphor coating on the top of high-energy blue light, a working and cost-effective white LED was produced (George et al., 2013).

The usage of artificial light for horticulture started long time ago in the era of incandescent and gas discharge lamp; however there have been several negative outcomes that have rendered it to be thought not beneficial. These negative outcomes have been fixed with the current day LED, such as a longer lamp shelf-life, potential for new designs, energy saving, rapid cycling between on and off cycle, the ability to do spectral mixing, and the lack of heat generated from the lamp itself. Since the LED system is adjustable up to certain degree, it can trigger the beneficial acclimation response of plants. These adjustable qualities are quantification of photon (PFD), quality (wavelength), duration of lighting (milliseconds to weeks), and automatic timing of lights (Pocock, 2017).

2.1.1 Structure and Principle of LED

To date, there are two types of LED available in the market, namely dual in-line package or the high-power light emitting diodes as can be seen in Figure 2.1. The most basic design of LED comprises an LED chip inside a housing made from epoxy or plastic or other heat resistant material and metal wire for the movement of electrical current. The semiconductor chip is roughly $1mm^2$ in size and contains a specific impurities or dopants. There are two types of dopants in one chip: the n-type and the p-type. The n-type dopant is normally an element with

high number of valence electrons, while the p-type is element with high number of empty slots in its valence shell. The two dopants' crystals will have to be fused together, and it is called as the "p-n heterojunction".

Figure 2.1: Dual in-line (left) and high power (right) LED (Gupta & Agarwal 2017)

In a complete circuit, the electricity will enter the p-type dopants first and cross the p-n heterojunction to the n-type dopant as can be seen in Figure 2.2. This movement will cause the electron to flow in opposite direction from the n-type towards the p-type. These electrons will then fill the vacant slot in the valence shell as mentioned above, and this phenomenon is known as the "electron-hole pairing". As the electron that moves to the slot has higher energy compared to the acquired orbital, the excess energy will be released as electromagnetic radiation specific to certain wavelength or colour (Gupta & Agarwal 2017).

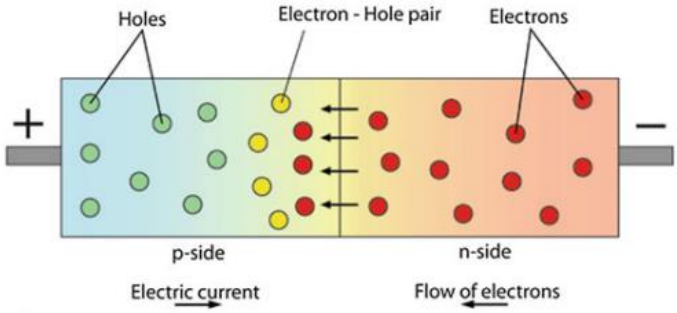

Figure 2.2: The p-n heterojunction (Gupta & Agarwal 2017)

2.2 Photosynthesis

As mentioned by Boyle (2004), despite the intense sunlight received by the plants, only roughly 50% will be utilized by the plants, and comprises mostly wavelength region from 400 to 700 nm. Biologically, plants have specialized photoreceptors in all parts that can capture the photons from the sunlight and convert it to chemical energy by the process of photosynthesis. The photoreceptors also contain accessory pigment known as the carotenoids. The photo

receptors in plants are different from the animal, where they are only limited to the specific organ like the eyes. Plants have photoreceptors embedded inside all tissue throughout the tissue (Galvão & Frankhauser, 2015).

There are several types of photoreceptor proteins that are involved in the absorption on lights. These photoreceptors absorb lights not only in photosynthetically active radiation (PAR), but also at far-red and ultraviolet region. The first protein, cryptochrome, absorbs lights at the UV-A and blue light region; between 340 nm and 520 nm. This photoreceptor is special compared to the others as it has two different absorption maxima, 375 nm and 450 nm. This is due to the availability of 2 different chromophore, 5,10-methyltetrahydrofolic acid (MTHF) which functions at lower wavelength, while flavin at higher wavelength (Fuller et al., 2016). Cryptochrome not only responsible for the morphological aspect of the plants, but it also plays a vital role in numerous circadian clock function such as seedling development and de-etiolation (Fox et al., 2012). Phytochrome is another type of photoreceptor in plants. This hydrophilic compound is mainly active in the red and far-red region; however, it also absorbs near the blue region. Its primary function mainly involves in the hormone production pathway in plants, such as gibberellins, auxins, ethylene, jasmonates and abscisic acid (Casal, 2000). Due to the fact that the UV-radiation is capable to damage the DNA and proteins, plants naturally have UVR8, photoreceptor proteins which main function is to trigger the gene expressions that are vital for the production of protective pigments such as anthocyanin (Thoma et al., 2020). The absorbance spectrum for both chlorophyll *a* and *b* can be seen in Figure 2.3.

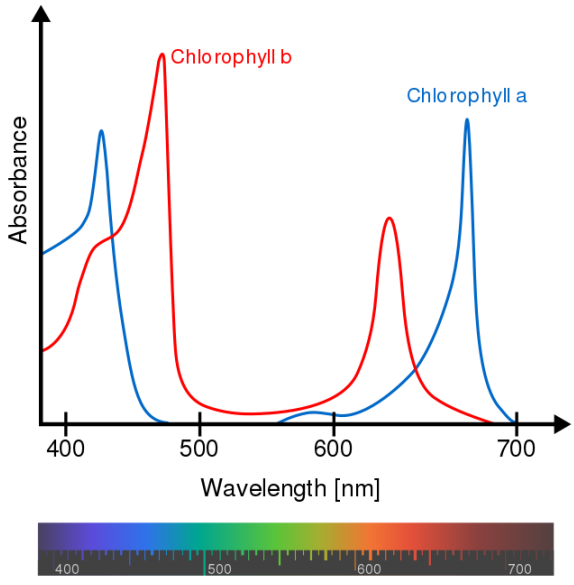

Figure 2.3: Spectrum absorbance of chlorophyll-a and b (Boyle, 2004).

There are two different types of chlorophyll: *a* and *b*, which detect different spectrum of light. Chlorophyll-*a* absorbs light maximally at 430 and 675 nm, while chlorophyll-*b* is at 453 and 642 nm. The lighting condition is extremely important to plant that is not only used during photosynthesis. It also is involved in numerous other biological functions of the plants, such as photomorphogenesis, phototropism and photoperiodism. Photomorphogenesis is defined as the plant's biological development which relies heavily on light, specifically at the far-red radiation of 730-735 nm, such as plants cells differentiations into different component in plants. Phototropism is the ability of plants to detect and move towards the light source in order to get maximum light. Plant's phototropism will be triggered by light in 400-500 nm wavelength region. Meanwhile, photoperiodism refers to the plant's ability to detect light and day cycle and regulate itself to suit properly (Gupta & Agrwal, 2017).

Chlorophyll-*a* and *b* works together to absorb light at different wavelength which will be converted to chemical energy. Structurally, chlorophyll-*b* is differentiated by a functional group which is bound to the porphyrin ring and more soluble in polar solvent compared to chlorophyll-*a*. Both chlorophyll groups have Mg^{2+} at the center, making it hydrophilic and ionic, and also hydrophobic due to the presence of a ring which has a carbonyl group on its long hydrocarbon tail (Sumanta et al., 2014).

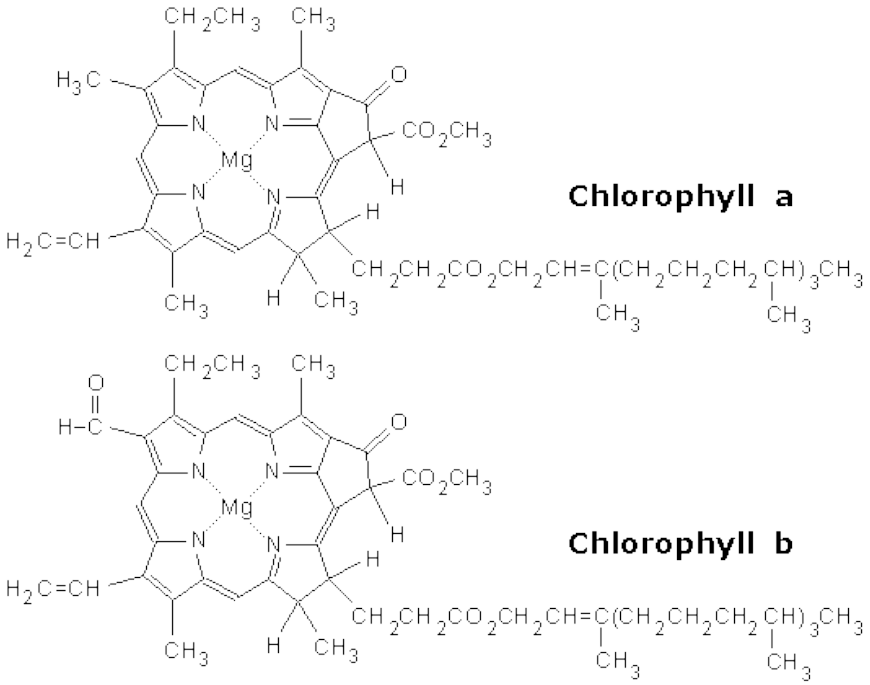

Figure 2.4: Chemical structure of chlorophyll.

During photosynthesis, the top of the leaf will absorb the blue and red light from the light spectrum and are more efficient in facilitating photosynthesis in this area compared to the green light. Thus, green light will be transmitted deeper into the leaf and absorbed by chlorophyll at the lower surface (abaxial) of the leaf. Because of this, the green light is much more efficient to CO_2 fixation compared to the blue and red lights (Terashima et al., 2009). There are also accessory pigments available inside the photoreceptors, namely the carotenoids; and other than light absorption, they also help in protecting the photoreceptors by dissipating excess energy and quenching the chlorophyll to prevent them from becoming too over excited (Ruban, 2015).

2.3 Phenolic acids and Flavonoids

Plants produce chemicals known as secondary metabolites as byproducts of plant's biological processes. Some of the most important secondary metabolites produced by the plants are phenolic acids and flavonoids (Kim et al., 2003). Phenolic acids are important as it affects plant's taste, flavor, and medicinal properties; thus, increased phenolic content can increase plant's nutritional and medicinal quality (Tomas-Barberan & Espin, 2001). Phenolic acids are characterized as compound with phenol groups in it. In plants, phenolic compounds could appear as monophenol, diphenol, and more; but most abundant are the polyphenols. Similarly, flavonoids are compounds that contain polyphenol, and all flavonoids share the same basic structure C6-C3-C6 skeleton. Flavonoids were classified into 6 different subgroups: flavones, flavonols, flavanones, flavan-3ols, isoflavones and anthocyanidins (Manach et al., 2004).

Phenolic acids and flavonoids are well known for their antioxidant activities. Antioxidant is important in order to combat oxidative stress which in excess will cause conditions and diseases such as inflammations, autoimmune diseases, cancers and many more. In order to protect body from free radicals, human, animals, and plants need antioxidants to avoid cellular damaged caused by imbalance of antioxidant-free radical level (Kukic et al., 2006). This research was focusing on 4 different compounds: rosmarinic acid and caffeic acid (phenolic acids) and eupatorin and sinensetin (flavonoids) as they were found to be present in the studied plant leaves (Muhammad et al., 2011). It is this study's ultimate goal to examine how these compounds concentration respond to the lighting conditions imposed onto the plants.

Phenolic compounds are found abundant in plants and are synthesized by the plants through the Shikimate pathway. Shikimate pathway was first discovered by the discovery of shikimic acid, first isolated from the flower of *Illicium anisatum*, or commonly known as shikimi by the Japanese. Shikimate pathway begins with the production of chorismate from

phosphoenolpyruvate and erythrose 4-phosphate through a 7-reaction processes, catalysed by 6 different enzymes. Chorismate will later be catalysed by chorismate mutase to form prephenate, which later will be catalysed to arogenate. Arogenate is the direct precursor of tyrosine and phenylalanine, which are amino acids vital for production of numerous secondary metabolites such as flavonoids, anthocyanins, phenylpropanoids and etc (Tzin & Galili, 2010).

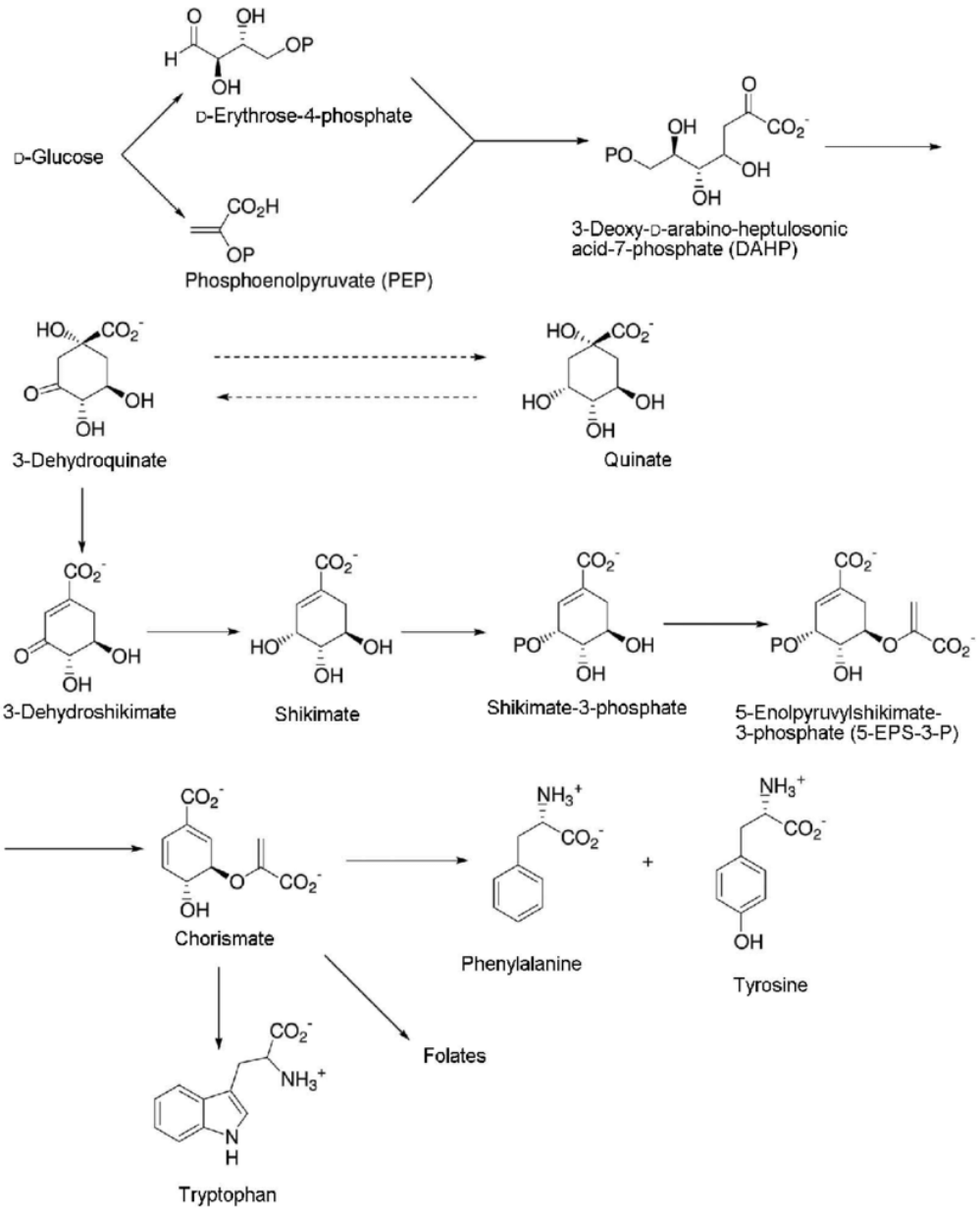

Figure 2.5 Shikimate pathway. (Borah, 2015)

2.4 Medicinal potentials of *O. stamineus*

2.4.1 Phenolic content and antioxidant

Over the years, due to the popularity of *O. stamineus*, extensive studies have been conducted on the phenolic contents of it. Over 20 different phenolic compounds were isolated from *O. stamineus* alone such as lipophilic flavones, flavonol glycosides and caffeic acid derivatives. A few examples of compounds found were rosmarinic acid, 2,3-dicaffeoyltartaric acid, sinensetin, 30-hydroxy-5,6,7,40-tetramethoxyflavone, 5-hydroxyl-6,7,30,40-TMF,5,6-dihydroxy-7,40-dimethoxyflavone, eupatorin, tetramethyl scutellarein, camphor, menthone, d-terpineol, isomenthone and many more (Ashraf et al. 2018).

There are four different compounds to be analyzed for this study, based on works of Akowuah et al. (2004) and Muhammad et al. (2011): rosmarinic acid, caffeic acid, sinensetin and eupatorin. Akowuah et al. (2004) which focused on comparing all *O. stamineus* all around Malaysia found that concentration of these compounds was 5.10 – 29.90%, 0.05 – 0.69%, 0.22 - 1.76%, and 0.34 – 3.37% of total dry leaf weight, respectively. They also found that the lowest total phenolic content came from Pasir Puteh, Kelantan with 6.69 mg caffeic acid equivalent /g dry weight; while the highest was recorded by sample from Parit, Perak with 10.20 mg caffeic acid equivalent/g dry weight. However, the author mentioned that sample from different locations showed considerable variation in antioxidant activities, perhaps due to numerous factors such as fertility of the soil, age of the plants and multiple way of sample sourcing. Meanwhile, Muhammad et al. (2011) only focused on rosmarinic acid and reported the concentration of 44.00 ± 1.879 µg of rosmarinic acid per mg of dry weight (4.40%; w/w).

Several studies have showed that *O. stamineus* has magnificent antioxidant activity. In a study conducted by Akowuah et al. (2005) for scavenging activity using 1,1-diphenyl-2-picrylhydrazyl, they reported that *O. stamineus* potency is comparable to standard antioxidants such as quercetin. They also reported that acetone extract's antioxidant activity was significantly higher compared to aqueous methanol, methanol, and chloroform extracts. Their findings were further supported by Awale et al. (2003) that also reported highest radical scavenging activity in 70% aqueous acetone extract against methanol, aqueous methanol and chloroform. However, in 2007, Yam et al. found that 50% methanol extract of *O. stamineus* was able to give significant in-vivo antioxidant activity.

An interesting research by Abdelwahab et al. (2011) tried to correlate the antioxidant activity and phenolic content of *O. stamineus* with antiapoptotic activity. They found that chloroform extraction has higher flavonoid content while aqueous methanolic extract has higher phenolic content and antioxidant activity. They also reported that cells pre-treated with

O. stamineus extract has lower H_2O_2-induced apoptosis death. This outcome showed that *O. stamineus* has antiapoptotic effect and might be directly related to its phenolic content.

This study also performed extraction efficiency experiment in order to determine the best solvent that should be use in extracting the phenolic contents of *O. stamineus*.

2.4.2 Antimicrobial activity of *O. stamineus*

Ethanolic and aqueous extracts of *O. stamineus* were shown not to have any effect on Gram negative bacteria; specifically, *Escherichia coli* and *Klebsiella pneumonia* in disc diffusion method. However, in Gram positive bacteria, both extracts were able to inhibit growth of *Staphylococcus aureus* and *Streptococcus agalactiae*. Ethanolic extracts' inhibition zones were 6.8 ± 0.09 mm on *S. aureus* and 6.5 ± 0.09 mm on *S. agalactiae*; while aqueous extracts' inhibition zones were 10.5 ± 0.20 mm and 8.1 ± 0.07 mm for respective microbacteria. A further test on aqueous extracts of *S. aureus* and *S. agalactiae*'s minimum inhibitory concentration (MIC) found that the respective MIC values were 1.56 mg/mL and 3.13 mg/mL; while the minimum bactericidal concentration (MBC) values were 3.13 mg/mL and 6.25 mg/mL respectively (Alshawsh et al., 2012).

2.5 Previous Studies on the Effects of Lighting on Plant

2.5.1 The effect of lightings on growth

As demonstrated in the previous studies by other researchers, the rate of photosynthesis, growth and morphogenesis of a plant depends heavily on the lighting conditions (Averceva et al., 2009). The idea of using artificial lighting system with the optimum wavelength has been mulled by farmers and green house operators, in order to create a better harvest in a short period of time (Kozai, 2007). Traditionally for indoor planting, normal lighting system has been used, such as sodium lamps, fluorescence lamps, and incandescent lamps; but this proves to be inefficient due to short lifetime, heat generated and high usage of energy (Astolfi et al., 2012).

There have been several studies on the effect of light emitting diode (LED) lighting on the growth of a plant species. A study on *Lactuca sativa* in Japan shows that the fresh weight, dry weight and photosynthesis rate are the highest in the red LED lighting, comparable with the fluorescent light. However, the number of leaves is the highest in the mixture of blue, green and red lighting at 33.4%, 33.9% and 32.7% respectively (Shimizu et al., 2011). Another study conducted in Taiwan for hydroponic *Lactuca sativa* shows that the addition of white light to red and blue light brings positive result on the growth, development, nutritional content and appearances (Lin et al., 2013).

In a study conducted on 'Green Oak Leaf' lettuce by Chen et al. (2014), among the parameters that was chosen to identify the effect of LED on plant's growth were plant height and leaf number. After 50 days of exposure, the researchers reported that lettuce grown under fluorescence light with supplemental LED light (either blue or red) were statistically tallest and have the greatest number of leaves compared to monochromatic red, blue, mixture of red and blue, and monochromatic fluorescence. These observations were also supported by other usual growth parameters such as fresh weight, dry weight, stem length, and stem diameter. They concluded that fluorescence light, when combined with red or blue LED resulted in improved morphology and greater biomass than monochromatic red, blue, fluorescence or red-blue mixture. Lili et al. (2019) reported that "Favorita" potato plantlets grown under monochromatic red had the greatest height compared to monochromatic blue and red-blue mixtures lighting conditions. Similarly, this result also was supported by Puspa et al. (2008) who observed greatest shoot elongation and internode length under monochromatic red light for grape vines. In the discussion, they argued that the use of LED will trigger photomorphogenic pigments, which is responsible for photoreception and regeneration.

2.5.2 The effect of lightings on secondary metabolites

Research conducted to test the effect of light intensity to the flavonoids production found that different species of plants would react differently at the same light intensity. Plants such as *Ginkgo biloba* (Leng et al., 2002) and *Erigeron breviscapus* (Su et al., 2006) favor condition with higher light intensities and will produce more secondary metabolites; while plants such as *L. litseifolius* prefer 40% shading condition and produce fewer secondary metabolites at higher light intensities (Li et al., 2016). This finding of *L. litseifolius* was in agreement with another research conducted on *Piper aduncum* where plants growing at 100% irradiance have significantly lower flavonoids compared to those planted under 50% irradiance (Pacheco et al., 2014).

Studies such as performed by Bantis et al. (2016) on two different cultivars of *Ocimum basilicum* have shown that the total phenolic content have increased significantly in plants under blue LED treatment compared to fluorescence light; however, the growth rate seems to be different depending on the cultivar. In terms of plant growth, a study by Snowden (2015) found that the dry mass, leaf area index and stem elongation to be inversely correlated with the percentage of blue lights subjected upon the plants (11 – 18%). He stipulated that the increasing blue light would increase the cyptochrome, which causes the plants to become overstimulated and placing them under stress, thus reducing growth. The study also has shown that the

concentration of chlorophyll increased significantly with increasing blue light in most species studied. The chlorophyll content is also affected by the photosynthetic photon flux density (PPFD) level for blue light; and he concluded that the highest chlorophyll content mostly occurred around 20-30% of blue light treatment.

Several groups also focused on studying the effect of different PPFD values on plants. In research conducted by Dou et al. (2018) of growing sweet basil under five different PPFD of 160, 200, 224, 290, and 310 $\mu mol \cdot m^{-2}.s^{-1}$, they reported that the plants under 290 and 310 $\mu mol \cdot m^{-2}.s^{-1}$ had the significantly highest phenolic and flavonoid content compared to other treatment group. Similar to other researchers before, Dou et al. (2018) inferred that the synthesis of phenolic compounds including phenolic acids and flavonoids is greatly enhanced under strong UV and visible light treatments. Sweet basil under the treatment of 290 and 310 $\mu mol \cdot m^{-2}.s^{-1}$ also have the lowest total chlorophyll content, together with plants under PPFD value of 224 $\mu mol \cdot m^{-2}.s^{-1}$. They argued that this could be due to the possession of chloroplast that is highly adapted to higher PPFD values. These so-called adapted chloroplasts had higher photosynthetic quantum conversion rate, thus explains the reduction of total chlorophyll content.

A lot of studies on the effect of lighting conditions on plants focused on the seedlings or green vegetables such as lettuce (Stutte et al., 2009, Li & Kubota, 2009, Samuoliene et al., 2012), kale (Lefsrud et al., 2008), mustard (Tarakanov et al., 2012), and cabbage (Mizuno et al., 2011). These studies that used lettuce and other leafy plants, found that red light (658-660 nm) increased the phenolic content of the leaves (Li & Kubota 2009). Study by Stutte et al. (2009) found that the usage of far-red light was beneficial for plant's growth which was seen by increased total biomass and elongation of leaves but suppressed anthocyanin content and antioxidant potential. Samuoliene et al. (2012) published a study on the effect of red light together with white light and found that there was an increase of TPC (28.5%), sugar levels (52.0%) and antioxidant activities (14.5%); but exhibited reduction in ascorbic acid concentration of baby green leaf lettuce. In another research on kale by Lefsrud et al. (2008), they found that there was an increase of chlorophyll-*a* and *b* for plants that have been exposed to red light. On contrary, Mizuno et al. (2011) found that chlorophyll content was the highest under fluorescence lamp compared to blue and green lights for "Kishun" cabbage, but no significant difference was observed for "Red Rookie" cabbage for all lighting treatments.

On the matter of the effect of monochromatic blue light, research done by Mizuno et al. (2011) found that "Kishun" cabbage has exhibited increase in growth of main stem and petiole, while "Red Rookie" cabbage only exhibited increase in petiole length, but not the main stem.

Tarakanov et al. (2012) did a research on the effect of 75% of red light at 660 nm and 25% of blue light at 460 nm on Indian mustard and found that it exhibited delayed or inhibited flowering of the plants. These researches have argued that different plants might have a different reaction to different spectrum of lights. Red light might be beneficial on certain plants, but blue light has been found to be beneficial to some other species too. Red light (650-665 nm) falls within the absorption peak of the chlorophyll; thus, the higher percentage of red light would trigger higher level of photosynthesis. Sabzalian et al. (2014) however argued that using blue and red light would actually increase growth further due to the fact that the stomatal opening for CO_2 uptake is controlled by the blue photoreceptors.

2.5.3 The effect of lighting on *O. stamineus*

There has not been much study done on the effect of lights on *O. stamineus*. A study conducted in 2012 found that there was a steady decrease of secondary plant metabolites of interest, phenolics and flavonoids in *O. stamineus*, when it is being exposed to increasing irradiance of normal light of different shadings: 0%, 20%, 40% and 60%, which were equivalent to 900, 675, 500 and 225 μmol m^{-2} s^{-1} (Ibrahim & Jaafar, 2012). They stipulated that at low irradiance, there will be a decrease in photosynthesis and total biomass but will have an increase in secondary metabolite productions. The study was further supported by another study from another group of researchers where they tested growing *O. stamineus* using different light intensity of white light (Affendy et at., 2010). The study concluded that *O. stamineus* grows well under 50% light intensity, thus implying that *O. stamineus* grows well under shaded condition compared to direct sunlight. This study however was contradicted by findings by Farhan et al. (2012). Based on the findings that the leaves had significantly higher total phenolic content (TPC) and antioxidant activity compared to stems and roots, Farhan et al. (2012) compared the readings of the leaves on plants planted under open environment and those grown under shaded conditions (50% and 70% shaded). They noted that leaves of plants under open environment had significantly higher TPC and antioxidant activity compared to the plants grown under 50% and 70% shaded condition. They also reported that similar finding was observed in young seedlings grown under similar conditions.

However, these two studies only focus on the irradiance and intensity of the light instead of focusing on the specific spectrum of blue, red, far-red, and green light as the present study is aimed to do.

Chapter 3
Project Description and Objectives

3.1 Problem Statements

The focus of the study is to try to find a balance between the two: growth and secondary metabolites production, by using LED at different percentages of four different coloured lights as mentioned above. Past studies have found that growth and secondary metabolites production have an inverse relationship as discussed in the previous chapter, so we would like to study at which wavelength we can get maximum secondary metabolites productions without sacrificing growth.

3.2 Research Question

Albeit there has been a lot of research which found that red light will affect growth while blue light will affect secondary metabolite production (as discussed in the previous chapter), there has not been much research done on medicinal herb. *O. stamineus* is one of the popular medicinal herbs that normally grow outdoor. In this research, we would like to understand the following:

1. Is *O. stamineus* suitable to be grown indoor under controlled condition?

Furthermore, the previous research which focused on growing *O. stamineus* indoors was using white light as the source of light at various intensity (Affendy et al., 2010). However, in view of the previous studies which have found the different role played by red and blue LED to plants, we would like to determine:

2. What is the proportion of red and blue light that is most suitable to grow *O. stamineus* indoor to maximize growth without sacrificing the production of secondary metabolites of interest, compared to the white light and outdoor plant?
3. Can the similar effects also be observed in mature *O. stamineus*?

As plants subjected to the different light quality will directly affect the action of chlorophyll and its subsequent byproduct carbohydrate as discussed in previous chapter, we also would like to determine:

4. How will the relative proportion of red and blue light affect the production of chlorophyll and carbohydrate in *O. stamineus*?

It is hypothesized that different proportion of red and blue light, while keeping a relatively low and fixed proportion of green and far-red lights, will affect the growth and secondary metabolites production on *O. stamineus*.

3.3 Research Objectives

1. To determine extraction efficiency using different solvents combination for extracting *O. stamineus*.
2. To determine the effect of different lighting conditions towards the growth of *Orthosiphon stamineus*.
3. To determine the effect of different lighting conditions towards the total phenolic content, antioxidant activity, chlorophyll and carbohydrate contents and antimicrobial activity of *O. stamineus*.
4. To establish correlations among biochemical traits under different lighting conditions imposed onto *O. stamineus*.

Chapter 4
Methodology

4.1 Planting and Growing Conditions

Plant that was used in the research is *Orthosiphon stamineus*, or locally known as "Misai Kucing". The white flower variety of the plants were purchased from Sungai Buloh Nursery, and smaller cuttings were propagated by using water rooting method. The white variety was chosen as the purple variety was unavailable during the study. Only 5 cm of the branches with young shoot on top were selected, placed in a beaker of tap water to encourage root growth for 2 weeks, before being planted into the growth medium. Total of these 36 two-week old plants were used in this study (Six plants were used per light treatment until the 11th week). The growing medium was using combination of clay, silt, and sand at the ratio of 1:1:1 respectively to create a loamy medium. Organic fertilizer NPK (15:15:15) was used to provide nutrients to the young plants. The fertilizers were applied three days before the planting day, at 1 t/ha (Zaharah 2005). The following calculation of fertilizer was used to calculate the amount of the fertilizers to be added (Johnston & Askin, 2005):

$$kg\ fertilizer\ per\ ha = kg\ element\ per\ ha \times \frac{100}{\%\ element\ in\ fertilizer}$$

Each pot was filled with 1.5 liter of soil mixture and fertilized with 7 g of NPK fertilizer (15:15:15) at the beginning of the study based on the above formulae. Detailed calculation is available in Appendix G. The pots were in the shape of inverted cone frustum, measuring 20 cm (top diameter) x 11 cm (height) x 14 cm (bottom diameter).

Six different growth conditions were planned for this study. Four of the conditions were indoor in the growth cabinets, with constant temperature and humidity (22.0 ± 2.3°C and 65.1 ± 4.9% respectively), while two were grown outdoor under partially shaded area (30.2 ± 2.5°C) on the rooftop and under direct tropical sunlight (3-4 hours daily). Six plants (two-week old) were planted for each of these conditions, making it 36 plants in total. The plants for the LED and the fluorescence light treatment were placed on the planting racks, where each rack has two tiers. Six plants were placed per tier, to ensure maximum exposure to the lights. The lights were placed inside the growth chamber, and the rate of photon released per square meter per second (photosynthetic photon flux density, PPFD) were determined using the lighting passport

(ASENSETEK (China), ALP-01) as can be seen in Figure 4.1. The LED lights were produced by P-Plus Sdn. Bhd. with the advice from Osram (M) Sdn. Bhd. The light tubes were checked individually using the lighting passport to ensure each one is at the right frequency. The light tubes were placed 70 cm from the base of each growth chamber, and were arranged in the following arrangement: green, blue, red, far-red, green, blue, red, and far-red.

Figure 4.1: Lighting Passport, ASENSETEK, ALP-01

The plants grown indoor were watered once every two days (morning) at fixed volume of 50 mL to avoid water seeping out of the pots and to keep the humidity in the room constant. Those in rooftop and outdoor were watered daily (morning) to replicate real outdoor growing condition. In the growth chamber with different LED combinations, a heat-resistant plastic tray was placed under each plant, to ensure the water will not seep down to the lamps on the tier below. The plants were subjected to the lights for 12 hours per day (8.00 am until 8.00pm). Sunblock curtains were used to separate one lighting conditions from the next to avoid light spillage. The dimension of each growth chamber was 91.5 cm (length) x 46.0 cm (width) x 77.5 cm (height). Figure 4.2 shows the growth cabinet which was modified to house two growth chambers (top and bottom).

Figure 4.2: The four tiers growth cabinet.

Two cycles of investigation were carried out for young plants (starting from 2-week old) with each cycle taking place for four months in total from preparation to harvest. The first round of growing phase was carried out from February until May 2019 while the second phase took place between November 2019 until February 2020. For the mature plants (~15-week old), one round of investigation under different lighting conditions was carried out. The mature cycle was carried out between June – July 2019. A detailed look on all the conditions is summarized in Table 4.1 and Figure 4.3 are as follows:

Table 4.1: Light treatments percentage for all growth conditions.

Conditions	**Blue LED**	**Red LED**	**Green LED**	**Far Red LED**	**PPFD[a] (µmol/m²s)**
35B40R	35%	40%	15%	10%	120 ± 5
25B50R	25%	50%	15%	10%	120 ± 5
15B60R	15%	60%	15%	10%	120 ± 5
FLUO	Fluorescence light (in-door)				40 ± 3
SRT	Shaded rooftop area (glasshouse)				35 ± 3
SUN	Sunlight (open)				800 ± 50

[a]PPFD of each treatment was in the wavelength range 380 –780 nm. PPFD and spectral scans were measured at 30 cm from lighting sources at nine points (except for SRT and SUN).

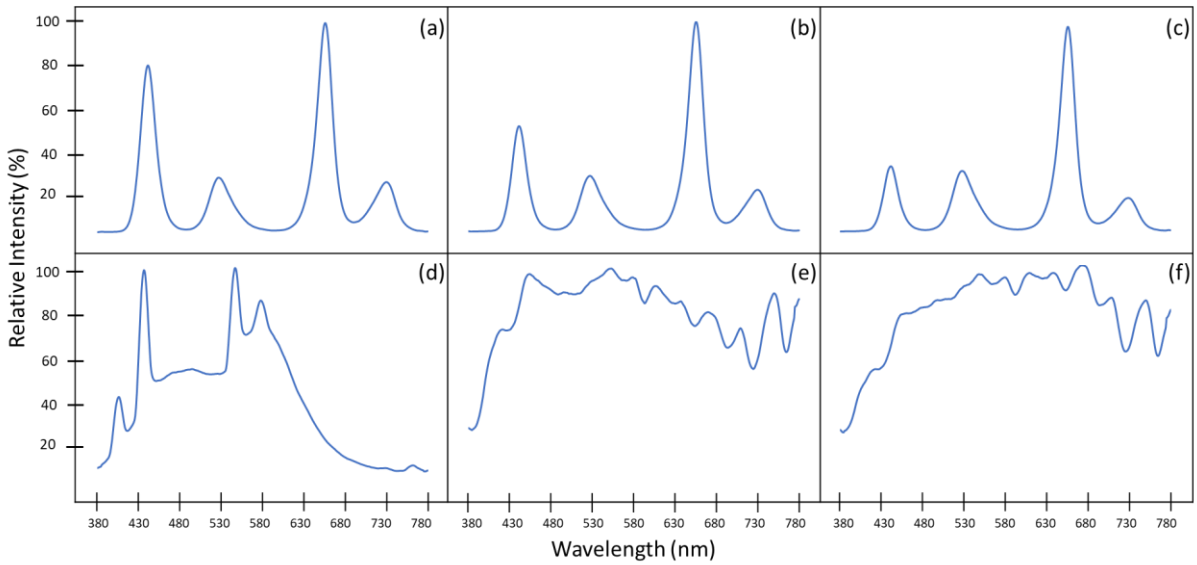

Figure 4.3 Spectral scans for each condition[a]

[a]Relative spectral distribution of blue, green, red, and far-red used in this study: (a) 35B40R, (b) 25B50R, (c) 15B60R, (d) FLUO, (e) SRT, (f) SUN.

Note that as shown in Figure 4.3, spectra of lights from SUN, SRT and FLUO treatments do not have distinct and well-resolved bands, although FLUO light has a high proportion of blue and green, and very low proportion of red and far-red.

4.2 Growth Measurement

There are two parameters that were used in this study to determine growth. The first was the height of the plants. It was measured from the top of the top the top-most shoots of each plant. The measurement was taken in centimeter and was recorded every week.

The second measurement that this study used to measure growth was by calculating number of leaves every week. The leaves that were calculated must be healthy, separated from the shoots and over 1 cm in length. As for mature leaves, those with browning more than 50% of the total leaf area was excluded.

These two measurements were chosen to determine the growth of the plants as these are the non-destructive growth measurement that can be applied to plants with continuous study model, compared to other commonly used measurement (destructive) such as fresh and dry weight, root mass, and root-shoot ratio.

4.3 Extraction Efficiency Test

After 11 weeks of light treatment, sample extraction and extraction efficiency determination were performed using method described by Chong & Lim (2012). Fresh leaves (0.5 g) were weighed and grinded into fine particles using liquid nitrogen in metal mortar and pestle. A total of 25 mL of solvents were added and let swirl in room temperature for one hour with an orbital shaker. Two concentrations of methanol (Chong & Lim, 2012) and ethanol (Ibrahim & Jaafar, 2012): 80% and 100%, were used as solvents, to determine the suitable solvent that yielded the most phenolic contents. After one hour, the solutions were filtered using “vacuum” suction and kept in glass bottle for further testing. To determine the extraction efficiency of the first extraction, the process of extraction was repeated for the second and third time for the same plant material. The efficiency of the extraction was determined by comparing percentage of phenolic content in the first, second and third extraction. These percentages were used in subsequent study to adjust the TPC from the first extraction in order to reflect the actual TPC values.

4.4 Sample Extraction

4.4.1 Standard Leaf Extraction

Fresh leaves (0.5 g) were crushed using liquid nitrogen and 25 mL solvent was added together. The mixture was then swirled for 1 hour in room temperature using orbital shaker at 150 rpm. The solution was extracted using vacuum pump and kept in glass container at -20ºC until experimental assays can be performed.

4.4.2 Chlorophyll Extraction

Fresh leaves (0.5 g) were washed with tap water and followed by distilled water and dabbed to dry with tissue paper. Methanol (10 mL) was added, and the sample was digested using tissue homogenizer. The mixture was then centrifuged at 10,000 rpm at 4ºC for 15 minutes. A total of 0.5 mL of the supernatant was added with 4.5 mL methanol and the absorbance was determined using spectrophotometer.

4.5 Antioxidant Content - Total Phenolic Content (TPC)

The total phenolic contents of *O. stamineus* extracts were determined using Folin-Ciocalteu assay as described by Chan et al. (2007). Reagents that were used in this assay are 10% Folic-Ciocalteu's reagent and 7.5% sodium carbonate (w/v). 750 µL and 600 µL of the respective reagents were added into 24-well microplate which had 150 µL of extracts in triplicates. The microplate was left in room temperature for 30 minutes away from the light before being read using Tecan microplate reader at 765 nm, at 50 beams per second.

The total phenolic content was expressed in terms of GAE in mg per 100 grams fresh leaves. The calibration equation that was used to determine the content of phenolic in the sample were determined by preparing a standard calibration curve using gallic acid as standard solution. The calibration equation used for gallic acid was $y = 0.006x + 0.0229$ (R^2=0.9976), where x represents the concentration of gallic acid in mg/L and y represents absorbance.

4.6 Antioxidant Activity

4.6.1 Free Radical Scavenging (FRS)

Free radical scavenging test is a well-known test to determine the activity of antioxidant. 2,2-diphenyl-1-picrylhydrazyl (DPPH), a stable free radical was used to determine the antioxidant activity of *O. stamineus*. The method used was as described by Chong & Lim (2012) where different dilutions of 75 µL extract samples (20%, 40%, 60%, 80%, and 100%) were used. FRS was expressed as ascorbic acid equivalent antioxidant

capacity (AEAC) in mg ascorbic acid per 100 g sample; where the calculation of AEAC was using IC_{50} of sample and ascorbic acid in the following formulae: $IC_{50(ascorbic\ acid)}/IC_{50(sample)}$ x 10^5, where IC_{50} ascorbic acid was determined to be 0.00389 mg/mL.

The control solution was prepared by using ascorbic acid of multiple concentrations (1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, 12.0, 13.5, and 15.0 mg/ L). The protocol to run the standards were exactly how the samples were run: by adding 1 part of standard to 2 parts of DPPH. From these readings, standard curve of percentage of scavenging activity was created, and IC_{50} of the standards were determined. The standard curve equation obtained for the scavenging activity of ascorbic acid was $y = 12.991x$ ($R^2 = 0.9669$), where x represents the concentration of ascorbic acid in mg/L and y represents absorbance.

4.6.2 Ferrous Ion Chelating Activity (FIC)

The determination of FIC was using the method as mentioned by Chan et al. (2007), with modification by Chong & Lim (2012). Samples were prepared in different concentrations (blank, 20%, 40%, 60%, 80%, and 100%) of 75 µL and added to 75 µL iron (II) sulfate (0.1 mM) in triplicate. 75 µL ferrozine (0.25 mM) were added and the mixture was left for 10 minutes in room temperature. The absorbance of the mixture was then measured at 562 nm and expressed as percentage of chelating effect CC_{50} in mg/mL, using the following formulae:

The results were expressed as percentage of chelating effect using the following formulae:

$$\%\ chelating\ effect = \left(1 - \frac{Absorbance\ (sample)}{Absorbance\ (control)}\right) \times 100\%$$

4.7 Chlorophyll Content Determination

The determination of chlorophyll content was done using method as mentioned by Sumanta et al. (2014) with slight modification. 0.5 g of fresh leaf samples were washed thoroughly with tap water followed by distilled water and dabbed to dry with tissue. The leaves were then homogenized using tissue homogenizer with 10 mL of methanol. The mixture later centrifuged at 10,000 rpm for 15 minutes at 4^0C. 0.5 mL of the supernatant was added to 4.5 mL of solvent. The solution was mixed thoroughly and read in the spectrophotometer at different absorbance. The absorbances that were used were 665 nm for chlorophyll-*a* and 652 nm for chlorophyll-*b*. The following equations were used to determine the content of chlorophylls:

Table 4.2: Equation used by Porra et al (1989) for chlorophyll determination.

Solvents	Equations (µg/mL)
Methanol	Chl-a = $16.29A_{665} - 8.54A_{652}$
	Chl-b = $30.66A_{652} - 13.58A_{665}$
	Total Chl = $22.12A_{652} + 2.71A_{665}$

4.8 Sugar Content

The determination of soluble sugar was done using phenol-sulfuric method as first described by Dubois et al. (1955) but further refined and tested by Chow & Landhausser (2004) in order to find a rapid but reliable method for determining sugar content in woody plant tissues. 200 mg of leaves were added into 5 mL of 80% ethanol in a test tube. The test tube was then placed in 95°C water baths, with the mouth of the test tube covered with a glass marble for 10 minutes. The solution was then topped up to make up to 5 mL and centrifuged at 2500 rpm for 5 minutes. 0.5 mL of the solution was added into 1 mL of 2% phenol solution; followed by rapid addition of 2.5 mL of concentrated sulfuric acid. The test tube was then incubated in dark for 30 minutes before being read in spectrophotometer at 490 nm by using glass cuvette.

Concentration of sugar was determined by comparing the absorbance against a pre-prepared 7-point calibration point standard curve that was using pure glucose as standard. The standard concentrations used were 2, 4, 6, 8, 10, 15, and 20 µg/ mL. The standard curve equation obtained for the glucose content was $y = 0.0627x$ ($R^2 = 0.9966$), where x represents the concentration of glucose in µg/ mL and y represents absorbance.

4.9 Antimicrobial Activities

The concept of microbial inhibition concentration (MIC) was mentioned by Patton et al. (2006) where MIC is the concentration where no growth is observed. MBC is defined as minimum bactericidal concentration, which is the lowest concentration of a compound required to kill a particular bacterium.

4.9.1 Disc Diffusion Assay

Method that was used for anti-microbial activity determination was as explained by Bauer et al. (1966) by using disc diffusion assay and broth dilution method as mentioned by Balouiri et al. (2016). Preparation of media followed the method as described by Bakht et al (2011). Mueller Hinton broths were prepared for culture activation and Mueller Hinton agar (MHA) for the culturing and growing of

microorganism of interest. The prepared nutrient agar undergo sterilization before being poured into sterile petri dishes and let cool for one hour. The plates were kept for one day to see any contamination appearing, and those without were used for innoculation.

Three species of Gram-positive bacteria and two species of Gram-negative bacteria were cultured and used for the disc diffusion testing, namely *Staphylococcus aureus* (ATCC 700699), *Streptococcus pyogenes* (ATCC 19615), *Enterococcus faecalis* (ATCC 700802), *Escherichia coli* (ATCC 10798), *and Klebsiella pneumoniae* (IMR 140808). Methanol was used for the preparation of *O. stamineus* stock (50 mg/ mL) in sterile condition. The prepared disks were allowed to dry completely, to remove all the methanol used during preparation stage. Innoculated plates will have six discs (6mm) on it in this antimicrobial testing. Three different volumes of stock solution were placed on the disc: 0.25, 0.50, and 1.00mg/mL. Antibiotic chloramphenicol commercial disc (30 µg) was used as positive control for both Gram-positive and Gram-negative, while methanol was used as negative control. Prepared plates were placed in the incubator for 24 hours at 37^0C before zone of inhibition was measured in millimeters.

4.9.2 Broth Dilution Method

This study also performed antimicrobial activity using broth dilution method as described by Chun-Hoong et al. (2010) with slight modification by Balouiri et al. (2016). Flat bottom 96-well plates were used in this method. Microorganisms of choice remain the same as determined above, and they were cultured in Mueller Hinton Broth (MHB) for 24 hours. After 24 hours, inoculums were adjusted to 0.5 McFarland using spectrophotometric method. Later, the inoculums were diluted by 100 times.

Preparation of extracted powder from *O. stamineus* was prepared to give a final concentration of 1mg/mL in 200 µL of MHB in the first well. 100 µL of MHB were added from well 2 until well 11, and 200 µL were added to well 11 to serve as positive control. Serial dilutions were conducted from well 1 where 100 µL were transferred to the next well until well 11 where the last 100 µL were discarded. 100 µL of the prepared inoculum were added to each of the well. The plates were then incubated at 37°C for 24 hours and MIC values were determined by determining the concentration where the growth of bacteria were not observed.

The following day, the MBC values were determined by using spot growth test on MHA by placing a 2 µL of the solution from the well where MIC value was identified onto a plate to determine growth. The plates were then incubated for another 24 hours. The MBC were determined by determining the last spot that showed growth.

4.10 HPLC Analysis of Plant Extracts

The phenolics concentration in *O. stamineus* extracts from plants grown under different lighting conditions were determined by using reverse-phase high performance liquid chromatography (HPLC). Dried extract was used in the quantitative HPLC analysis of phenolic compound. Several standards were chosen to determine the changes in concentration of the phenolics under different lighting conditions. Based on the study on *O. stamineus* done by Muhammad et al. (2011), the standards used were: rosmarinic acid, caffeic acid, sinensetin and eupatorin. The standards were used to determine the retention time (R_T) of each compound of interest and was also used to prepare standard curves for concentration determination. The solvents that were used were ultrapure water as Solvent A and HPLC grade acetonitrile as Solvent B. Both solvents were added with 0.05% trifluoroacetic acid in order to create slightly acidic condition as it will improve resolution of peaks. This effect was mentioned in numerous studies, such as demonstrated by Li et al. (2010) in their study of addition of mobile phase additives to the separation of *Herba Artemisiae Scopariae*. Detector was set to 320 nm; on-board thermostat was set to 30°C and post-run was set to 6 minutes.

Method used was based on Muhammad et al. (2011), with slight modification to increase sensitivity and achieve proper separation of peaks. The method was further validated using commercial standards. Table 4.3 lists the gradient condition that was used:

Table 4.3: Gradient condition of the HPLC method.

Time (min)	Solvent A (%)	Solvent B (%)
0.00	70	30
2.00	70	30
10.00	50	50
20.00	5	95
22.00	5	95
24.00	70	30

*Solvent A= water; Solvent B = acetonitrile

4.11 Statistical Analysis

Analysis of comparison for the various parameters among the different lighting conditions was done using IBM SPSS Statistics 24 software (IBM Corporation) with one-way ANOVA and Tukey HSD post-hoc test analysis. Pearson correlation was used to check the correlation between the parameters with $r = 0$ indicating no correlation, while $r = 1$ and $r = -1$ represent strong positive and negative correlation respectively. The P value of <0.05 was considered statistically significant. All the data were presented as mean ± standard deviation where applicable.

Chapter 5

Results

5.1 Growth of *O. stamineus* under different lighting conditions

Upon continuous daily exposure of the plants under different light conditions, difference in term of height and number of leaves can be observed among the lighting conditions. Plants under condition 35B40R significantly demonstrated the tallest values from week seven onwards until reaching week 11, followed by treatment 15B60R, with values lower than the previous condition but similar at week 11. Plants exposed to the rest of the lighting conditions exhibited similar shorter stature with the lowest value displayed under the condition of SRT, followed by FLUO. The height's growing trend for all the conditions can be observed in Figure 5.1.

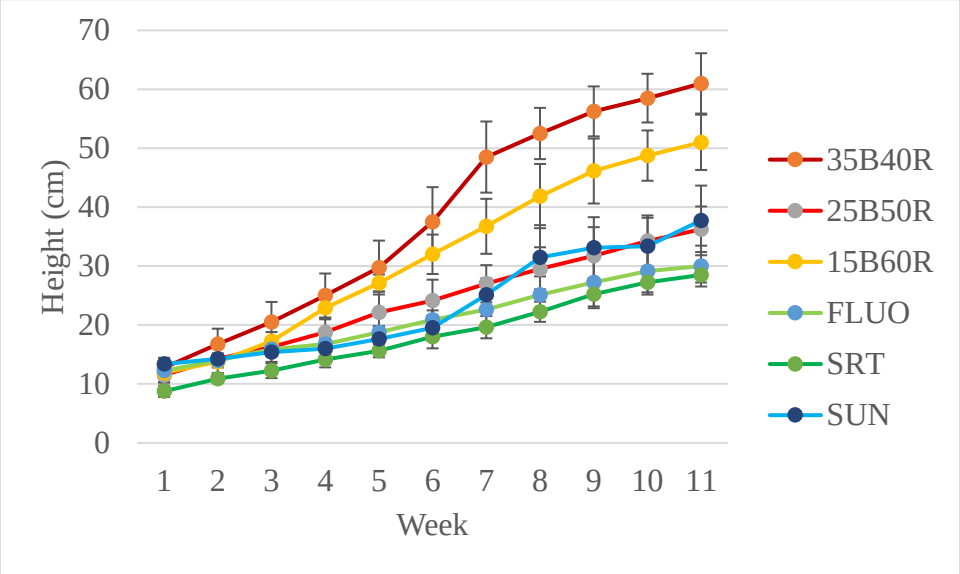

Figure 5.1 Weekly average of plant's height for each condition (bars represent standard deviation, n= 6).

Another parameter that we used to determine growth of *O. stamineus* was the number of leaves. From Figure 5.2, at the end of week 11, we observed that condition SRT had the highest number of leaves, followed SUN while condition FLUO had the lowest number of leaves. Morphologically, it was evident that plants placed under the shaded condition of the rooftop were lower in height but had more leaves compared to plants under other conditions.

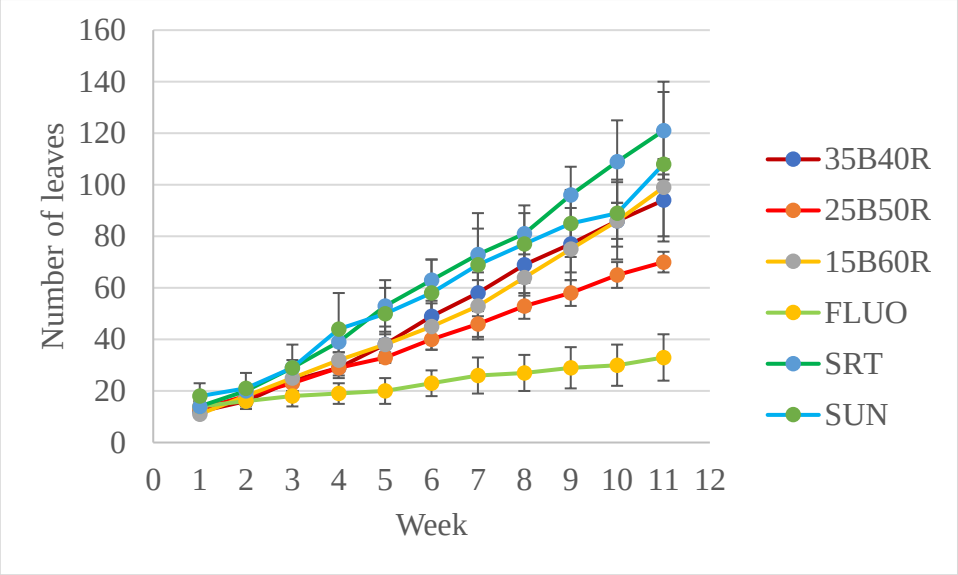

Figure 5.2 Weekly average of number of leaves for each condition (bars represent standard deviation, n = 6).

Figure 5.3 Pictures taken prior to treatment (left), week 2 (middle) and at the end of week 11 (right).

5.2 Solvent Selection and Extraction Efficiency

Table 5.1 shows that 80% methanol has the significantly highest total phenolic content extracted with 819.1 ± 9.4 mg GAE/100g fresh leaves, followed by pure methanol, pure ethanol, 60% methanol, and 80% ethanol with readings of 772.9 ± 22.7, 725.8 ± 19.6, 692.3 ± 15.2, and 673.3 ± 27.5 mg GAE/100g fresh leaves, respectively.

In addition, extraction efficiency tests were also conducted to determine the percentage of extraction collected in every round of extraction. The test was performed only for the first three rounds, as for the subsequent extraction the TPC was too little that it can be considered as negligible. Based on the test, 80% methanol also recorded the highest percentage of phenolic content extracted during the first round (83.4%), followed by 60% methanol, pure methanol, pure ethanol, and 80% ethanol with readings of 82.2%, 81.2%, 80.7%, and 75.8% respectively.

In all the TPC determinations in the subsequent work, 80% methanol was therefore chosen as the extraction solvent.

Table 5.1 Total phenolic content (TPC) and percentage extraction of *O. stamineus* using different solvents.

Solvent	TPC (mg GAE/ 100g fresh leaves)			Percentage extraction (%)		
	1st extraction	2nd extraction	3rd extraction	1st extraction	2nd extraction	3rd extraction
80% Ethanol	673.3 ± 27.5[e]	135.6 ± 5.2	79.5 ± 3.4	75.8[b]	15.3	8.9
100% Ethanol	725.8 ± 19.6[c]	124.9 ± 23.8	48.4 ± 8.6	80.7[a]	13.9	5.4
60% Methanol	692.3 ± 15.2[d]	117.2 ± 12.3	32.8 ± 5.2	82.2[a]	13.9	3.9
80% Methanol	819.1 ± 9.4[a]	114.9 ± 7.4	47.8 ± 1.7	83.4[a]	11.7	4.9
100% Methanol	772.9 ± 22.7[b]	120.0 ± 7.6	58.9 ± 4.2	81.2[a]	12.6	6.2

*The values are presented as mean of three replicates ± standard deviation.
**The values followed by the same superscripted letter are not statistically significant ($P<0.05$).

5.3 Total Phenolic Content of *O. stamineus* Under Different Lighting Conditions

In this study, the highest level of TPC was presented by 25B50R (2725 ± 163 mg GAE/ 100g fresh leaves), which was significantly higher compared to other plants subjected to other lighting conditions. This result was followed by 35B40R, 15B60R, SUN, SRT, and FLUO, with TPC recorded at 2019 ± 97, 1919 ± 148, 1663 ± 176, 1433 ± 80, and 943 ± 70 mg GAE/ 100g fresh leaves, respectively.

Total phenolic content was significantly increased as the percentage of blue light decreases, and red light increases as can be seen in Table 5.2. However, this gain was reversed when as the percentage of blue light further decreases, and red light increases. Meanwhile, the phenolic content for all three test groups was significantly higher compared to the three control groups: FLUO, SRT and SUN. Among the control groups, SUN was the highest and FLUO had the lowest phenolic content.

Table 5.2 Total phenolic content of *O. stamineus* after 11 weeks of growing phase.

Condition	TPC (mg GAE/ 100 g fresh leaves)	Adjusted TPC (to 100%) (mg GAE/ 100 g fresh leaves)
35B40R	1684 ± 81[b]	2019 ± 97
25B50R	2273 ± 136[a]	2725 ± 163
15B60R	1601 ± 123[bc]	1919 ± 148
FLUO	790 ± 82[e]	943 ± 70
SRT	1195 ± 66[d]	1433 ± 80
SUN	1387 ± 146[cd]	1663 ± 176

*The values are presented as mean of three replicates ± standard deviation. Adjusted TPC (to 100%) is the adjusted TPC calculated based on the extraction efficiency of the first extraction (i.e. sum of three successive extractions)
**Values followed by the same superscripted letter are not statistically significant ($p < 0.05$)

5.4 Total Phenolic Content of Mature *O. stamineus* Under Different Lighting Conditions

Additionally, a separate batch of mature (15-week old) plants were subjected to five of the lighting conditions for a duration of four weeks. In total, 15 plants were used (three plants per each condition) for this additional analysis. The aim for this test was to observe if the changes that were observed in the plants subjected to lighting conditions from young also can be replicated in adult plants in shorter period of time. The results for this test can be observed in Table 5.3.

Table 5.3 Total phenolic content of mature *O. stamineus* after four weeks under the lighting conditions.

Condition	TPC (mg GAE/ 100 g fresh leaves)	Adjusted TPC (to 100%) (mg GAE/ 100 g fresh leaves)
35B40R	1263 ± 10[b]	1578 ± 13
25B50R	1577 ± 70[a]	1972 ± 89
15B60R	1121 ± 8[c]	1401 ± 11
FLUO	585 ± 40[e]	731 ± 50
SUN	783 ± 30[d]	978 ± 38

*The values are presented as mean of three replicates ± standard deviation. Adjusted TPC (to 100%) is the adjusted TPC calculated based on the extraction efficiency of the first extraction (i.e. sum of three successive extractions)
**Values followed by the same superscripted letter are not statistically significant ($p < 0.05$)

After four weeks exposed under the lighting conditions, 25B50R was found to have the highest TPC compared to the rest. 35B40R and 15B60R were not different from each other; however, they are lower than 25B50R. Meanwhile, FLUO recorded the second lowest TPC amongst the group and our control group, SUN, had the significantly lowest compared to other light treatments.

5.5 Antioxidant activity of *O. stamineus*

Table 5.4 shows the IC_{50} and AEAC values for *O. stamineus* at the end of growing period for all lighting conditions. The highest scavenging activity was significantly demonstrated by condition 25B50R, with IC_{50} value of 0.173 ± 0.003 mg/ mL. This reading was followed by condition 35B40R, 15B60R, SUN, SRT, and FLUO; with readings of 0.228 ± 0.010, 0.251 ± 0.004, 0.296 ± 0.011, 0.354 ± 0.006, and 0.461 ± 0.005 mg/ mL respectively at varying significance levels.

Table 5.4 Antioxidant activity of *O. stamineus* at the end of growing phase.

Condition	**IC50 (mg/ mL)**	**AEAC (mg ascorbic acid/ 100g fresh sample)**
35B40R	0.228 ± 0.010^{d}	1713 ± 71^{d}
25B50R	0.173 ± 0.003^{e}	2252 ± 34^{e}
15B60R	0.251 ± 0.004^{cd}	1548 ± 26^{cd}
FLUO	0.461 ± 0.005^{a}	843 ± 19^{a}
SRT	0.354 ± 0.006^{b}	1144 ± 18^{b}
SUN	0.296 ± 0.011^{c}	1317 ± 50^{c}

*The values are presented as mean of three replicates ± standard deviation.
**Values in each column followed by the same superscripted letter are not statistically significant ($p < 0.05$)

The IC_{50} gradually decreased as the percentage of blue light (35%) decreases and red light increases, suggesting increasing potency of the extract to inhibit 50% of the radicals. Similar to phenolic content, this effect however reversed as the percentage of blue light (25%) further decreases and red light increases. Phenolic content and activity were observed to be highly correlated as elaborated in subchapter 5.11, Correlation Study.

5.6 Ferrous Iron Chelating Activity

Figure 5.4 shows that the plants under 25B50R exhibited the highest chelating capability compared to those under different lighting conditions although the value was not significantly different to those of 35B40R and 15B60R. Meanwhile, FLUO was found to have the significantly lowest chelating capability; followed by plants under SUN and SRT.

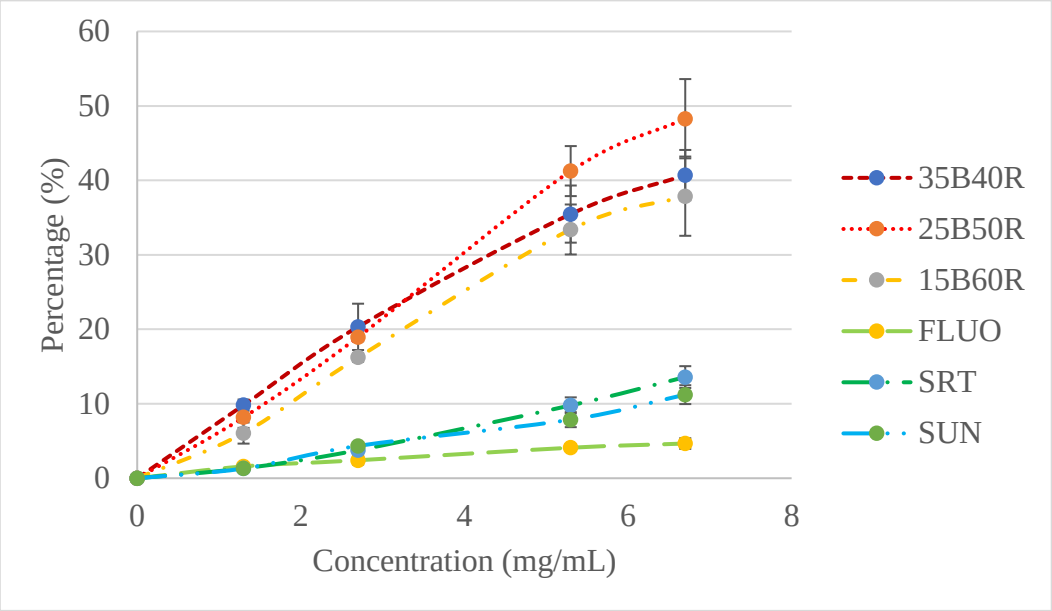

Figure 5.4 Ferrous iron chelating activity of *O. stamineus* (bars represent standard deviation, n= 3).

This result, combined with scavenging activity shows that 25B50R lighting condition not only enhances the primary antioxidant activity, but also the secondary antioxidant activity. All three of lighting conditions used in this study have significantly higher chelating activity compared to the control groups. However, the chelating activity was too low that the CC_{50} was not determined in all lighting groups and controls.

5.7 Chlorophyll Content

The highest content of chlorophyll a was reported by *O. stamineus* grown in SRT condition (2.964 ± 0.040 µg/ mL); followed by FLUO (2.606 ± 0.037 µg/ mL), SUN (2.490 ± 0.060 µg/ mL), 15B60R (2.444 ± 0.098 µg/ mL), 35B40R (2.299 ± 0.219 µg/ mL), and 25B50R (1.577 ± 0.105 µg/ mL). Meanwhile, the content of chlorophyll b was reportedly high in condition FLUO, SRT, SUN and 15B60R, with readings of 1.074 ± 0.023, 0.982 ± 0.050, 0.921 ± 0.016 and 0.892 ± 0.062 µg/ mL, respectively. However, these readings were not significantly

different amongst them as can be observed in Table 5.5. The lowest chlorophyll b content was recorded by condition 35B40R (0.126 ± 0.023 µg/ mL) and followed by 25B50R (0.149 ± 0.041 µg/ mL). Similar to the highest values, these lowest values were also not significantly different between them. As for the total chlorophyll content, the rank from highest to lowest is like chlorophyll a content: SRT, FLUO, SUN, 15B60R, 35B40R, and 25B50R. SRT's total chlorophyll was the highest at 3.946 ± 0.099 µg/ mL, and the lowest was 25B50R at 1.726 ± 0.061 µg/ mL. (Note: 1 µg/ mL is equivalent to 20 µg/ g of fresh leaf).

Table 5.5 Chlorophyll content of *O. stamineus* after 11 weeks of growing phase.

Condition	Chlorophyll (µg/ mL)			Chl a/b ratio
	Chl-a	Chl-b	Total Chl	
35B40R	2.299 ± 0.219^{d}	0.126 ± 0.023^{c}	2.425 ± 0.070^{d}	18.25
25B50R	1.577 ± 0.105^{e}	0.149 ± 0.041^{c}	1.726 ± 0.061^{e}	10.58
15B60R	2.444 ± 0.098^{bd}	0.892 ± 0.062^{b}	3.336 ± 0.063^{c}	2.740
FLUO	2.606 ± 0.037^{bc}	1.074 ± 0.023^{a}	3.669 ± 0.072^{b}	2.426
SRT	2.964 ± 0.040^{a}	0.982 ± 0.050^{ab}	3.946 ± 0.099^{a}	3.018
SUN	2.490 ± 0.060^{bcd}	0.921 ± 0.016^{ab}	3.411 ± 0.025^{c}	2.704

*The values are presented as mean of three replicates ± standard deviation.
**Values followed by the same superscripted letter in the same column are not statistically significant ($p < 0.05$)

Initially, total chlorophyll was reduced as the blue light decreases and red light increases. However, this reduction was reversed as blue light further decreases and red light increases. Overall, the control groups were higher compared to LED lights treated groups. However, chlorophyll a/b ratio was observed to be reduced as the percentage of blue light decreases and red light increases.

5.8 Sugar Content

Table 5.6 shows that the highest sugar content was reported by SRT (5.913 ± 0.387 mg/ g), followed by FLUO (4.311 ± 0.296 mg/ g), 35B40R (3.937 ± 0.419 mg/ g), SUN (3.932 ± 0.418 mg/ g), 15B60R (3.403 ± 0.203 mg/ g), and 25B50R (2.094 ± 0.385 mg/ g). This result was quite similar to the chlorophyll content: 25B50R was significantly lowest amongst all the group while SRT was significantly the highest. Control groups were observed to be higher compared to LED treated group, except 35B40R.

Table 5.6 Sugar content of *O. stamineus* at the end of growing phase.

Condition	Sugar (mg/ g fresh sample)
35B40R	3.937 ± 0.419^{b}
25B50R	2.094 ± 0.385^{e}
15B60R	3.403 ± 0.203^{d}
FLUO	4.311 ± 0.296^{bc}
SRT	5.913 ± 0.387^{a}
SUN	3.932 ± 0.418^{bc}

*The values are presented as mean of three replicates ± standard deviation.
**Values followed by the same superscripted letter are not statistically significant ($p < 0.05$)

5.9 High Performance Liquid Chromatography (HPLC)

Pure standards (Sigma Aldrich) were used to determine the retention time for each compound. These standards were prepared in 80% methanol and run using the same method as samples in order to determine the retention time. Based on the standard's chromatogram, the retention time for caffeic acid, eupatorin, rosmarinic acid, and sinensetin were found to be 5.028, 17.297, 7.304, and 16.773 minute, respectively. The concentration of each respective compounds is summarized in Table 5.7.

Table 5.7 Rosmarinic acid, caffeic acid, eupatorin, and sinensetin content.

	Concentration (mg/ g leaves)			
	Caffeic Acid	**Ros. Acid**	**Eupatorin**	**Sinensetin**
35B40R	0.435 ± 0.010^{d}	4.108 ± 0.066^{b}	ND	0.010 ± 0.001^{e}
25B50R	0.511 ± 0.010^{b}	8.739 ± 0.098^{a}	ND	ND
15B60R	0.569 ± 0.006^{a}	3.646 ± 0.034^{c}	$0.009 \pm 0.001^{a\backslash b}$	0.069 ± 0.007^{b}
FLUO	0.463 ± 0.001^{cd}	1.295 ± 0.002^{e}	0.021 ± 0.001^{a}	0.104 ± 0.001^{a}
SRT	0.491 ± 0.002^{bc}	2.786 ± 0.004^{d}	0.006 ± 0.001^{c}	0.056 ± 0.001^{c}
SUN	0.555 ± 0.023^{a}	2.856 ± 0.038^{d}	0.009 ± 0.001^{b}	0.031 ± 0.001^{d}

*The values are presented as mean of three replicates ± standard deviation.
**Values followed by the same superscripted letter in the same column are not statistically significant ($p < 0.05$)
***ND: Not Detectable

Rosmarinic acid in 25B50R was found to be the highest (8.739 ± 0.098 mg/ g), double that of the amounts found in 35B40R (4.108 ± 0.066 mg/ g), 15B60R (3.646 ± 0.034 mg/ g) and the control groups. The lowest amount was reported by FLUO (1.295 ± 0.002 mg/ g), followed by SRT (2.786 ± 0.004 mg/ g) and SUN (2.856 ± 0.038 mg/ g). However, the same trend was not observed in caffeic acid content. 15B60R was found to have the highest amount of caffeic acid followed by SUN, 25B50, SRT, FLUO, and 35B40R with readings of 0.569 ± 0.006 mg/g, 0.555 ± 0.023 mg/ g, 0.511 ± 0.010 mg/ g, 0.491 ± 0.002 mg/ g, 0.463 ± 0.001 mg/ g, and 0.435 ± 0.010 mg/ g respectively.

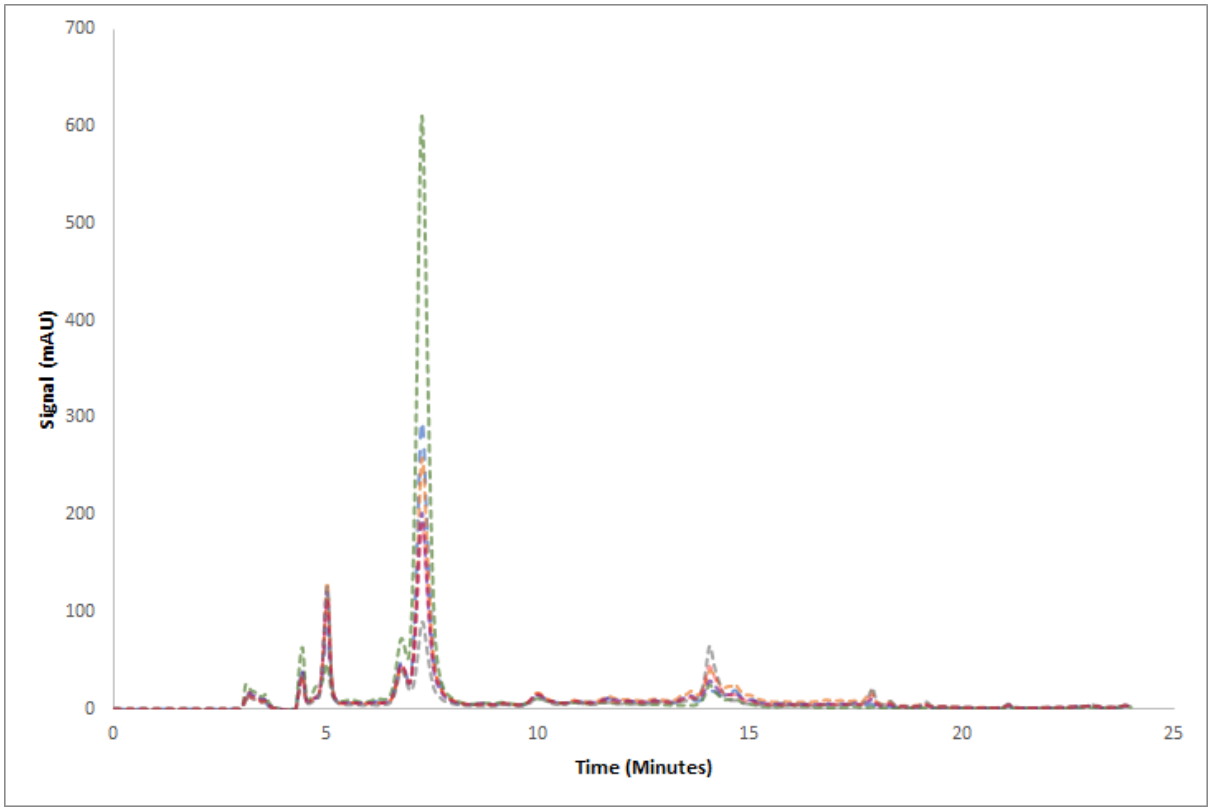

Figure 5.5 Overlapped HPLC's chromatogram for all conditions
(Note: 35B40R: blue, 25B50R: green, 15B60R: brown, FLUO: grey, SRT: red, SUN: purple)

There were no clear trends observed in the concentration of caffeic acid. However, rosmarinic acid's trend was similar to the one observed in phenolic content. Initially, rosmarinic acid was greatly increased as the percentage of blue light decreases to 25% and red light increases, but the trend was reversed when blue light decreases to 15%. There was no clear trend observed for both eupatorine and sinensetin. However, condition FLUO was found to have the highest significant amount of both of these compounds compared to other LED

treated groups and controls. The overlapped HPLC's chromatogram of all the lighting conditions is shown in Figure 5.5.

5.10 Antimicrobial activity

As illustrated in Table 5.8, the extracts had no zone of inhibition for both Gram-negative microorganisms, *E. coli* and *K. pneumoniae*.

Table 5.8 Inhibition zone for antimicrobial activity test.

Tested strain	**Zone of inhibition (mm)**						
	35B40R	**25B50R**	**15B60R**	**FLUO**	**SRT**	**SUN**	**Control**
Escherichia coli (ATCC 10798)	0 -	0 -	0 -	0 -	0 -	0 -	24.0 ± 1.2 +++
Klebsiella pneumoniae (IMR 140808)	0 -	0 -	0 -	0 -	0 -	0 -	8.0 ± 0.2 ++
Staphylococcus aureus (ATCC 700699)	11.8 ± 0.2 +++	13.0 ± 0.4 +++	12.3 ± 0.1 +++	8.1 ± 0.2 ++	10.0 ± 0.2 ++	11.5 ± 0.3 +++	31.0 ± 1.3 +++
Streptococcus pyogenes (ATCC 19615)	9.2 ± 0.1 ++	10.4 ± 0.2 +++	8.3 ± 0.2 ++	6.5 ± 0.1 +	8.3 ± 0.3 ++	9.8 ± 0.2 ++	15.1 ± 0.7
Enterococcus faecalis (ATCC 700802)	0 -	0 -	0 -	0 -	0 -	0 -	30.0 ± 0.9 +++

Key: (-): no inhibition; (+): weak inhibition (<8mm); (++): modest inhibition (8 mm ≤ x ≤ 10 mm); (+++): strong inhibition (> 10 mm); all readings were inclusive of 6mm disc diameter.

However, a more significant antimicrobial activity can be observed in Gram-positive microorganisms selected. Both *S. aureus* and *S. pyogenes* had inhibition zone, ranging from modest to strong inhibition. Bigger zones of inhibition were also observed in *S. pyogenes*, where 25B50R (10.4 ± 0.2 mm) had the biggest zone of inhibition and the only one with strong inhibition (> 10 mm); while FLUO had the smallest zone with 6.5 ± 0.1 mm and the only one

with weak inhibition (< 8 mm). Similar observation was reported in *S. aureus*, with the biggest zone observed in 25B50R (13.0 ± 0.4 mm) while the smallest was observed in FLUO (8.1 ± 0.2 mm). Control used was chloramphenicol (30 µg/ disc).

Table 5.9 Minimum inhibitory concentration for antimicrobial activity test.

Tested strains	**Minimum Inhibition Concentration (mg/ mL)**					
	35B40R	**25B50R**	**15B60R**	**FLUO**	**SRT**	**SUN**
Escherichia coli	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000
Klebsiella pneumoniae	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000
Staphylococcus aureus	0.125	0.0625	0.125	0.500	0.250	0.250
Streptococcus pyogenes	0.500	0.125	0.500	0.250	0.250	0.250
Enterococcus faecalis	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000	> 1.000

Table 5.10 Minimum bactericidal concentration for antimicrobial activity test.

Tested strains	**Minimum Bactericidal Concentration (mg/ mL)**					
	35B40R	**25B50R**	**15B60R**	**FLUO**	**SRT**	**SUN**
Staphylococcus aureus	0.125	0.125	0.250	1.000	0.500	1.000
Streptococcus pyogenes	1.000	0.125	1.000	0.250	0.500	0.500

These observations were further clarified by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) test. The results for these two tests are summarized in Table 5.9 and Table 5.10. The MIC and MBC values for 25B50R was observed to be the lowest for both *S. aureus* and *S. pyogenes*.

5.11 Correlation studies

Correlation test between TPC and IC_{50} has showed that they are inversely correlated with coefficient of correlation value (R) at -0.9658 (Figure 5.6). A highly positively correlation was also seen between TPC and AEAC with coefficient of correlation (R) value of 0.9952 (Figure 5.7). The high amount of rosmarinic acid in 25B50R was consistent with the high antioxidant content and activity. Correlation test showed that they are highly correlated at R=0.9482 (Figure 5.8). However, the caffeic acid content showed no correlation to the total phenolic content at R=0.043. The strong correlations among TPC, IC_{50}, AEAC, and rosmarinic acid further proved that not only rosmarinic acid is the major constituent of phenolic compound contained in *O. stamineus* and directly affected by the growing conditions the plants subjected to, but they also play a major role in providing scavenging capabilities in *O. stamineus*. This however does not discredit the fact that the other phenolic compounds are also involved in scavenging activity.

Another correlation tested was between TPC and chlorophyll content (Figure 5.9). Interestingly, they were inversely correlated with R=-0.8993, meaning that as the chlorophyll content reduced, the TPC content increased. This is in line with the fact that the secondary metabolites increase as the plant is under stress, in this case the diminishing number of chlorophylls. This study also tested the correlation between TPC and sugar content. TPC was found to be negatively correlated with sugar content. However, the correlation is the weakest amongst all the parameters that we tested, with coefficient of correlation (R) only at -0.7545 (Figure 5.10). Another observation is that a good correlation exists between sugar content and total chlorophyll (Figure 5.11).

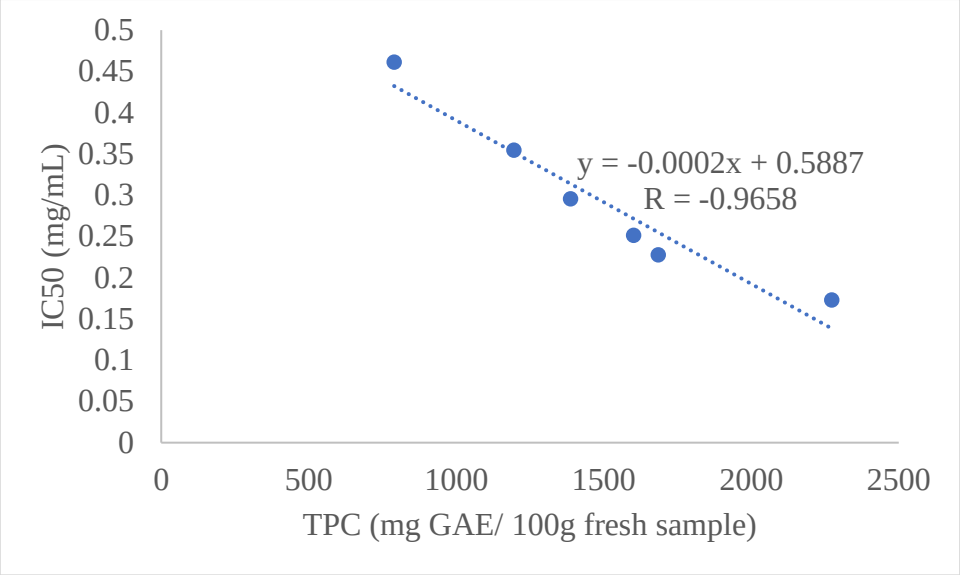

Figure 5.6 Correlation between TPC and IC_{50}.

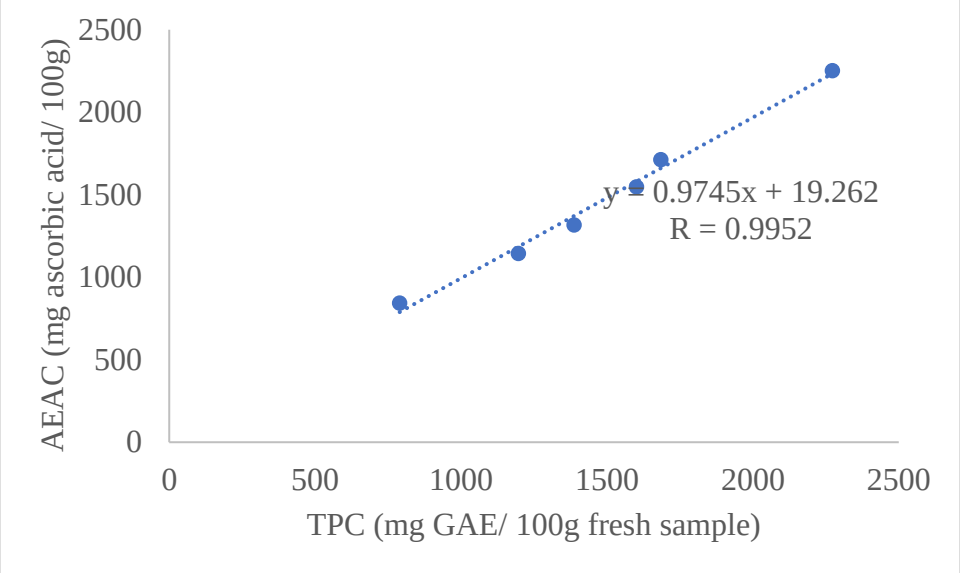

Figure 5.7 Correlation between TPC and AEAC.

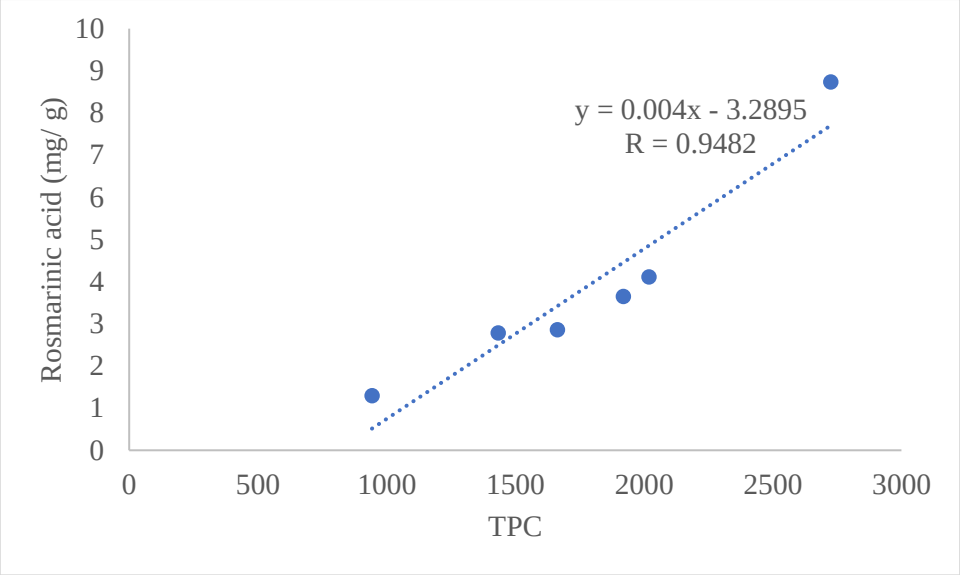

Figure 5.8 Correlation between total phenolic content and rosmarinic acid content (TPC is adjusted to 100%).

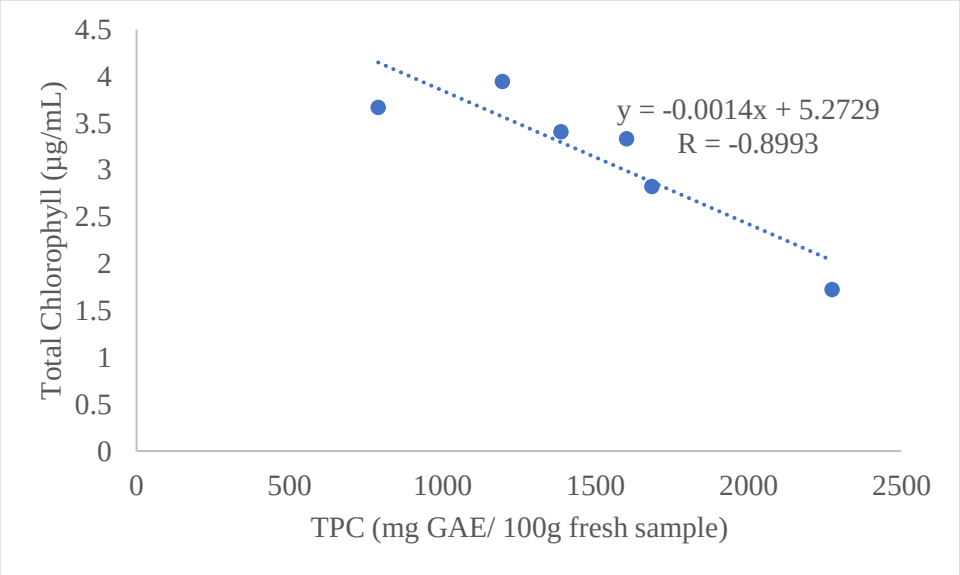

Figure 5.9 Correlation between TPC and total chlorophyll.

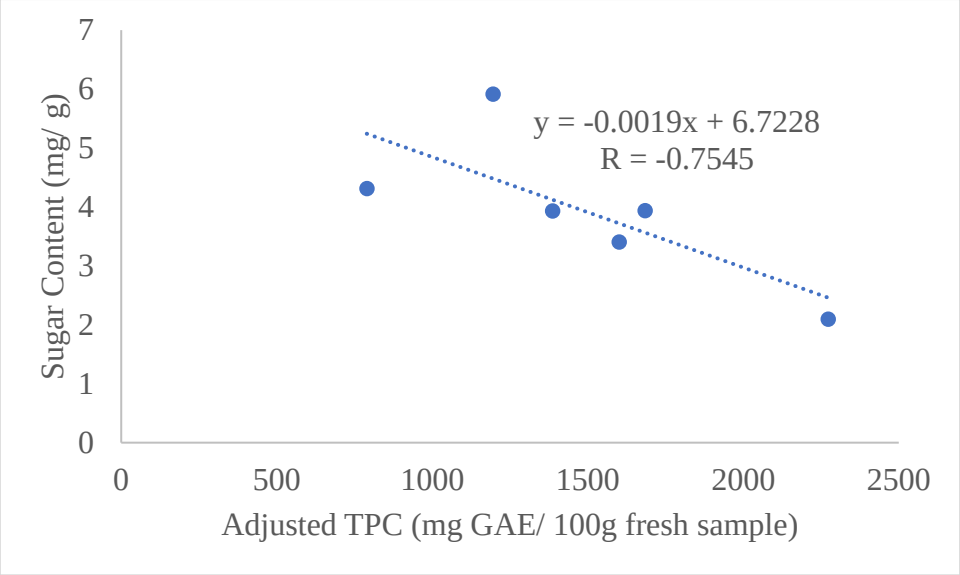

Figure 5.10 Correlation between TPC and sugar content

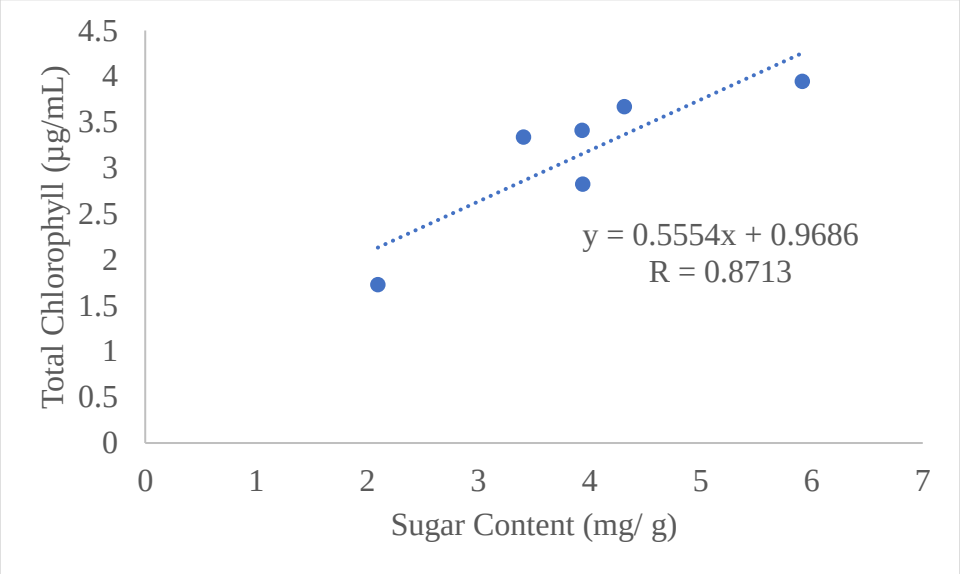

Figure 5.11 Correlation between sugar content and total chlorophyll

Chapter 6
Discussions

6.1 Solvent Selection and Extraction Efficiency

Over the years, numerous solvents have been used by researchers to extract the phytochemicals and antioxidants from all parts of plants such as shoots, leaves, roots, stems, flowers, and fruits. Among the most used polar solvents are dimethylformamide (He et al. 2017), methanol (Akowuah et al., 2004; Chew et al., 2009; Ho et al., 2010; Chong & Lim, 2012, Fiutak et al., 2019), ethanol (Ibrahim & Jaafar, 2012; Ibrahim et al., 2013; Ibrahim et al., 2017; Olatunde et al., 2018), acetone (Lobiuc et al., 2017), and water (Abdullah et al., 2012).

In addition, the extracted compounds vary greatly depending on the type of solvents chosen and the concentration used. In a study conducted by Ho et al. (2010) on the extraction procedure of *Orthosiphon stamineus* using different concentration of methanol (0%, 25%, 50%, 75%, 100%), they found out that 50% methanol was more effective in recovering rosmarinic acid compared to the other concentration. In a similar experiment using 50% and 100% methanol to extract phenolic content, Chong & Lim (2012) found out that 100% methanol was able to extract more phenolic content from *Vitex negundo* and *Vitex trifolia* compared to 50% methanol, thus it was chosen as the appropriate solvent for subsequent extraction processes in their study.

In the present study, the highest phenolic content recovered was by using 80% methanol during the first hour's extraction. The same solvent also recorded the highest percentage of extraction efficiency compared to the other solvents of varying concentrations. Thus, 80% methanol was chosen as the extraction solvent in *O. stamineus* in this study.

6.2 Growth of *O. stamineus* under different lighting conditions

Plant's growth mostly occurred in plants under lowest blue light treatment, as the shade-avoidance responses occurred, which may be due to under-stimulated cytochrome and will contribute to lower leaf area index and growth in general (Snowden 2015). However, in our study we found that 35B40R and 15B60R which has the highest and the lowest blue light, had maximum height compared to other light treatment. Meanwhile, SRT which was found to be the shortest in term of height has the greatest number of leaves compared to other groups.

Riikonen et al. (2016) found that Norway spruce grown under high pressure sodium vapor (HPS) lamp were taller compared to plants grown under various percentages of far-red,

red, green, and blue light. Similarly, they also reported that Scots pine grown under HPS was the tallest, but it was closely followed and not significantly different by 25B/F_{FR} treatment group (25% blue and various percentages of other light spectrums). This is an interesting observation as both HPS and 25B/F_{FR} have nothing in common. HPS had only 6% of blue light and over 40% for both green/yellow and red light; while 25B/F_{FR} had 25% blue, 1% of green/yellow, and a whopping 70% of red light. This could suggest that not only one type of light can induce the stems to elongate, but other combination of lights too.

There has been a lot of previous attempts by other researchers trying to relate the morphogenesis and growth of plants to red and blue light treatment. In recent times, some research also mixed green/yellow and far-red light after several findings reported the importance of these spectra too. The red and the blue light seem to be affecting plants differently. The elongation of stems was at a maximum for plants under red light treatment for both Chrysanthemum (Kim et al., 2004) and grape (Puspa et al., 2008). However, Heo et al. (2002) reported that marigold had the maximum stem elongation under monochromatic blue light, while Jao et al. (2005) reported that blue LED enhanced the height of *Zantedeschia jucunda*. Meanwhile, there were reports of the inhibitory effect of lights as well. For instance, *Rehmannia glutinosa* was found to have its stem elongation inhibited by red light (Hahn et al. 2000) while blue light was able to reverse the leaf growth promoted by red light for *Cymbidium sp* (Tanaka et al., 1998).

The varying results of plant's growth between species under different lighting conditions may be explained by the report of synergistic effect between the cytochrome and phytochrome that may account for variation of results seen in various species (Kim et al. 2004).

The present study found that the decreasing blue light reduced the height of the plants. These reductions however were reversed as the blue light further reduced. However, as we were also using different percentage of red light, this effect cannot be attributed to blue light alone. There is possibility that red light also contributed to stimulating the stem elongation as the blue light decreases as mentioned by Kim et al. (2004) and Puspa et al. (2008). A further in-depth study of the effect of red and blue light separately is needed in order to confirm this hypothesis.

6.3 Antioxidant content and activity

The quality of lights has been long discussed among the researchers that it is one of the regulators of the metabolism in higher plants, including the production of antioxidants. Phenolic compounds, contained mostly in fruits, vegetables and herbal plants, are vital in

providing unique taste, flavour, and a lot of health benefits (Tomas-Barberan & Espin, 2001). There are a lot of different types of phenolic compounds from various species of plants; but they are mostly identified by the presence of hydroxylated aromatic rings (Mandal et al., 2010). Phenolic compounds are important in regulating the growth of the plants, and mostly produced as a response towards environmental stressors such as light, temperature, ambient, and many more (Valentine et al., 2003). In present study, 25B50R was found to have the highest total phenolic content, the lowest IC50 concentration, and the highest chelation capabilities compared to the other conditions. Additionally, we also found that similar trend of TPC values between the groups can be observed by exposing adult plants under the lighting conditions for 4 weeks. The TPC between the lighting conditions were significantly different; and the similar trend to the one planted from young plants can be observed here too: TPC increased as the percentage of blue light increases but reduced as the percentage of blue light increased over 25%. By examining this result, we can conclude that the changes in the phenolic content in *O. stamineus* treated under different lighting conditions were apparent even after 4 weeks, despite the readings are lower than the one exposed for 11 weeks. We can also conclude that the effect can be seen in adult plants as well as the young plants.

Bantis et al. (2016) reported that the highest TPC of *Ocimum basilicum* was found in condition with relatively lower blue light (20%) and higher red light (35%) compared to control condition (fluorescence light). They also found that TPC under this condition was 3.7 times higher compared to control, while our current study was close to 3 times higher compared to fluorescence light. Other similar studies conducted on different species such as lettuce (Stutte et al., 2009, Li & Kubota., 2009, Samuoliene et al., 2012), kale (Lefsrud et al., 2008), mustard (Tarakanov et al., 2012) and cabbage (Mizuno et al., 2011) have showed that plants produce more phenolic content in red light condition. However, there has been findings by other researchers that suggest the increasing phenolic content is condition and species dependent. Liu et al. (2016) for instance, found that pea sprouts grown under blue light had the highest total phenolic and flavonoid content compared to those grown under the red light. Interestingly, Lobiuc et al. (2017) reported contrasting result of total phenolic content for 2 different cultivars of basil: the green cultivar was reportedly having higher content under 2R:1B treatment (more red light), while the red cultivar was the highest under 1R:1B and 1R:2B. They concluded that the green cultivar was most stimulated by higher ratios of red light in phenolic synthesis, while the red cultivar, by the higher ratios of blue light. They hypothesized that the differences between the cultivars might be explained by the different regulatory mechanism of PAL in red and green tissues.

Similar to the total phenolic content, we hypothesized that the percentage of red light is also playing an important role in the production of phenolic compound, thus affecting the antioxidant activity as well. Manivannan et al. (2015) reported that the scavenging activity of plants under red lights was lower than blue lights but was not significantly different. The effect of lighting towards radical scavenging capability of plants seems to be species dependent. In another study conducted by Lee & Hyun (2010), they found that barley grown in dark condition has the highest scavenging activity followed by blue light. This is a good indicator that different plants react differently towards different lighting conditions. However, our research also found that as the increasing red light and decreasing blue light seems to be accompanied by an increase in phenolic production, the effect was reversed as the intensity of red light further increased and blue light decreased.

As discussed above, different plants reacted differently towards light treatments. Bian et al. (2015) in their review paper discussed about the effect of lights on carotenoids, phenolic compounds, and anthocyanins. They concluded that UV light is more effective in maximizing the phenolic content in plants; mainly because the phenolic compounds have higher absorption towards UV radiation, thus plants produced more phenolic compounds to protect itself from photodamage. Ghasemzadeh & Ghasemzadeh (2011) discussed the possible pathway that is involved in the accumulation of phenolic compounds. They theorized that the production of phenolic compounds is regulated by gene controlling phenylalanine ammonia-lyase, a catalyst that is involved in the shikimic pathway by converting phenylalanine to phenolic compounds. By using examples such as demonstrated by Hisaninato et al. (2001) and Keski-Saari (2005), they concluded that the phenolic contents were significantly reduced by inhibiting the PAL activity by using 2-aminoindan-2-phosphonic acid (AIP); thus, suggesting the role of PAL in plants under stress.

6.4 Chlorophyll content

Cope et al. (2014) hypothesized that based on previous studies, at higher proportion of blue light, the photoreceptor activity should be suppressed, thus chlorophyll production would also be reduced. However, the findings of their research were contrary to that hypothesis: the chlorophyll content was the highest at 92% blue light compared to 0.3%. Similarly, Fan et al. (2013) found that the chlorophyll content in Chinese cabbage was higher for those under the red and white light, where the chlorophyll precursor was significantly higher in 100% blue light compared to 0%.

In the present study, we found that the total chlorophyll content reduced as the percentage of blue light decreases from 35% to 25%. However, as the percentage of blue light further reduced and percentage of red light further increased, the percentage of total chlorophyll increased significantly, suggesting a shift from suppression to increased production of chlorophyll. This could be due to the effect of red light towards the photoreceptor proteins, specifically, phytochrome that induced the production of chlorophyll under red light. However, as noted by Landi et al. (2020) that despite red light having the highest quantum yield for CO_2 fixation, majority of plants are unable to survive on red lights alone. They also noted that too much red light and the imbalance of red:blue light ratio may contribute to reduction of plant's photosynthesis capabilities due to unresponsive stomata, reduced chlorophyll production, and many more.

Our observation contradicted both Cope et al. (2014) and Fan et al. (2013) up to a certain degree. This was further supported by several other journal reports. For instance, Heo et al. (2010) reported that the amount of chlorophyll b increased for both *D. amoena* and *F. elastic*a which were under blue light treatment compared to those under natural light. They also reported that it was the red light that reduced the production of chlorophyll b. As for chlorophyll a, they also reported that blue light treatment reduced the content compared to natural light in *D. amoena*. However, there were no changes to chlorophyll a content of *F. elastica* reported for all light treatment.

Lobiuc et at. (2017) reported that green cultivar of basil had their chlorophyll a and b contents increased as the blue to red light ratio increases. However, there were no significant changes reported for the red cultivar of basil in all light treatments. These findings suggested that the effect of lighting conditions towards the chlorophyll contents appeared to be cultivar and species dependent.

The results of present research, combined with previous studies such as conducted by Cope et al. (2014), Fan et al. (2013), Heo et al. (2010) and Lobiuc et al. (2017) suggested that the production of chlorophyll is not purely regulated by photoreceptor proteins only, but also by other unknown biochemical interactions. The relationship between photoreceptors, lighting conditions, and chlorophyll production is yet to be fully understood. It is worth noting that none of the abovementioned research has studied the effects of the different combinations of red, blue, green, and far-red as this research had done.

6.5 Sugar content

Through our study, we found that the sugar content in plants initially reduced as the percentage of blue lights decreases and red light increases. However, as the blue light was further decreased and red light increased, the effect was found to be reversed. There have been quite a number of reports that studied the effect of lighting conditions towards the carbohydrate contents of other species. For instance, Wang et al. (2009) performed the test on *C. sativus* that was subjected to different colours of LEDs. They found that plants subjected to blue LED has the highest total soluble sugar content, followed by purple and white. Meanwhile, plants subjected to green, yellow, and red LEDs had the lowest sugar content, and not significantly different from each other.

A rather similar observation was also reported by Jiang et al. (2019) that experimented on the effect of red and blue LED combinations and compared it against fluorescence light for 'Favorita' potato (*Solanum tuberosum* L.) cultured in vitro. They reported that plants under 100% blue light was higher while 100% red had the lowest soluble sugar content compared to florescence light. But another interesting observation was that the sugar content was increasing in concentration as the percentage of red light reduced while blue light increased even when compared against the 100% blue light. This goes to prove that blue and red light, when used at the correct percentage, can increase the sugar content in plants.

Interestingly our finding of the sugar content seems to be in line with the chlorophyll content in our analysis. This is actually contradicting results reported by Kadleček et al. (2003) on *Nicotiana tabacum L.* that higher sugar content would reduce chlorophyll content, thus reduce photosynthesis activity. It was suggested that sugar actually play important role in plant's metabolism such as the regulation of photosynthesis, growth, and development. Sugar availability has been connected to defense mechanism and secondary metabolite production. According to Koch (1996) high level of sugars would decrease the photosynthesis activity based on the concept of balance between sugar production and consumption. However, in their experimental results of the effect of sugars on *Arabidopsis thaliana*, Eckstein et al. (2011) found that their results were contradicting the theories and experimental works that show high level of sugars increase the photosynthesis rates. Our results also suggested that the same relationship as shown in the correlation study: the sugar contents for *O. stamineus* under lighting conditions are highly correlated to the chlorophyll content (Figure 5.10).

6.6 High Performance Liquid Chromatography

The true mechanism of why 25B50R has the highest rosmarinic acid with moderate amount of caffeic acid is not fully understood. Researchers previously have noted that caffeic acid is the precursor of rosmarinic acid (Trócsányia et al., 2020). Stress induced by the lighting conditions may be causing more production of rosmarinic acid from caffeic acid, but further studies need to be conducted to prove this hypothesis and better understanding of the possible biochemistry pathway that was happening in *O. stamineus* when subjected to different lighting conditions.

A rather similar experiment was also conducted by Lobiuc et al. (2017) but they mainly focused on the effect of red and blue lights on red cultivar of *O. basilicum*, compared to white light. Their control's result was similar to this study; the white light had the lowest content of rosmarinic acid. They concluded that the rosmarinic and caffeic acid was lower in condition with more red light, and increased as percentage of blue light increases. They argued that as a protective mechanism against blue light, reactive oxygen species such as rosmarinic acid and caffeic acid was produced due to the action of cytochrome P450. They also concluded that plant's response to blue and red light is dependent on various factors such as species and cultivation conditions. This statement is true as demonstrated by Shiga et al. (2009) where they reported observation which contrasted to that of Lobiuc et al. (2017): total phenolic content and rosmarinic acid were significantly higher for plants under white light compared to blue and red light. This is further supporting Lobiuc et al.'s argument that the response is species dependent.

However, Iwai et al. (2010) reported that red perilla grown under 80% red light and 20% blue light followed by irradiation with UV-A was found to have caffeic and rosmarinic acid increased by a factor of 7.9 and 6.6 respectively compared to greenhouse grown plants. They hypothesized that the increase of caffeic and rosmarinic acid may act as compensators to scavenge the ROS generated from the activity of cytochrome P450 and monooxygenase involved in the biosynthesis of coumaric acid from *trans*-cinnamic acid. Similarly, Nguyen & Oh (2020) observed that green perilla had the highest rosmarinic and caffeic acid under the mixture of red, green, and blue light with additional far-red light. Meanwhile, the red cultivar of perilla had the highest rosmarinic acid and caffeic acid under monochromatic red light. This also supported other researcher's theory that the effects of lighting condition are species and cultivar dependent.

6.7 Antimicrobial activities

The observation of no zone of inhibition for both Gram-negative microorganisms, *E. coli* and *K. pneumoniae* is in line with the result reported by Ho et al. (2010).

In line with observation observed in disc diffusion assay result, the inhibition value was higher than 1mg/ mL for *E. coli, K. pneumoniae,* and *E. faecalis*. This finding is in line with Ho et al. (2010) that *E. coli* and *K. pneumoniae* were not reacting to *O. stamineus*'s extracts. As for *E. faecalis*, the strain that was used were a vancomycin-resistant strain, thus it may be interfering with the antimicrobial activity of the extracts.

Our result that 25B50R had the lowest MIC and MBC value was in line with increased total phenolic content and antioxidant activity. Similar to antioxidant content and activity, the antimicrobial activity potency of *O. stamineus* in our research increases as the blue light decreases and red light increases. However, as the blue light further decreases and red light increases, the effect is reversed. This observation can be seen from the minimum inhibitory concentration readings. The same trend can be observed on the minimum bactericidal concentration test on *S. pyogenes,* but not on *S. aureus*.

To the best of our knowledge no other studies have experimented on the effect of different lighting conditions on the antimicrobial activities of plants. The present study could be the first reported work in the area.

Chapter 7

Conclusion & Future Studies

As demonstrated by this study and other past research works, there are limitless potentials in improving yields and nutritional values that make herbal plants important in our life. However, as different species reacted differently towards stress, scientists are racing against each other to find out the responses of different plant species (some even different cultivars) towards varying stress factors.

In this study, we focus on the effect of lighting conditions towards the phenolic, sugar and chlorophyll contents, antioxidant activities, and antimicrobial activities of *O. stamineus*. However, there are several questions that are yet to be answered, such as:

1. What is the biochemical pathway triggered in *O. stamineus* when it is subjected to LED lightings?
2. What genes are expressed (more or less) compared to the one grown naturally?
3. What is the mechanism of action of antimicrobial properties of *O. stamineus*?

Most available studies currently only focused on the effect of blue and red lights towards plants. In this study, we incorporated both green and far-red ligh, but not individually. Thus, it would be beneficial to observe the effects of these 4 lights on *O. stamineus* separately, and in combination where green and far-red lights are varied. Only then we can manipulate and optimize the suitable percentage of different colored lights to be used in order to gauge the maximum nutritional potential of *O. stamineus* without sacrificing growth.

For future studies, we recommend these to be conducted:

1. Compare gene and protein expression of *O. stamineus* in normal and treated conditions.
2. Treat the plants under individual and combination of lights.
3. Discover the mechanism of action of *O. stamineus* as an antimicrobial agent.

O. stamineus is a widely popular plant that has been used for centuries. We believe that with the help of science, its potential could be further realized.

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Appendix A

Standard Curves

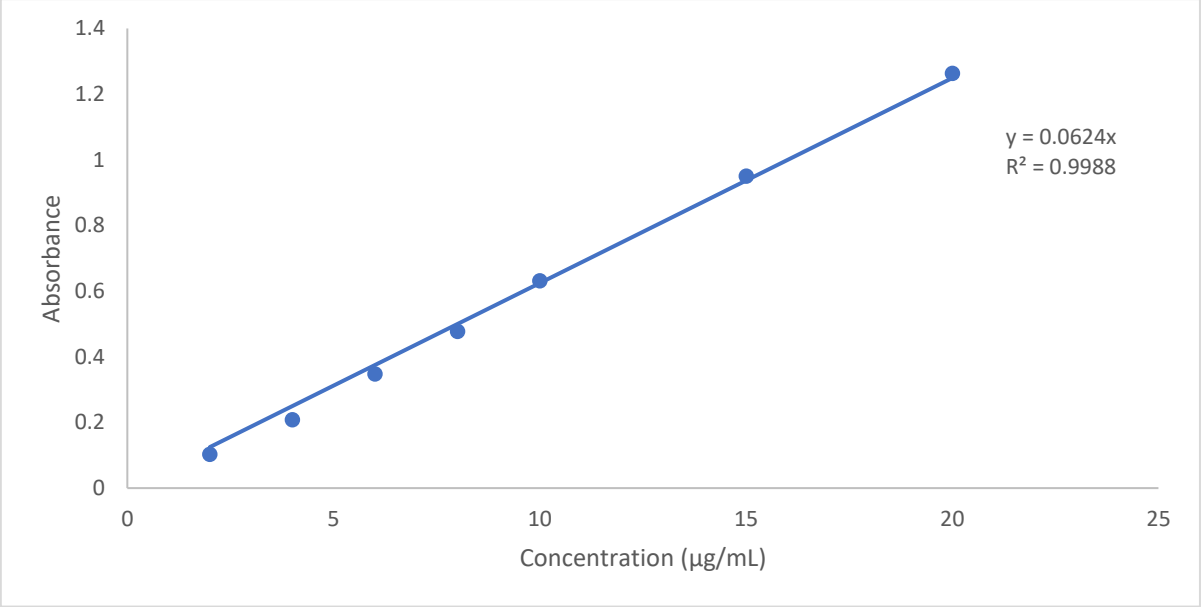


Figure A1. Standard curve of absorbance at 490 nm for sugar content determination using glucose as standard.

**Note: concentration stated above is the actual concentration in 4.0 mL solution)*

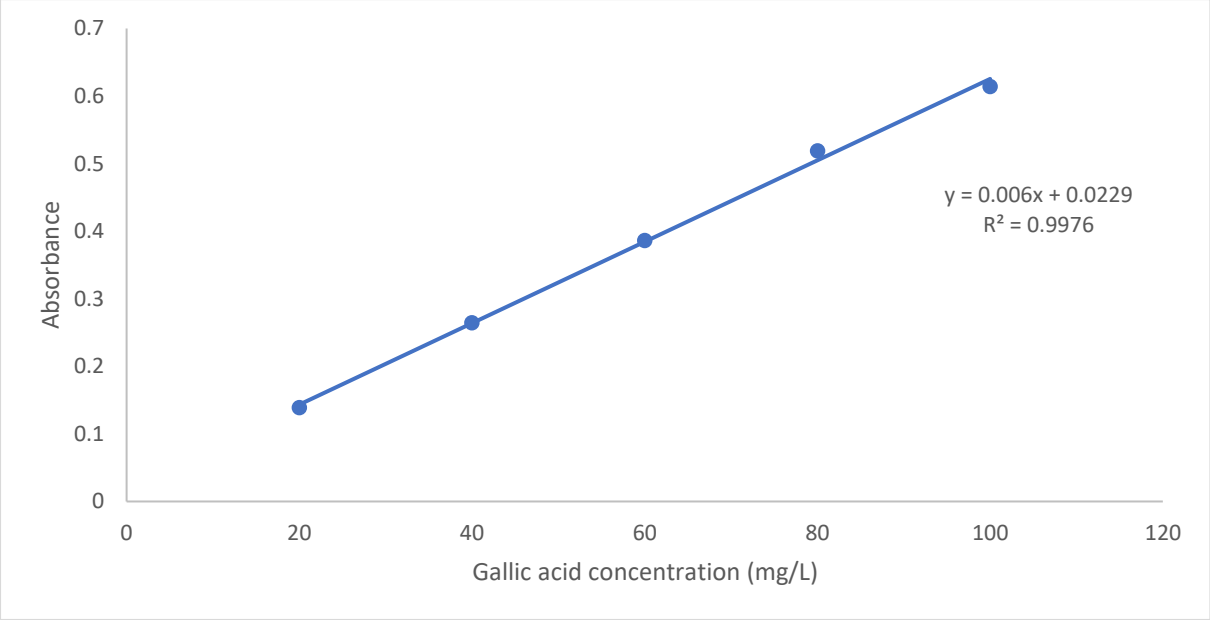


Figure A2. Standard curve of absorbance at 765 nm for total phenolic determination using gallic acid as standard.

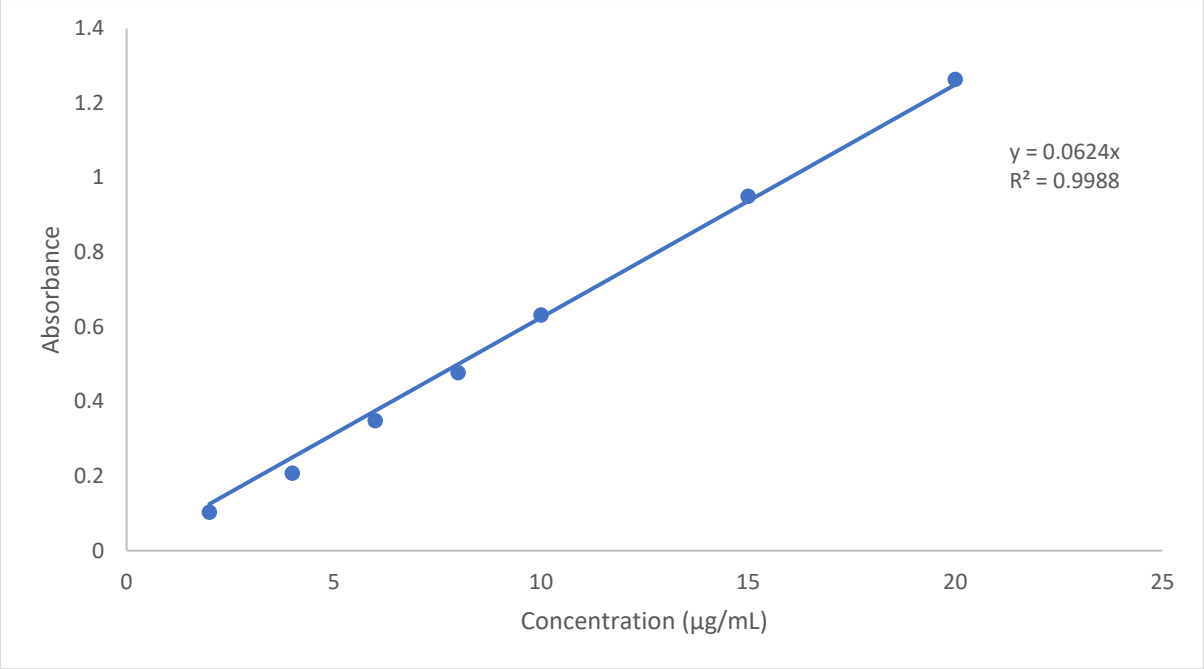


Figure A3. Standard curve of absorbance at 517 nm for percentage of scavenging activity for DPPH free radical scavenging activity.

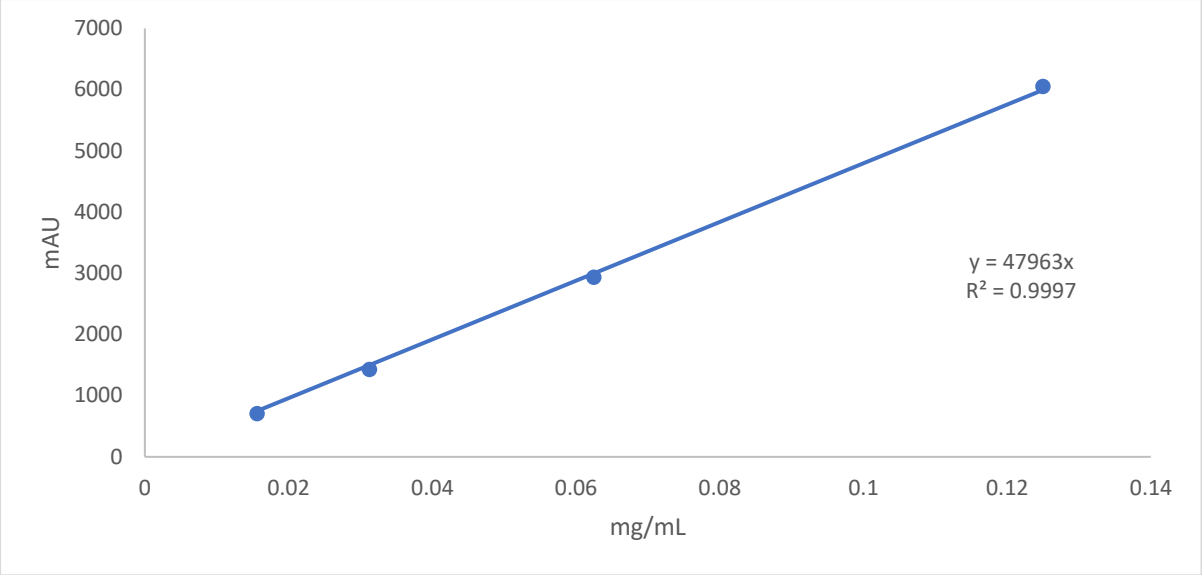


Figure A4. Standard curve of rosmarinic acid obtained by using high performance liquid chromatography.

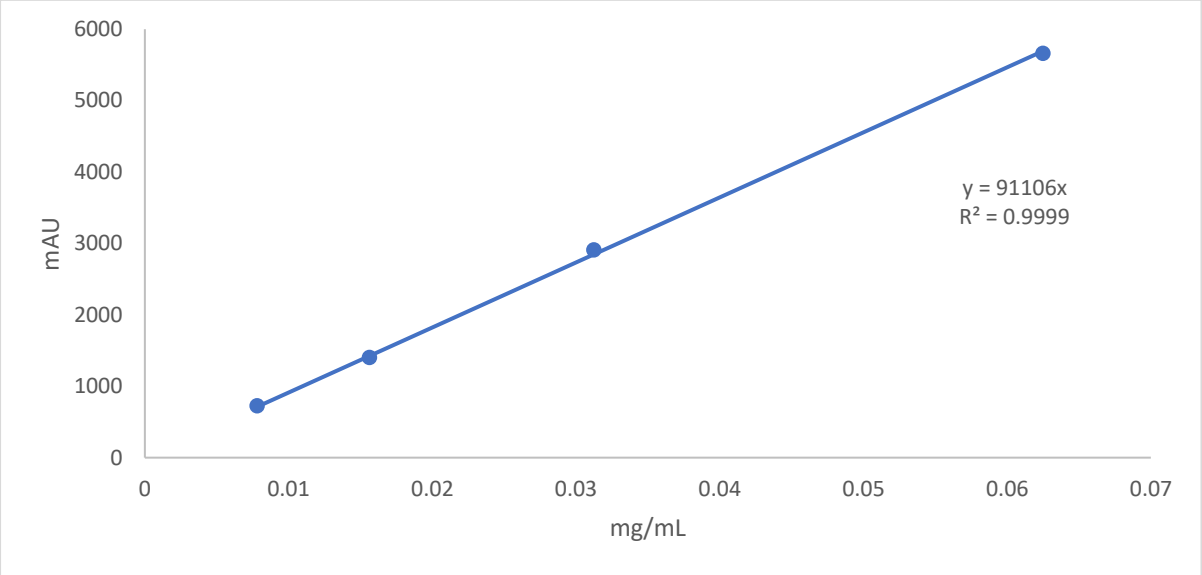


Figure A5. Standard curve of caffeic acid obtained by using high performance liquid chromatography.

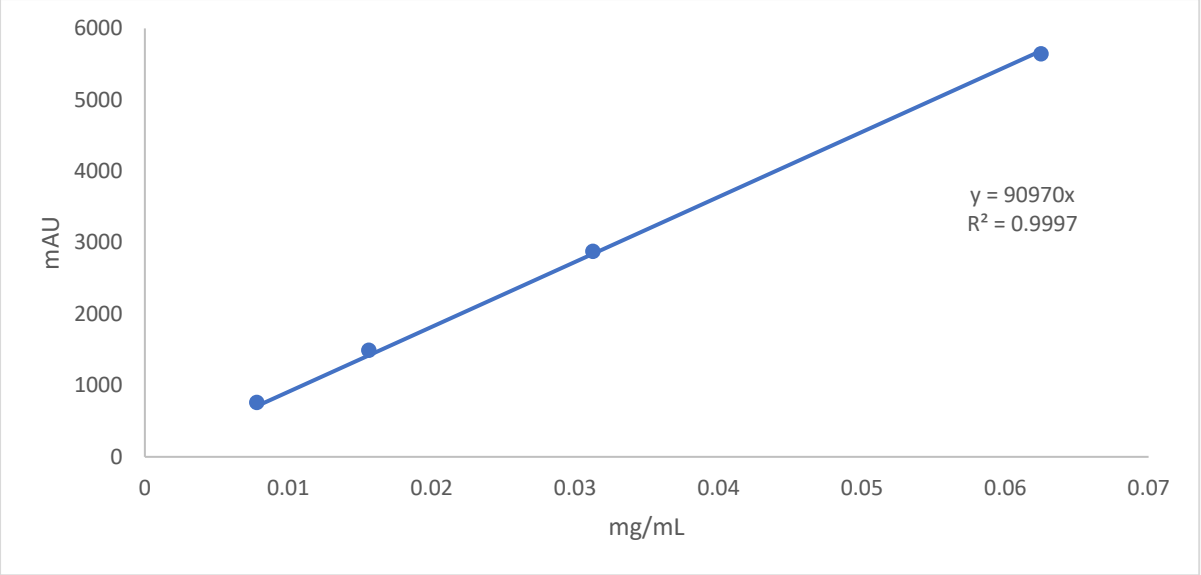


Figure A6. Standard curve of sinensetin obtained by using high performance liquid chromatography.

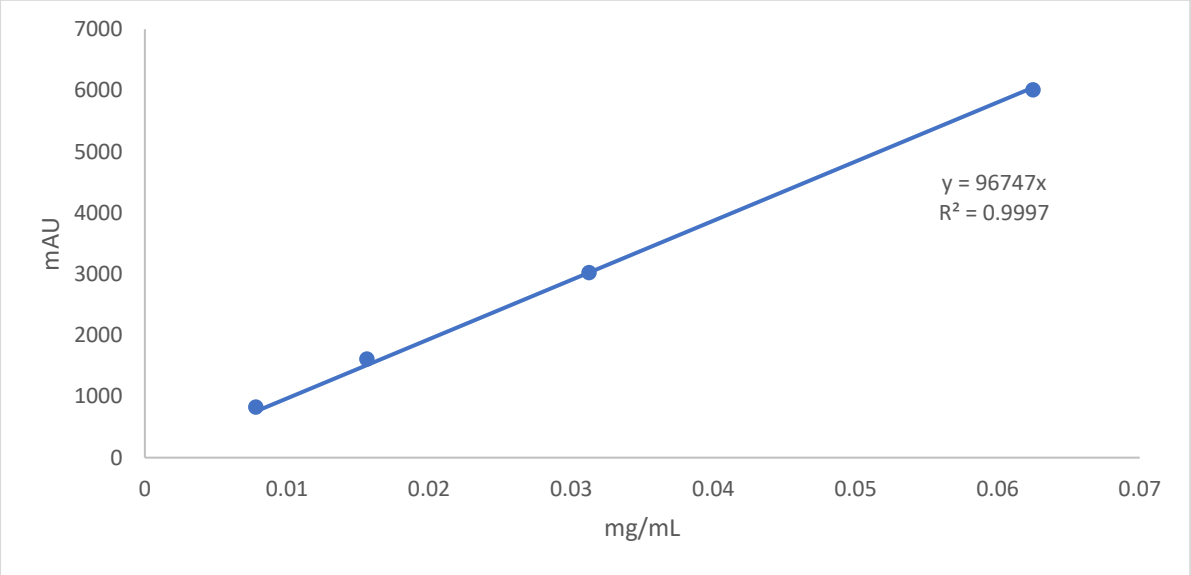


Figure A7. Standard curve of eupatorin obtained by using high performance liquid chromatography.

Appendix B

High Performance Liquid Chromatography Chromatogram

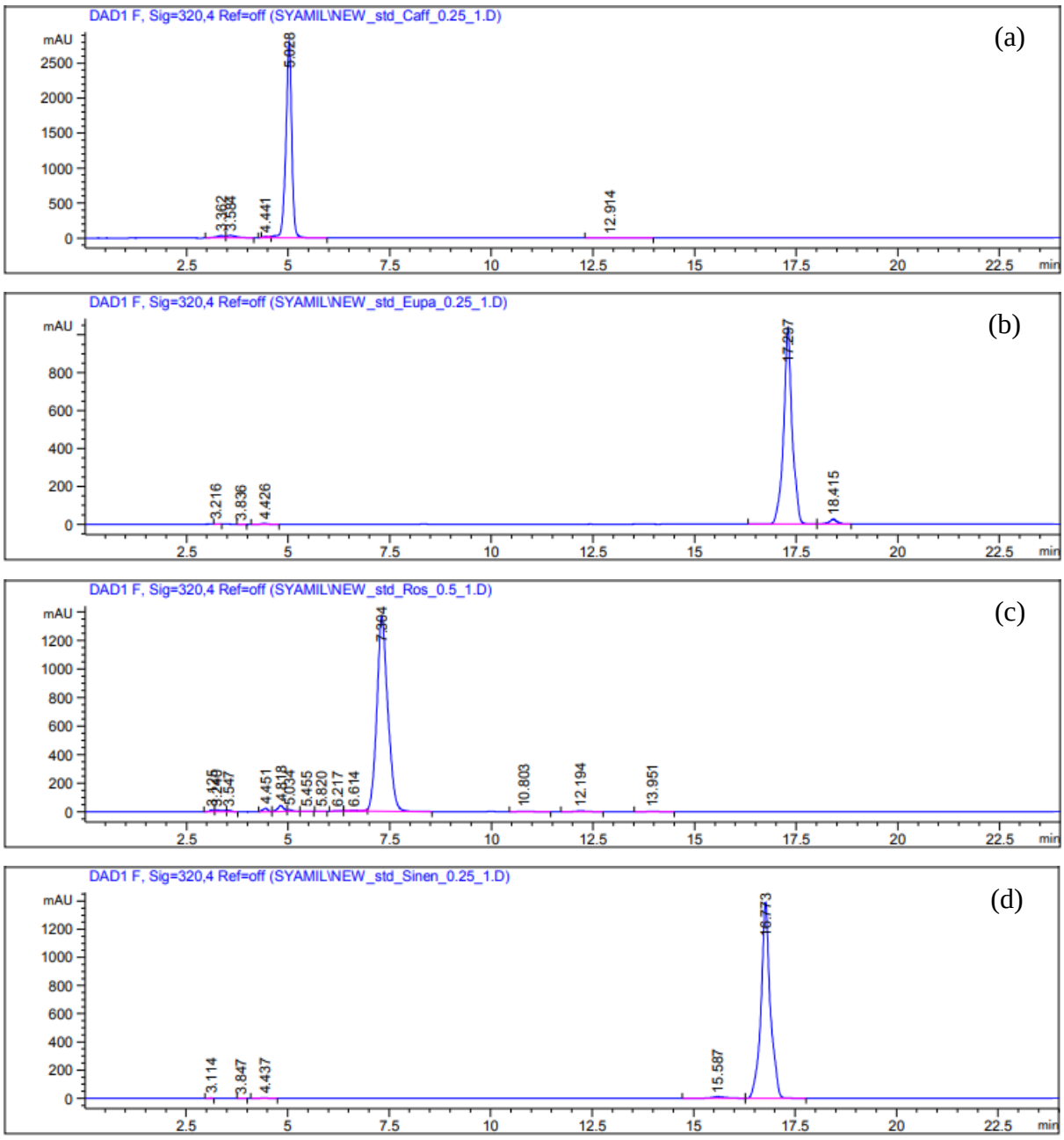
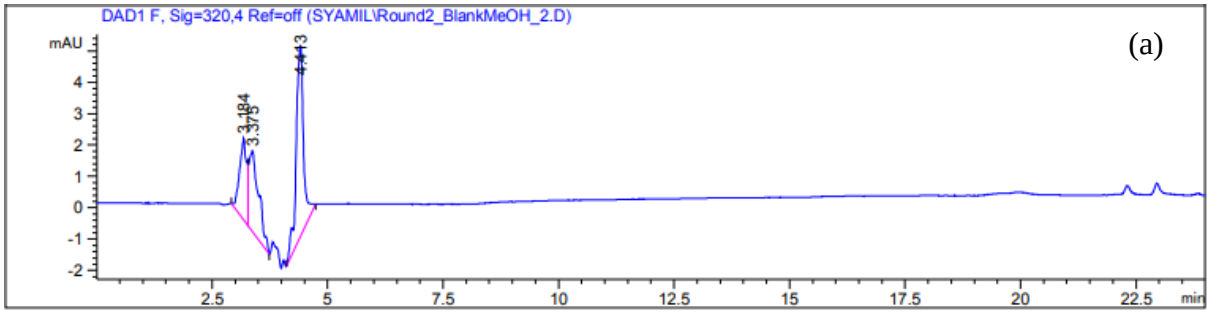
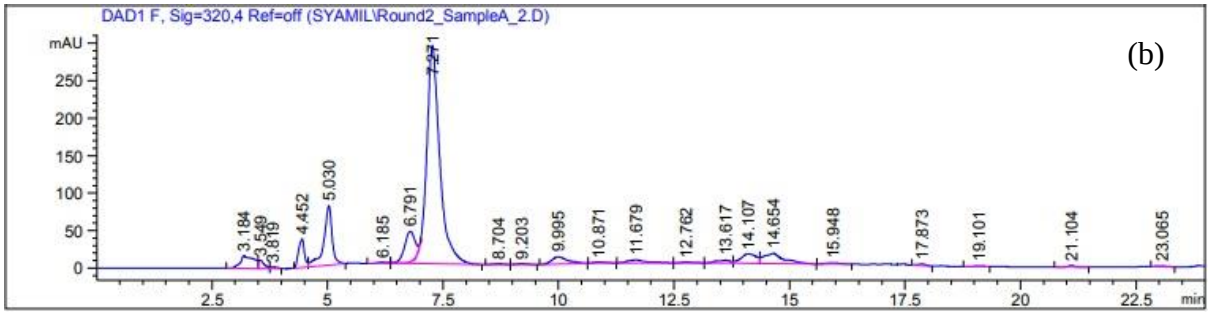
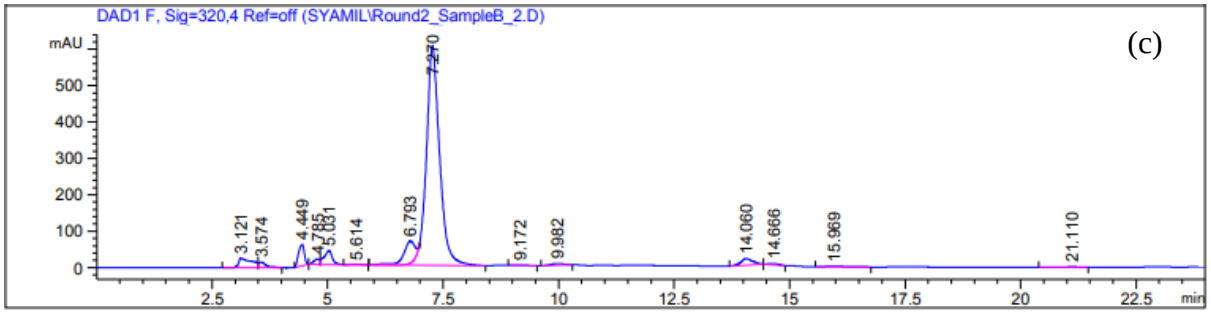
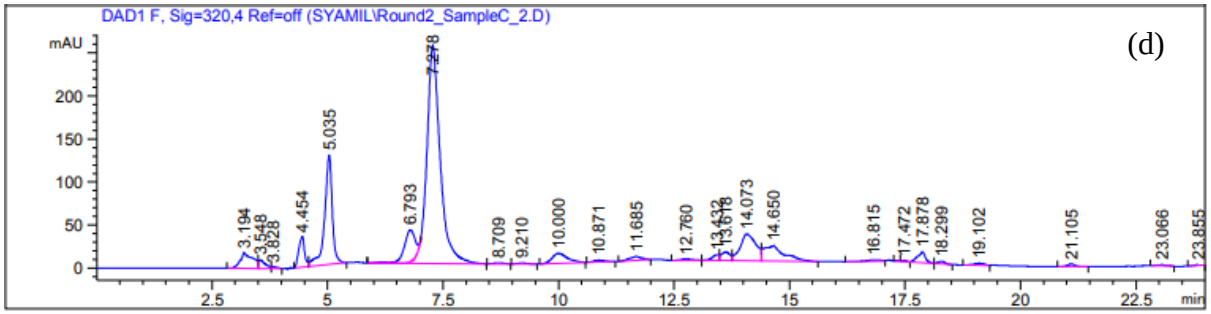


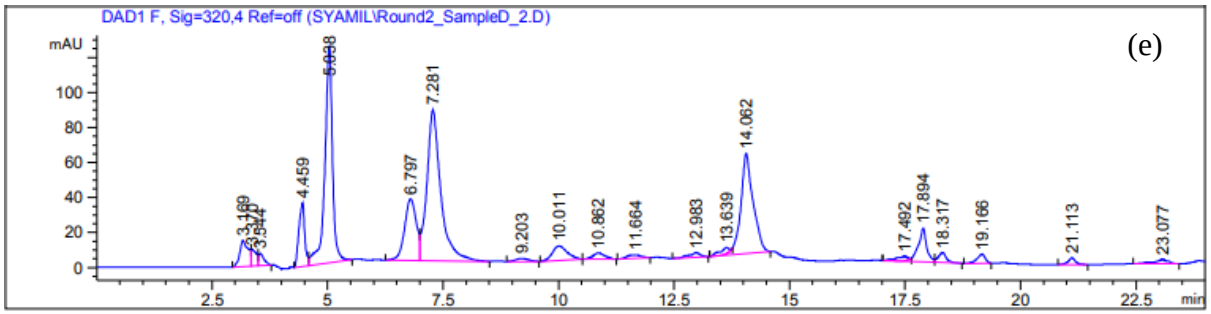
Figure B1. HPLC chromatogram and retention time for caffeic acid (a), eupatorine (b), rosmarinic acid (c), and sinensetin (d).


DAD1 F, Sig=320,4 Ref=off (SYAMIL\Round2_BlankMeOH_2.D)
(a)
mAU
min


DAD1 F, Sig=320,4 Ref=off (SYAMIL\Round2_SampleA_2.D)
(b)
mAU
min


DAD1 F, Sig=320,4 Ref=off (SYAMIL\Round2_SampleB_2.D)
(c)
mAU
min


DAD1 F, Sig=320,4 Ref=off (SYAMIL\Round2_SampleC_2.D)
(d)
mAU
min


DAD1 F, Sig=320,4 Ref=off (SYAMIL\Round2_SampleD_2.D)
(e)
mAU
min

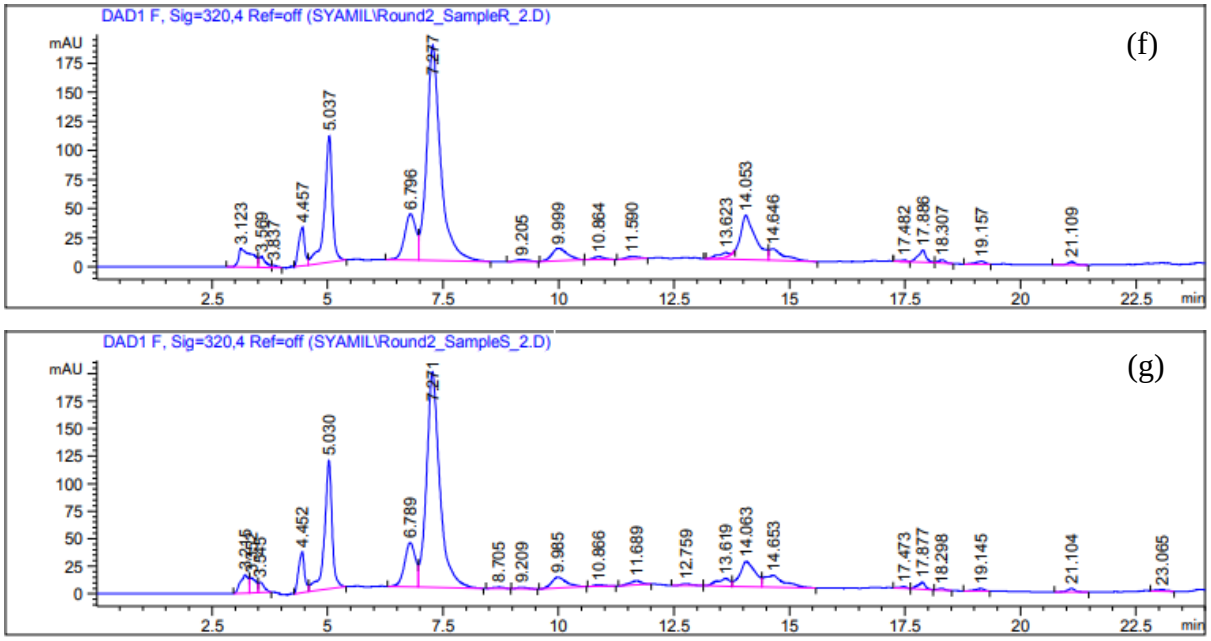


Figure B2. HPLC chromatogram for rosmarinic acid, caffeic acid, eupatorin and sinensetin in all conditions (a: blank; b: 35B40R; c: 25B50R; d: 15B60R; e: FLUO; f: SRT; g: SUN).

Appendix C

Growing Cabinet

Figure C1. Process of building, modifying, and adjusting the growth cabinets.

Appendix D

MIC Zone of Inhibition

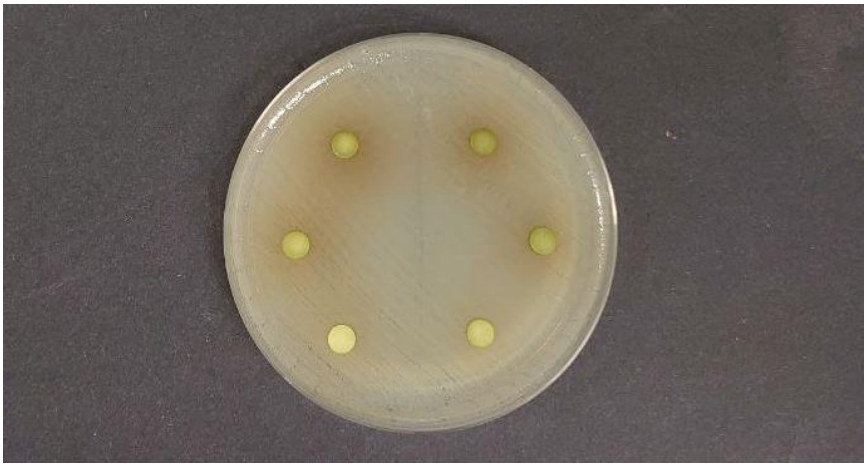


Figure D1. Zone of inhibition of *O. stamineus* extracts from different lighting conditions on *Klebsiella pneumoniae*.

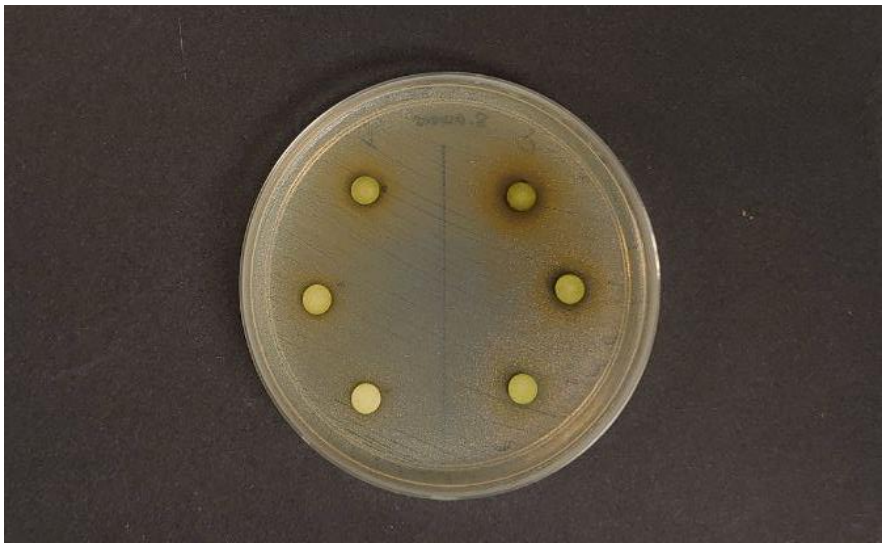


Figure D2. Zone of inhibition of *O. stamineus* extracts from different lighting conditions on *Staphylococcus aureus*.

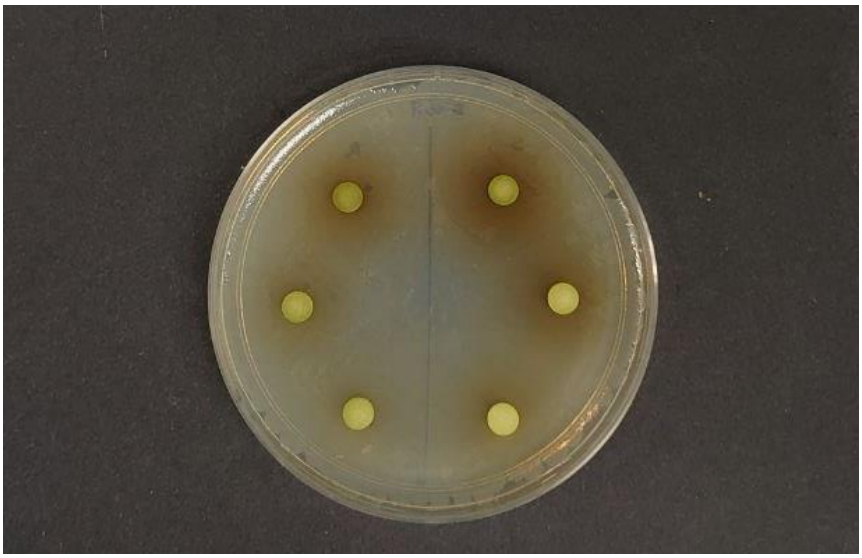


Figure D3. Zone of inhibition of *O. stamineus* extracts from different lighting conditions on *Escherichia coli*.

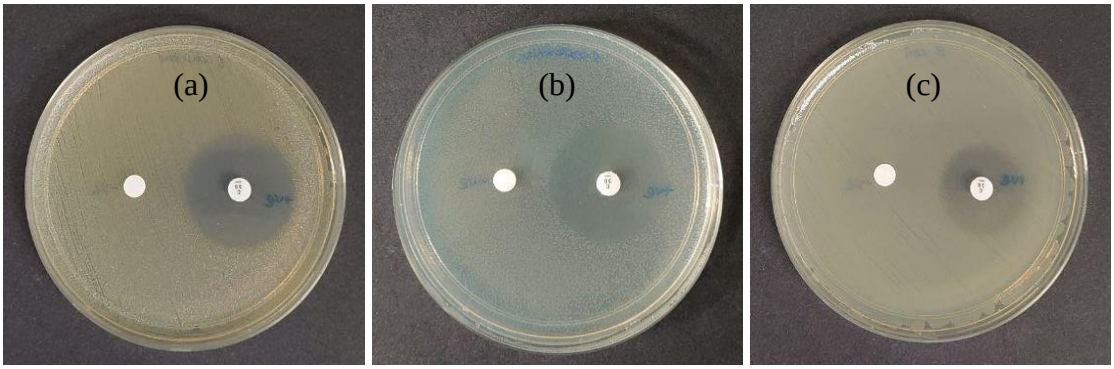


Figure D4. Zone of inhibition of *O. stamineus* extracts for positive and negative control on *Staphylococcus aureus* (a), *Klebsiella pneumoniae* (b), *and Escherichia coli* (c).

Appendix E

Growth Pictures

Figure E1. Beginning of planting phase (all conditions).

Figure E2. The end of growing phase for 35B40R.

Figure E3. The end of growing phase for 25B50R.

Figure E4. The end of growing phase for 15B60R.

Figure E5. The end of growing phase for FLUO.

Figure E6. The end of growing phase for SRT.

Figure E7. The end of growing phase for SUN.

Appendix F

List of Chemicals and Instrument

Table F1. Chemicals, Reagents, and Instruments.

Assay	Chemical	Brand
Phenolic Compound Extraction	Liquid nitrogen	Alpha Gas
	Methanol	Merck
Total Phenolic Content	Folin-Ciocalteau reagent	Merck Millipore
	Anhydrous sodium carbonate	Fisher Scientific
	Gallic acid	R&M Marketing
Free Radical Scavenging (FRS)	2,2-diphenyl-1-picryl-hydrazyl-hydrate	Sigma-Aldrich
	Methanol	Merck
	Ascorbic acid	Fisher Scientific
Ferrous Iron Chelating (FIC)	Iron (II) sulphate	HmbG Chemicals
	Ferrozine	Acros Organics
Chlorophyll content	Methanol	Merck
Sugar content	Absolute ethanol	Friendemann Schmidt
	Phenol	Merck
	Sulfuric acid	Fisher
	Glucose	Merck
HPLC	Acetonitrile	JT Baker
	Trifluoroacetic acid	Fisher
	Rosmarinic acid	Sigma Aldrich
	Caffeic acid	Sigma Aldrich
	Sinensetin	Sigma Aldrich
	Eupatorin	Sigma Aldrich
Antimicrobial	Mueller-Hinton agar	Oxoid
	Mueller-Hinton broth	Oxoid
	Methanol	Merck

Table F2. Chemicals, Reagents, and Instruments.

Assay	**Instrument**	**Brand & Model**
Antioxidant assays	Microplate reader	Tecan Spark 10M
	Spectrophotometer	Unico 2100
Chlorophyll content	Spectrophotometer	Unico 2100
	Refrigerated centrifuge	Eppendorf 5910R
	Homogenizer	Wiggens D-500
Sugar content	Spectrophotometer	Unico 2100
	Waterbath	Julabo TW20
Plant extraction	Orbital shaker	Protech Model 719
	Vacuum pump	Eyela aspirator A-3S
	Filter paper	Fisher brand P5
HPLC	Rotary evaporator	Eyela N-1001S-W
	Freeze-dryer	Labconco Freezone 4.5 Plus
	Refrigerator (-80°C)	Sanyo MDF-U4086S
	HPLC machine	Agilent Technologies 1260 Infinity
	C_{18} column	Agilent Poroshell 120 EC-C18 column (4.6 × 150 mm, 2.7 µm)

Appendix G

Sample Calculation

The following sample calculations are only shown for one replicate.

1) Fertilizer calculation.

Fertilizer brand: Baja Serbajadi Hijau

Percentage of elements:

1. Nitrogen: 15%
2. Phosphorus: 15%
3. Postassium: 15%

Recommended rate of fertilizer: 1tonne/ha (Zaharah 2005).

Calculations using calculation of Johnston & Askin (2005):

$$kg\ fertilizer\ per\ ha = kg\ element\ per\ ha \times \frac{100}{\%\ element\ in\ fertilizer}$$

$$= 1000kg\ N\ per\ ha \times \frac{100}{15}$$

$$= 6666.667kg\ of\ N\ per\ ha$$

Converting to kg/m²:

$$= \frac{6666.667\ kg\ per\ ha}{100m \times 100m} = 0.6667\ kg\ per\ m^2$$

Converting to g/m²:

$$0.6667\ kg\ per\ m^2 \times 1000 = 666.667\ g\ per\ m^2$$

Converting to g/cm²:

$$\frac{666.667\ g\ per\ m^2}{100cm \times 100cm} = 0.06667\ g\ per\ cm^2$$

Polybag's surface area:

$$Area = length\ x\ width$$

$$= 11cm \times 9.5cm = 104.5\ cm^2 per\ pot$$

So, amount of fertilizer per polybag:

$$104.5\ cm^2 per\ pot \times 0.06667\ g\ per\ cm^2 = 6.9667\ g\ per\ pot$$

6.9667g of fertilizer need to be placed in each pot, containing all NPK at the same ratio.

2) Total phenolic content calculation

Absorbance: 0.2288

Mass of sample: 1g

Standard curve: $y = 0.006x + 0.0229$

$$Extract\ concentration = \frac{0.2250 - 0.0229}{0.006} = 33.68194\ mg/L$$

$$Dilution\ factor = 33.68194\ mg/L\ \times 10\ = 336.8194\ mg/L$$

$$In\ 50mL\ of\ extract = 336.8194\ \times \frac{50}{1000} = 16.84097\ mg\ GAE/g$$

$$In\ 100g\ extract = \frac{16.84097}{1}\ \times 100g = 1684.097\ mg\ GAE/100g$$

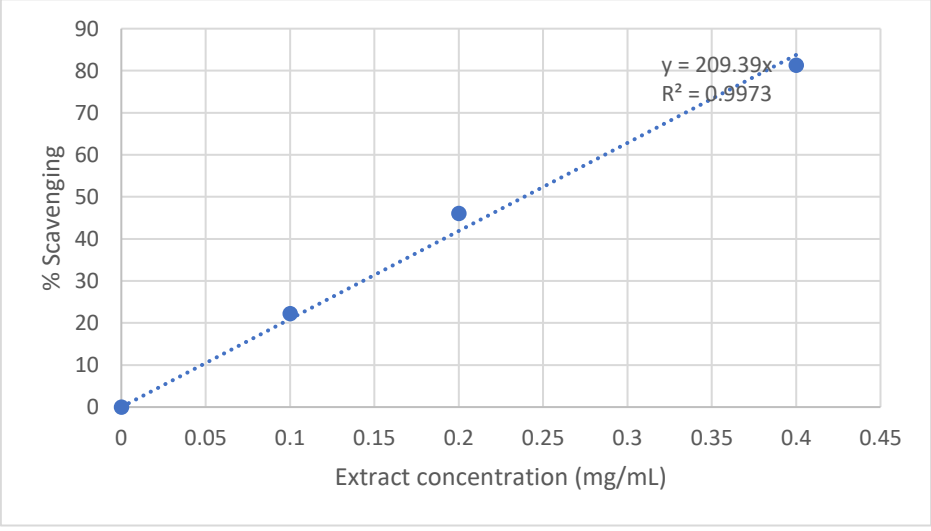
3) Calculation of DPPH free radical scavenging activity

Extract volume	Blank	0.015mL	0.030mL	0.060mL	0.075mL
Absorbance	0.6080	0.5488	0.4758	0.3424	0.2883

$$1mL\ extract\ concentration = \frac{500mg}{25mL} = 20mg\ of\ sample$$

$$If\ 0.075\ mL\ of\ extract\ used, \frac{20 \times 0.075}{3} = 0.5mg/mL$$

$$\%\ scavenging = \frac{0.6080 - 0.5488}{0.6080} \times 100\% = \ 9.737\%$$



Graph of % scavenging activity against concentration of extract in one of the conditions.

Calculation of IC_{50}:

$$IC_{50} = \frac{50}{209.39} = 0.2388\ mg/mL$$

Calculation of AEAC:

$$AEAC = \frac{IC_{50(ascorbate)}}{IC_{50(sample)}} = \frac{0.00389}{0.2388} \times 10^5 = 1629.05\ mg\ AA/100g\ fresh\ sample$$

4) Calculation of sugar content

Mass of leaves = 0.2g in 5mL = 0.04g/mL = 0.02g/0.5mL

0.5mL sample added to 1.0mL phenol + 2.5mL sulfuric acid = 4.0mL solution.

Thus, 0.02g/4mL = 0.005g/mL

Absorbance: 0.1233

Equation: $y = 0.0624x$

$$x\ (sugar\ concentration) = \frac{0.1233}{0.00624} = 1.976\ \mu g/mL$$

Thus,

$1mL = 1.976\ \mu g = 0.005g$

$In\ 1g\ of\ sample = 395.2\ \mu g$

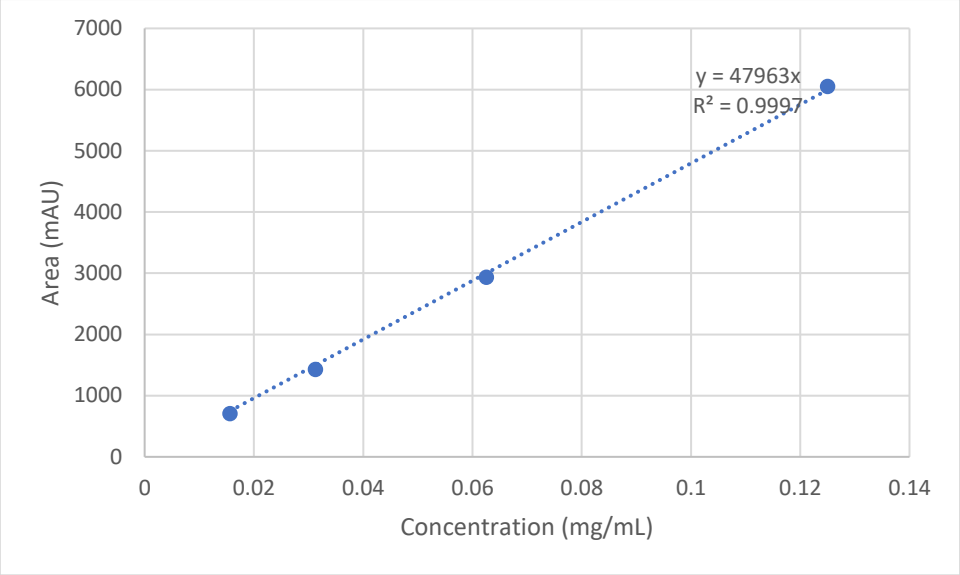
$Dilution\ factor = 395.2\ \mu g/g\ \times\ 10\ = 3952\ \mu g/g\ = 3.952\ mg/g$

5) Calculation of rosmarinic acid concentration

Area under the curve (based on HPLC) = 5730.56 mAU

Rosmarinic acid equation:

$$y = 47963x$$



$$Concentration\ of\ rosmarinic\ acid = \frac{5370.56}{47963} = 0.11197\ mg/mL$$

Concentration of HPLC sample: 1mg yield/ mL. Thus,

$$Concentration = 0.11197\ mg/\ mg\ yield$$

Extraction yield: 5 g leaves = 180 mg extract,

Thus, 1 g leaves = 36 mg extract:

$$1mg\ extract = 0.11197\ mg\ RA$$

$$36\ mg\ extract = 4.03092\ mg\ RA = 1g\ leaves$$

Thus,

$$Concentration\ of\ RA = 4.03092\ mg\ rosmarinic\ acid/\ g\ leaves$$

- If one is interested to express it as mg RA/g extract, divide the above value (4.03092 mg) by 0.036 to get 111.97 mg/g extract

6) Calculation of chlorophyll content

Absorbance at 665 nm & 652 nm:

	665	**652**
Blank	0.0971	0.0971
Sample	0.2846	0.1837
Actual	0.1875	0.0866

Equation for chlorophyll content (Porra et al., 1989):

Ch-a = $16.29A_{665} - 8.54A_{652}$

Ch-b = $30.66A_{652} - 13.58A_{665}$

Total Chl = $22.12A_{652} + 2.71A_{665}$

Calculation:

$$Chlorophyll\ a = 16.29(0.1875) - 8.54(0.0866) = 2.3148\ \mu g/mL$$

$$Chlorophyll\ b = 30.66(0.0866) - 13.58(0.1875) = 0.1089\ \mu g/mL$$

$$Total\ chlorophyll = 22.12(0.0866) + 2.71(0.1875) = 2.4237\ \mu g/mL$$