Antioxidant potential and phytochemical screening of mature leave of *Flemingia macrophylla* (willd.) Merrill (leguminosae)

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the report of this final year project entitled "**Antioxidant Potential and Phytochemical screening of Mature Leaves of *Flemingia macrophylla* (Willd.) Merrill (Leguminosae)**" by **KYRA ALYSSA BT SHAHROL NIZA**, matric number **F15A0064** has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agriculture Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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ABSTRACT

Flemingia macrophylla is herbal plant that usually been used for herbal bath. Normally it is found in tropical country and claims that the plant able to give relaxing effect. However, the plant can be categorized as underutilized herbal plant. Besides, the plant will be extracted by using glycerol and ethanol that act as a control to determine the effectiveness of using glycerol as extraction solvent. Thus, this study is conducted to determine the compound of *Flemingia macrophylla* by using different extraction solvent in order to popularize the herbal plant among the community. 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant radical scavenging was used to determine the antioxidant activity and ethanol extracts show more radical scavenging activity compared to glycerol extracts. Whereas the antioxidant content were determine by total phenolic and flavonoid content and results show ethanol extract more flavonoid compound while glycerol extract more phenolic compound. The presence of phytochemical group were determine by using Ferric chloride, Alkaline reagent, Wagner's and Salkowski's test and shown positive results for both ethanol and glycerol extracts. However, the usage of glycerol as an extraction solvent need to be consider since ethanol show better results in comparison.

Keywords: *Flemingia macrophylla, antioxidants activity, glycerol, phytochemical screening, antioxidants content*

ABSTRAK

Flemingia macrophylla adalah tumbuhan herba yang biasa digunakan untuk mandian herba. Biasanya ia ditemui di negara tropika dan tumbuhan ini dikatakan dapat memberikan kesan yang nyaman. Walau bagaimanapun, tumbuhan ini boleh dikategorikan sebagai tumbuhan yang kurang digunakan. Selain itu, tumbuhan ini diekstrak dengan menggunakan gliserol dan etanol yang bertindak sebagai kawalan untuk menentukan keberkesanan penggunaan gliserol sebagai pelarut ekstraksi. Oleh itu, kajian ini dijalankan untuk menentukan kompaun *Flemingia macrophylla* dengan menggunakan pelarut pengekstrakan yang berbeza untuk mempopularkan tumbuhan herba di kalangan masyarakat. Pemerangkap radikal bebas anti-oksida 2,2-diphenyl-1-picrylhydrazyl (DPPH) digunakan untuk menentukan aktiviti antioksida dan ekstrak etanol menunjukkan aktiviti pemerangkap radikal lebih tertinggi berbanding dengan ekstrak gliserol. Seterusnya, kandungan antioksida ditentukan oleh jumlah kandungan fenol dan flavonoid dan keputusan menunjukkan ekstrak etanol lebih banyak sebatian flavonoid manakala gliserol mengekstrak lebih banyak sebatian fenolik. Kompaun fitokimia ditentukan dengan menggunakan ferik klorida, reagen alkali, ujian Wagner dan Salkowski dan menunjukkan hasil positif untuk kedua-dua ekstrak etanol dan gliserol. Walau bagaimanapun penggunaan gliserol sebagai pelarut pengekstra perlu dipertimbangkan kerana etanol menunjukkan hasil yang lebih baik berbanding gliserol.

Kata kunci: *Flemingia macrophylla, activiti antioksida, gliserol, kumpulan fitokimia, kandungan antioksida*

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LIST OF ABBREVIATIONS

g	gram
min	minute
nm	nanometre
µg	microgram
µL	microlitre
mL	millilitre
mM	millimolar
M	molar
rpm	revolutions per minute
ft	foot
mg/ml	microgram per mililitre
BHT	Butylated hydroxy toulene
DPPH	2,2-diphenyl-1-picryhydrazyl
GAE	Gallic acid equivalent
QE	Quercetin equivalent
R^2	Correlation coefficient
SD	Standard deviation
Sig	Significant
SOP	Standard operation procedure
TFC	Total flavonoid content
TPC	Total phenolic content
UV-VIS	Ultraviolet visible

LIST OF SYMBOLS

ºC	Degree Celcius
%	Percent
≤	Less than or equal
±	Plus-minus
:	Ratio
µ	micro

CHAPTER 1

INTRODUCTION

1.0 Research Background

Flemingia macrophylla or commonly known as seringan are easily found in tropical country such in Malaysia, Indonesia and India. Each part of *F. macrophylla* contains different beneficial compounds that able to promote specific benefits. Traditionally in Malaysia, the roots of *F. macrophylla* contain various usages such as able to treat insomnia, epilepsy, inflammation and microbial infection. According to previous research conducted by Ghalot, Lal, & Jha (2012), the roots of *F. macrophylla* proven to act as anticonvulsant by possess anti-depressant action of the central nervous system.

Phytochemical also known as plant chemical is present in plant based diets that are non-nutritive components which are related in offer protection against chronic disease such as hypertension, diabetes and cancer (Arendt & Zannini, 2013). Research done by Kannika Panyaphu, Panee Sirisa-ard, Preeyawis Na Ubol & Sunee Chansakaow (2012), on postpartum herbal plant bath showed presence of antibacterial and antioxidant activities which able to fight against *Staphylococcus aureus* that normally attack wound during postpartum. Based on research done, phytochemical can be found in plants. Different range

of phytochemical can be comprised of tocols, phenolic acid and sterols (Arendt & Zannini, 2013).

Antioxidant is product naturally inside the body or can be obtained by consuming additional food that rich in antioxidant. Foods that are rich in antioxidant can be found in fruits, vegetables and coffee. Example of antioxidant compounds are, vitamin A, lycopene and flavanols. Antioxidant can prevent cell damage that caused by oxidants. Oxidant is free radicals that can be produced naturally by body and attack cell and could lead to severe disease such as cancer.

The purpose of extraction solvent is to separate active compound and feed in order to gain the desire compound. Extraction solvent that is normally used in the industry are ethanol and methanol. However, due to several circumstances regarding health issue, the communities is well aware and try to avoid using ethanol and methanol as extraction solvent. This study is conducted to find an alternative of extraction solvent by replacing ethanol to glycerol.

1.1 Problem Statement

Based on previous publication, it can be stated that study done on the leaves of *F. macrophylla* is limited. Studies are mostly done on the roots of *F. macrophylla* to find out the antioxidant compound and its health benefit of the roots. This research is conducted to provide further information on the benefit of *F. macrophylla* and the antioxidant properties

where products of *F. macrophylla* can be accept and practice by Malaysian especially for lady that undergo confinement process.

Besides, there are a lot of health products including cosmetic and health supplement that contained chemical substance that might be harmful to human's health. Thus there are an urge to search for non-toxic and agro-solvent as a substitution to commonly used organic solvent in product of health care product. Health issue that is concern by the worldwide community is the usage of alcoholic solvent such as ethanol that able to cause burning sensation and unconsciousness just by smelling it.

Next, due to unfamiliar usage of *F. macrophylla*, especially to youngsters, the plant is underrated and the beneficial of the plants towards health is not fully utilized. Thus, in order to popularize this herbal plant, further research need to be conducted.

1.2 Hypothesis

H_0 – There is no significance difference in the antioxidant potential of *F. macrophylla* by using different extraction solvent.

H_a – There is significance difference in the antioxidant potential of *F. macrophylla* by using different extraction solvent.

H_0 – There is no significance different on phytochemical compound between phenolic and flavonoid content.

H_a – There is significant different on phytochemical compound between phenolic and flavonoid content.

1.3 Objectives

2. To extract dried mature leaves of *F. macrophylla* by using different extraction solvent.
3. To compare antioxidant potential of *F. macrophylla* sample by using DPPH scavenging activity, total phenolic and total flavonoid content.
4. To determine the phytochemical group content in *F. macrophylla* mature leaves.

1.4 Scope of Study

This study was conducted to compare the antioxidant activity and content using various extraction solvent of fresh & dried *Flemingia macrophylla* mature leaves. Sample of *F. macrophylla* was purchased from the local market in Jeli, Kelantan. The samples were air-dried at 24˚C room temperature with good ventilation for a week and subjected to extraction using ethanol and glycerol solvent. Antioxidant activity will be conducted using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and estimation of total phenolic and flavonoid content was carried out. Plant phytochemical groups were also be analyzed using Ferric chloride, Alkaline reagent, Wagner's and Salkowski's test.

1.5 Significance of study

This study will be able to provide antioxidant potential as well as compound groups information on the underutilized traditional herbal specifically plant on the mature leaves part of *F. macrophylla*. These studies also able to prove the beneficial usage of the *F. macrophylla* that are claimed by elderly which able to treat specific disease based on the groups of compound found. Thus, further information provided will encourage the community to fully utilize this herbal plant. In order to popularize *F. macrophylla,* further product development is needed to fully utilize the plants and its beneficial compound.

CHAPTER 2

LITERATURE REVIEW

2.1 *Flemingia macrophylla*

The family name of *Flemingia macrophylla is* Fabaceae and the species genus is Flemingia. Common names for *F. macrophylla* are depend on the location. In Malaysia, it is known as seringan or meringan plant. In English it is called as luck plant or wild hops. In Vietnam, *F. macrophylla* are known as Top Mo Bong Tron or Kopa-kopa. Nepalese called it as Ghari Mamarkha or Barkuali Jhar. For French people, Sainfoin du Bengale is known as *F. macrophylla* (Gahlot, Lal, & Jha, 2013).

2.1.1 Plant botany

F. macrophylla can normally be found in tropical country such as in Malaysia, Philipines, Vietnam and Nepal. The plant requires full sunlight and moderate amount of water to survive. The lifespan of *F. macrophylla* could last up to several years. *F. macrophylla* has the characteristics of able to produce angiosperm and shrub which able to reach up to 1 meter. The branches is numerous, woody and covered

with villi. The shapes of the leaves are curving downward, oval and short, which has parallel and straight nerves. The flower is dull green in colour with small in shape that enclosed in a pair of bean shaped flower (Gahlot et al., 2013)

2.1.2 Traditional usage of *Flemingia macrophylla*

Locals claim that *F. macrophylla* contains various benefits that able to cure health issue. Nepalese used this plant to treat gastrointestinal disease by consuming 2 teaspoons of the root in form of paste, twice a day. In India and Southeast Asia, elderly will use *F. macrophylla* to treat rheaumatism which is disease that affect the joints and muscle area. In order to treat fever, Malaysian will use the leaves of *F. macrophylla* in their bath and they claim it able to offer the body temperature and give soothing effect. Malaysian believes that by consume *F. macrophylla*, it able to give more relaxation especially for hyperactive children and kids that encounter problem with sleep at night (Gahlot et al., 2013).

2.1.3 Medicinal properties of *Flemingia macrophylla*

Study done by Madan, Singh, Kumar, & Kohli (2010) on *F. macrophylla* showed *F. macrophylla* contain antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermis, Pseudomonas aeruginosa, Escherichia coli, Methicillin* resistant *Staphylocuccus aureus and Candida albicans.* Based on research finding, there are lack of

information on the antioxidant activity and pharmacognostical investigation of *F. macrophylla* on the roots and leaf parts. Based on Madan et al. (2010), preliminary phytochemical screening method of the selected part of the *F. macrophylla,* showed that there are presence of lipids, phenolic compound, carbohydrates, flavonoids, tannins and phytosterols. Plant that contains phenolic including flavonoids show that high in antioxidant properties and based on the phytochemical findings, it shows that different part of the plant are rich in polyphenolic compounds such s flavonoids, tannins and steroids that act as free radical scavenger.

Study by Gahlot, Lal, & Jha (2013), had investigated the potential of *F. macrophylla* by using the root parts on treating epilepsy, hysteria, insomnia and as pain reliever. Based on Gahlot et al. (2013) epilepsy is a spontaneous reaction due to recurrent seizures. Electroconvulsive shocks were induced in the animals to create seizures condition and specific amount of dosage were given to the mice and results showed that at dosage of 300mg/kg, it able to reduce seizures durations preliminary phytochemical screening methods was done and result showed that the part contained anticonvulsant. In brief Gahlot et al. (2013) indicate that roots of *F. macrophylla* which contained ethyl acetate enable anticonvulsant activity in lab mice.

2.2 Antioxidant

Antioxidant is a substance that able to lower down the risk of cell damage by counteracting the damage cause by oxidation. Types of antioxidant that can be found are phytochemicals, vitamins and enzymes. The most beneficial antioxidants can be found in plants due to constantly exposed to UV light. Examples of antioxidants that are widely known are glutathione, vitamin A and vitamin C and foods that are pack with antioxidant properties are vegetables, fish and whole grains. Glutathione able to donates electron or hydrogen atoms while vitamin A or also known as carotenoids function to break the chain of oxidation molecules. Food engineer capable to imitate pills or supplement that is pack with antioxidant however, recent study show that by taken extra vitamins do not indicate healthier cell compared to those who consume real food that high in antioxidant (Silva et al., 2011)

2.2.1 Mechanism of action

Abushouk, Ismail, Salem, Afifi, & Abdel-Daim (2017) stated, “Oxidative stress can be defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses”. There are few signs that able to detect oxidative stress which can be fatigue, decrease in eye sight, muscle or join pain, wrinkles

and grey hair. Oxidative stress can be reduce by free the mind from stress or consume healthy diet which lower down the sugar intake or processed food.

Oxygen is important for living things however, due to highly reactive atom which have the ability to potentially damage the cells is called free radicals. Free radical is capable of attack the health of the cell by damaging, spread disease and cause severe disorders. Examples of damage that can be done by free radicals are cancer, heart disease and decrease the functionality of immune system (Sen, Chakraborty, Sridhar, Reddy, & De, 2010).

Free radical can be controlled by various compound that known as antioxidant. Antioxidants have the characteristics of stabilizing or deactivate the free radicals before the damage cells spread to other part of the body. Antioxidant can be found in different kind of forms which are naturally produced by the body and external consumptions from food (Alara, Abdurahman, Abdul Mudalip, & Olalere, 2017). Antioxidant properties that can be found are Vitamin C, Vitamin E and beta carotene. Whereas in human body, enzymes such glutathione and catalase is known as natural antioxidant.

Function of antioxidant is to protect important cell such as DNA. This action can be achieved in several ways by bind the antioxidants substances to the oxidative molecule that will prevent the oxidative molecule interact with other cells. The free radical will be carried away from the body via bloodstream and kidney. Other than that, antioxidants able to repair the damage cell by donating hydrogen atoms or electrons (Halliwell, 1991).

2.2.2 Antioxidant activity determination

Antioxidant compounds are determined to ensure the plant extracted is presence with beneficial health properties by determine with different assay. According to Adhikari et al. (2018) had studies the antioxidant activities of polyphenol, flavonoid, and amino acid contents and has proven the activities of antioxidant and antimicrobial and widely used for cough reliever, decrease in blood pressure and remove mucus from lungs. The number of polyphenolic compounds are able to promote health which can be applied on food industries. ABTS radical scavenging activity also possesses vary results based on different type of cultivars used. It is shown that high ABTS radical scavenging activity in the peanut shell due to phenolic compounds (Yen, Duh, & Tsai, 1993).

Free radicals in human body can be dissolved by antioxidant (Erasto, Grierson, & Afolayan, 2007). Based on Alam, Bristi, & Rafiquzzaman (2013) shrub plant that grow up to 3m above the sea level have high pharmacological properties such as antioxidant, anti-leukemia, anti-microbial and anti-diabetes. Bioactive compounds such terpenes, alkaloids and flavonoids had been isolated from the extracts (Farombi & Owoeye, 2011). Study by Vashisth, Singh, & Pegg (2011) revealed that edible or non-edible plant products contain phenolic compound that able to fight against free radicals by minimizing the risk of disease and providing protection on harmful free radicals. According to Erasto et al., (2007), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay is an improve version of trolox equivalent antioxidant capacity (TEAC) assay (the ferryl myoglobin) in order to

determine the antioxidant activity in a number of ways. Improvements that have been made are ABTS assay able to perform the activity on both aqueous and lipophilic systems.

2.3 Plant phytochemical

Phytochemicals have the properties of prevention and protecting plant from disease that is not essential for consumption of human. However, recent research show that phytochemicals able to protect human from disease. Each phytochemicals group might work differently and the possible actions are antioxidant where it helps in protect the cells, hormonal action that functions to mimic the estrogens and anti-bacterial effect that present inside garlic. The easiest way to sustain phytochemical groups inside the human body is by consume nutritive food such as vegetables, fruit and whole grain. The purpose to identify the phytochemicals group is to help in searching plants that high in phytochemicals and for future development where the food industry able to come out with external food or supplement that compact with phytochemical groups.

Phenolic acids, flavonoids and tannins are known to be vital antioxidants which can be commonly found in vegetables, roots, nuts and barks (Sieniawska & Baj, 2016). Identification of phytochemical group is carry out to identify the beneficial component

Based on research conducted by Khanam, Wen, & Bhat (2015), *E. longifolia* is extracted by using methanol, chloroform and ethyl acetate that are good sources for different classes of compound. Due to the high polarity of the solvents, it is proven to be

effective to isolate active biological compound. Phytochemical can be high value compared to synthetic drugs in pharmaceutical industries since it is less expensive and easy to be obtained (Kok et al., 2000). Phytochemical compound able to reduce oxidative stress via several pathways which can be, by perform a potent free radical scavenging activity (Abushouk et al., 2017). Based on study conducted by, Koduru et al. (2018) three steps involved in phytochemical are, solvent media selected for extraction of phytochemicals, plant sample used for extraction and nucleation and growth study of silver nanoparticles (Ag NPs).

2.3.1 Phytochemical screening

Phytochemical screening is done to detect the present of alkaloids, tannins, saponins and steroid in plant. The qualitative analysis of phytochemical can be done with Dragendorff's test in order to detect the present of alkaloids. Orange red will appear if alkaloid is detected by adding potassium bismuth iodine solution to the extracted sample. Besides from Dragendroff's test, Mayer's test can be used with adding potassium mercuric iodide solution and cream colour precipitate indicate the presence of alkaloids (Iqbal, Salim, & Lim, 2015).

Salkowski's test able to detect the presence of steroids and terpenoids with appearance of reddish brown however with interference of coloration indicate presence of terpenoids by shake the extracted sample with chloroform followed with slowly tilt the test tube and pour concentrated sulfuric acid. Other alternative to detect the presence of steroids

and terpenoids is by using Liebermann-Burchard test. Extracted is shaken with chloroform and drops of acetic anhydride is added. The mixture will be boiled in water bath for specific period and instantly cool with ice water. With addition of concentrated sulphuric acid, appearance of brown ring will appear at the middle of two layers and upper layer will turn into green indicate presence of steroid while presence of terpenoids show deep red colour. (Iqbal et al., 2015)

Tannins are tested with stir distilled water with extraction and filter with filter paper. 5% of ferric chloride is added and black or bluish green indicate the presence of tannins. Saponins can be tested with adding distilled water to the extraction in a test tube. In a water bath of 5 min, frothing will form indicate the presence of saponins. (Iqbal et al., 2015)

2.3.2 Phytochemical detection and quantification

Phenolics compound is secondary metabolites in plant which contribute in pigmentation of plant and antioxidant. Folin-Ciocalteu is redox reagent that reacts with polyphenols in plant extraction that can be quantified by visible light spectrophotometry by forming blue complex (Slinkard & Singleton, 1977)

Flavonoids are considered as vital compounds in propolis. Propolis is a glue like substances that are made of pollens, plant resin and bee waxes that used by bees to protect

their bee hives. Based on Miliauskas, Venskutonis, & Beek (2004), estimation of total flavonoid content can be done by using Aluminum Chloride Colorimetric method.

Tannins are the secondary metabolites in plant and consists different chemical structure. Tannin can be group into hydrolyzable, gallotannins and condensed tannins. High concentration of tannins can be found in most part of the plants include bark, seed and roots. Existence of tannins able to protects the plants from microbial, animal and insect infection. Example plants contain tannins are *Acacia katechu (L.)* Wild *Fabaceae* and *Diospyros kaki* Thunb. *Ebenaceae* (Sieniawska & Baj, 2016)

2.4 Plant Extraction

Extraction is a process of separation from the active portions of plant by using selective solvents. Preparation of extraction can be in various ways which are infusions, fluid extracts and powdered extracts. Products that might obtain are in form of solid, semi-solid or liquid. The sample extracted will undergo further quantitative analytical. Extraction method can be applied in production of antibiotics, obtaining essential oil and in pharmaceutical industries.

Many extraction methods have been done in the science field and commonly used are maceration, infusion, soxhlet method and rotary evaporator. Maceration is the simplest method which grinded crude extract is place inside a beaker with solvent and leaves for few days, hours or minutes depending on achieving desire results. Aim of using rotary evaporator is to remove extracted solvent from the feed and only gain the crude extract. The

advantage of using rotary evaporator is it able to handle large quantity of solvent due to the constant rotation and large surface area of the flask.

In order to separate the compounds of *F. macrophylla*, solvent extraction technique is use since it is proven to be an excellent separation technique. The solvent extraction or also known as liquid-liquid extraction is use to extract the active compound of plant sample to determine the activity and content of antioxidant. (Ag et al., 1916) stated, polar compound is a molecule with distinct electrical charges in different region for example, positive charge in one region and a negative charge in another and non-polar compound composed of molecules that possess a symmetric distribution of charge, so that no positive or negative poles exist, and that are not ionizable in solution, for example hydrocarbons.

According to Akowuah, Ismail, Norhayati, & Sadikun (2005), different polarity extraction solvents were compared by run with DPPH and they claimed that the highest free radical scavenging activity is polar extracts which the solvent might contain highest concentration of caffeic acid. Caffeic acid is known for its antioxidant properties that can be found in coffee and several vegetables and fruit. However, the non-polar solvent use which is chloroform, did show any low activity and significantly different from the reference compounds.

2.4.1 Issue and challenges

Ethanol is clear, wine-like smell and liquid consistency. Ethanol or ethyl alcohol is cited by Occupational Safety and Health Administration (OSHA) and National Institute of Occupational Safety and Health (NIOSH) as hazardous substance. Effect of regularly exposed to ethanol may lead to damage of fetus if the concentration is too high and can cause dehydration to the skin and cause peeling, itch and redness to the skin. Other than that, it may affect human breathing system and irritate the nose and lungs. Due to high toxicity of ethanol, the urge to find other alternative way for extracting solvent is well considered.

In this study, ethanol is used as control while replacing glycerol as extraction solvent. Glycerol is non-toxic and cosmetic based material. Thus, it is more users friendly and accepted worldwide. Consistency of glycerol is viscous which are thick and sticky. Glycerol is polar solvent however, it is low solvent selectivity. Thus, desire compound need to be extract might not well extracted by glycerol.

2.5 Plant sample preparation

Fresh sample is sample that freshly picked from the plant and do not undergo any treatment processing. Handling fresh sample can be difficult due to the water content inside the plant might dry up to the air and the research conducted need to be done within 24

hours. Dry sample is where the water content inside the plant sample is being remove or reduce by air dry or oven dry method. The purpose of drying the sample is to reduce the water content inside the sample. By drying up the sample, it will able to be kept longer throughout the research and avoid product from deterioration. Without proper drying method, the tendency for mold to grow is higher and will cause damage to the sample (Li et al., 2018).

Equipment involved in drying method can be tray dryers, rotary dryers, vacuum dryers and freeze dryer. Other drying process can be done by air dry, microwave treatment and infrared drying which it able to improve or preserve the nutritional and functional values. Tray dryers involve in stacking the tray inside large insulated chamber, while vacuum dryers can be more costly but suitable to use on material that sensitive to heat. Freeze dryer used the sublimation process and out of dehydration technique used, freeze dryer have the highest quality yield (Chang, Lin, Chang, & Liu, 2006).

Chang, Lin, Chang, & Liu (2006), had compared the freeze dried and hot air dried tomato on the antioxidant properties of the sample. Based on the results, the fresh tomatoes contain lowest phenolic content whereas the tomato that undergoes hot air dried treatment, experience increase number of phenolic content. The fresh sample of tomatoes contains lowest amount flavonoids compared to the sample that undergo hot air dried treatment. According to Todd, (2014) the samples that undergo high and low temperature treatment could cause significantly increase in the total flavonoids content. The DPPH radical scavenging activity shows that tomatoes contain high properties of scavenging effect, where with treatment of hot air dried treatment give the highest DPPH radical scavenging activities followed by freeze dried treatment and fresh samples. Based on the results obtain

by Chang et al. (2006), it can be conclude that drying process of tomatoes could enhance the nutritional value of tomatoes by increasing the total phenolic and total flavonoids content.

Boudhrioua, Bahloul, Ben Slimen, & Kechaou (2009) had compared the total phenol contents and the colour of fresh and infrared dried olive leaves. Based on Boudhrioua et al. (2009), fresh olive leaves was easily exposed to chemical and physical reactions that can rapidly destroyed the value compounds of fresh leaves. It is crucial to removed or reduced the moisture contain of the leaves in order to improve the shelf life of the leaves. By doing so, it able to reduce the cost of the manufacturing company and avoid from any pollution happen.

Study conducted by Martín-García & Molina-Alcaide (2008) showed that drying process tend to remove the chemical analyzed compound such lignin and amino acid composition except for tannins. It is importance to maintain the colour of the olive leaves and phenols content in ensuring the quality attributes in applications industries of food. Based on the results obtained, olive leaves showed that increase in infrared drying temperature influenced the phenol content to be increased too. In addition, amount of phenols content in dried leaves remain higher compared to the fresh leaves. Due to the results obtained, it can be conclude that the drying treatment received by the sample contribute to rupture of cell wall and lead to release of phenol compounds. Thus, it is proven that samples of olive leaves that undergo infrared drying process might enhance the functional values by increasing parts of the total phenols. Besides that, destroy of cell wall contribute to oxidative and hydrolytic enzyme to be released. However, high temperature

treatment likely to destroy the enzymes and avoid loss of phenolic acid and lead to increase amount of phenolic content.

CHAPTER 3

METHODOLOGY

3.1 Materials

Chemical that were used in this study are distilled water, Folin-Ciocalteu reagent, sodium carbonate, iodine, potassium iodide, glycerol, ethanol, chloroform, concentrated sulphuric acid, sodium hydrochloride, ferric acid, quercetin, aluminium chloride and potassium acetate.

3.2 Equipment/Consumables

Equipment and consumables that were used in this study are 100 mL beaker, 250 mL beaker, 1000 mL beaker, 10 mL measuring cylinder, 100 mL measuring cylinder, 500 mL measuring cylinder, test tube, 517nm absorbance spectrophotometer, weighing scale, glass rod, 0.125µ filter paper, spatula, grinder, air-tight zipper bag, aluminium foil, magnetic stirrer, vortex, Eppendorf tube and cuvette.

3.3 Sample collection

F. macrophylla was collected from Ketereh, Kelantan. The mature and young leaves were separate and pack in plastic bag. The sample was transported to the laboratory and kept at room temperature.

3.4 Sample preparation

Amount of 100 g mature leaves sample of *F. macrophylla* was weight for evaluation. The sample was wash with distilled water twice. 6 g of dried sample was air dry for 5 days at room temperature with good ventilation. The sample was grinded for 1 min according to the methods described by Hossain, AL-Raqmi, AL-Mijizy, Weli, & Al-Riyami, (2013) with slight modification. The grinded sample of *F. macrophylla* was packed in seal zip lock plastic bag until extraction.

3.5 Extraction

In 1000 mL of beaker, with ratio 1:25, 6 g of sample with 150 mL of 70% glycerol was placed and stir using magnetic stirrer, at 400 rpm for 2 hours. According to the methods described by Apostolakis, Grigorakis, & Makris, (2014) with slight changes, the extract undergoes filtration by using filtration paper (0.125µ) and store at -20˚C until further

analysis. The extraction above was repeated by using 90% ethanol as comparison. The yield of extraction will be calculated as below:

% Yield= (Weight/Volume) X 100% = % (w/v)

3.6 Antioxidant Content

3.6.1 Total phenolic content

Total phenolic content of mature Flemingia macrophylla leaves were determined quantitatively by using Folin-Ciocalteu method. Buslima, Shaharudin, Tang, & Muhamad (2016) describe the method of using Folin-Ciocalteu reagent to determine the total content of phenolic by dilute the Folin-Ciocalteu with using dimethyl sulfoxide (DMSO) as a solvent. In a test tube, 1.5 mL of 10% Folin-Ciocalteu was prepared and 0.5 mL of extract was taken and vortexed. The mixture was incubated for 5 min. The mixture was added with 2 mL of sodium carbonate and undergo incubation process for another 2 hours at room temperature. By using UV-Vis spectrophotometer, the value of absorbance was recorded at 765 nm. Similar procedure is repeated by using gallic acid as a standard and graph was constructed with range of 6.25 to 400 µg/mL. The concentration of total phenolic content will be expressed in mg gallic acid and equivalent (GAE) per gram raw material.

3.6.2 Total flavonoid content

Total flavonoid content were determined by using aluminium chloride method. According to method describe by Iqbal et al. (2015) with slight modification, in determining flavonoid content, quercetin was used as a standard. The quercetin was prepared in the range of 6.25 to 400 µg/mL. Stock solution was prepared by dissolving 0.01 g of quercetin in 10 mL of methanol. 0.3 mL of the extracted sample was taken 150 µL of 0.3 M $AlCl_3$ hexahydrate. The mixture was incubated at room temperature (24˚C) for 5 min and followed by addition of 1 mL of 1 M NaOH. After incubate for 15 min, the absorbance spectrophotometer is taken place at 506nm. The concentration of flavonoid is express in mg quercetin equivalent (QE) per gram of raw material.

3.7 Antioxidant activity via DPPH radical-scavenging assay

According to Buslima et al. (2016) with using 2,2-diphenyl-picrylhydrazyl (DPPH) reagent with slight modification where the standard is prepare with different concentrations range from 6.25 µl/mL to 400 µl/mL of ethanol. BHT was used as a standard. 0.004 g of DPPH were diluted with 2 mL of ethanol and incubated for 30 min in dark room. 2 mL of ethanol and glycerol extracts were prepare and mixed with DPPH solvent. By using UV-Vis spectrophotometer, the absorbance reading was measured at 517 nm against the blank.

The graph of scavenging activity is plotted. The percentage of scavenging activity will be calculated as followed:

DPPH radical scavenging activity (%) = $[A_0 – A_1/ A_0] X 100\%$

A_0 - Absorbance without sample

A_1 – Absorbance with sample

3.8 Partial Chemical Analysis

3.8.1 Ferric Chloride test

Based on method of RNS Yadav (2011), 2 mL of 2% ferric chloride ($FeCl_3$) solution was mixed with young seringan leaves extract. A green-yellowish indicated the presence of phenols and tanins.

3.8.2 Test for flavonoids

According to Rao, Abdurrazak, & Mohd (2016), one to five drops of concentrated hydrochloric acid (HCI) were added to the sample extracts. Instant development of red colour indicates the presence of flavonoids.

3.8.3 Wagner's test

According to the method describe by Iqbal et al., (2015), 2 g of Potassium iodide and 1.27 g of iodine was dissolved with 5 mL of distilled water and dilute with 100 mL of distilled water. Few drops of the solution was added to the filtrate and brown colour precipitate appeared indicate the presence of alkaloids.

3.8.4 Salkowski's test

According to the method describe by Khanam et al., (2015), with slight modification, 0.5 mg of extraction was added in 5 mL of chloroform followed with concentrated sulphuric acid to form a layer. The presence of terpenoids will indicate by reddish brown colour.

3.9 Statistical analysis

All test were done in triplicate and mean value was taken for calculation. The experimental results were expressed using statistical analysis as mean ± standard deviation (SD) of three parallel measurements by using Microsoft Excel. Student's t-test was used as

comparison between two mean and difference is considered statistically significant when $p \leq 0.05$.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Preparation of extract

This study was done to determine the effect of different solvent extract on matured *F. macrophylla* leaves through antioxidant activity, flavonoid and phenolic content determination as well as phytochemical screening. The samples were dried for a week to remove all the moisture content in room condition at 24˚C with good ventilation. According to Jespersen, Zhang, & Huang, (2016), chlorophyll level will decrease significantly if exposed to heat stress, thus air dry method was used in this study. The dryness of the sample was determine by weighting the sample in the morning and evening, constant weight have verify the dryness of the sample. Based on previous studied by Sultana et al., (2009), it is reported that extraction technique might result in loss of natural antioxidants since the heat produced might accelerate their oxidation and other degenerative reactions. Hence, heating technique used in extracting is needed consideration. In this study, glycerol was used to find better alternative in replacing ethanol or methanol which could cause adverse effect in long run. The extract were left

liquid due to boiling point of glycerol is 290˚C make it more difficult to evaporate. Ethanol was used as a comparison to glycerol. Table 4.1 shows the colours of *F. macrophylla* leaves extracts. Ethanol extract appeared in intense green colour while glycerol extract appeared in green yellowish colour. The presence of green colour of the *F. macrophylla* leaves is due to chlorophyll content in the *F. macrophylla* leaves (Du et al., 2017).

Table 4.1 Colours of *F. macrophylla* leaves extracts.

Extracts	Colour
Ethanol	Green
Glycerol	Green yellowish

4.2 Determination of Free Radical Scavenging by using DPPH Assay (BHT)

Radical scavenging activity of plant extracts was determine by spectrophotometrically against DPPH (2,2-diphenyl-picrylhydrazyl). The purple colour of DPPH radicals will turn to light yellow when reacts with antioxidant compound, which has the ability to donate hydrogen and free radicals will reduced (Miliauskas et al., 2004).

The relation between BHT and samples were showed in Figure 4.1, the value obtained based on the concentrations of BHT as a standard are 6.25, 12.5, 25, 50, 100 and 200 µg/ml, under 517 nm absorbance of spectrophotometer detection. The graph plotted based on the concentration of BHT versus percentage of scavenging. The graph indicates the percentage of the scavenging increase as the concentration of BHT increase. Based on Figure 4.1, the percentages of scavenging are range from 21.28 to 76.81%. Figure 4.1

shows the calibration curve of BHT with correlation coefficient (R^2) value obtained is 0.9093 and the equation of the graph of scavenging activity is y = 0.2758x + 26.886.

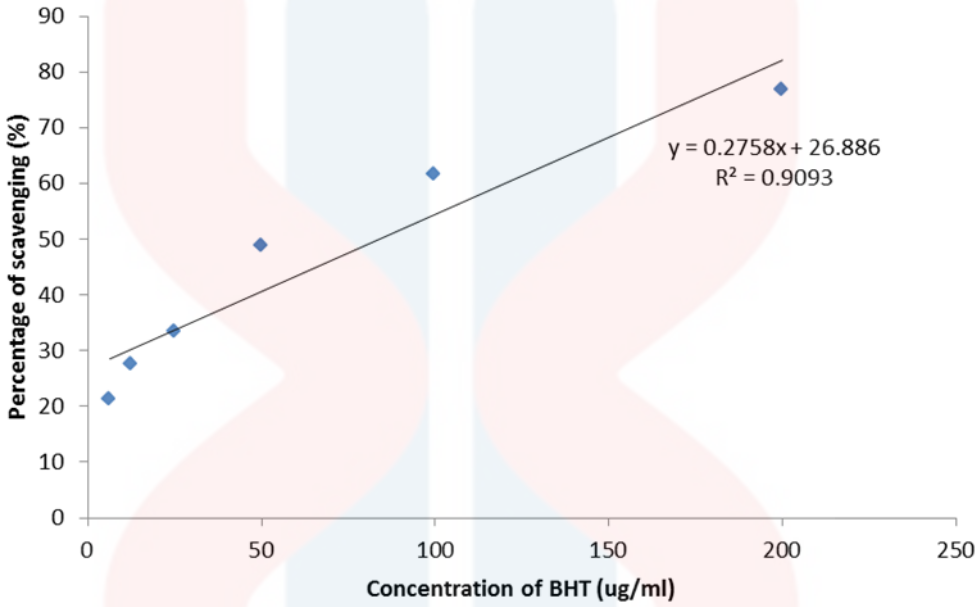

Figure 4.1 Graph of BHT calibration curve at 6.25 to 200 µg/mL.

BHT was used as a standard in this assay over ascorbic acids due to BHT able to provide large range of absorbance value compared to ascorbic acid. Several test have been tested with using ascorbic acids, gallic acids and BHT and it is found that BHT the most suitable to be used since the range was fall in the sample extracts range.

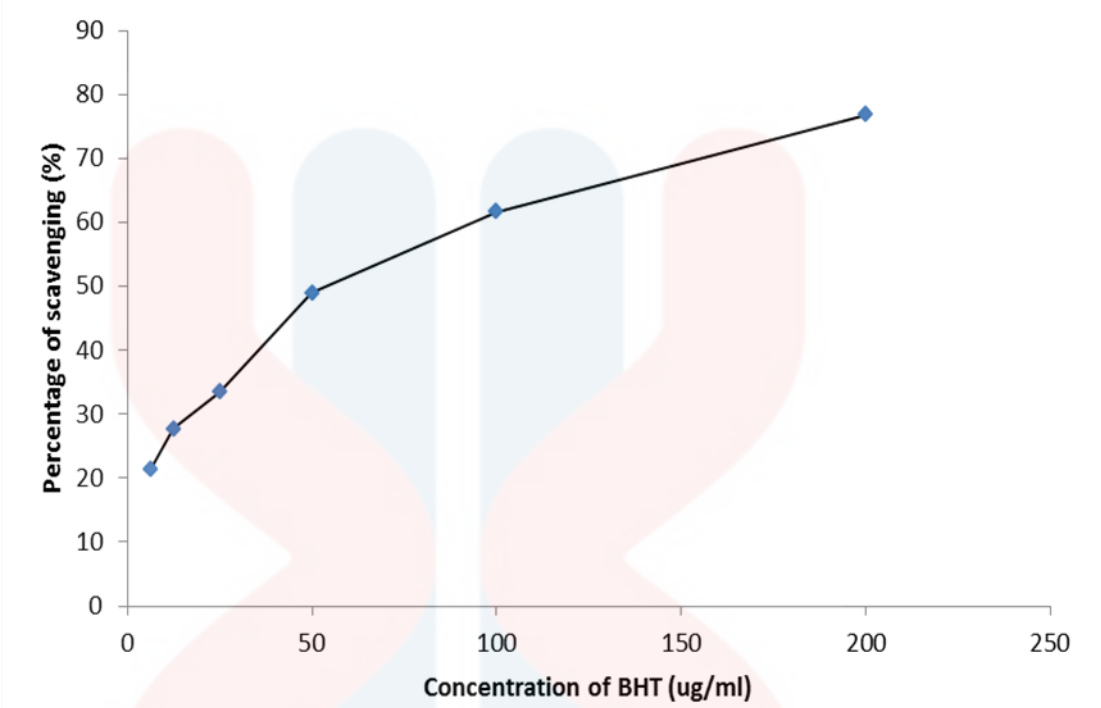

Figure 4.2 Graph of BHT equivalent curve against ethanol and glycerol of extract *F. macrophylla* (6 g raw material).

The antioxidant capacity for mg BHT equivalent per 1 g of raw material from sample was shown in Figure 4.3. The bar graph obtain was showed in appendix Figure A.1. The results showed obtain from samples are represented by mean ± standard deviation where ethanol extract exhibit (28.90 ± 1.69 mg BHTE/ g raw material) and glycerol extract (19.444 ± 1.889 mg BHTE/ g raw material). The factors of the amount of antioxidant compounds extracted from a plant sample is the efficiency of the extraction solvent in dissolving endogenous compounds (Sultana et al., 2009). The antioxidant activity of ethanol extract is higher compared to glycerol extract. Results clearly show that antioxidant of ethanol extract more effectively in scavenged free radical compared to glycerol extract (Alam et al., 2013).

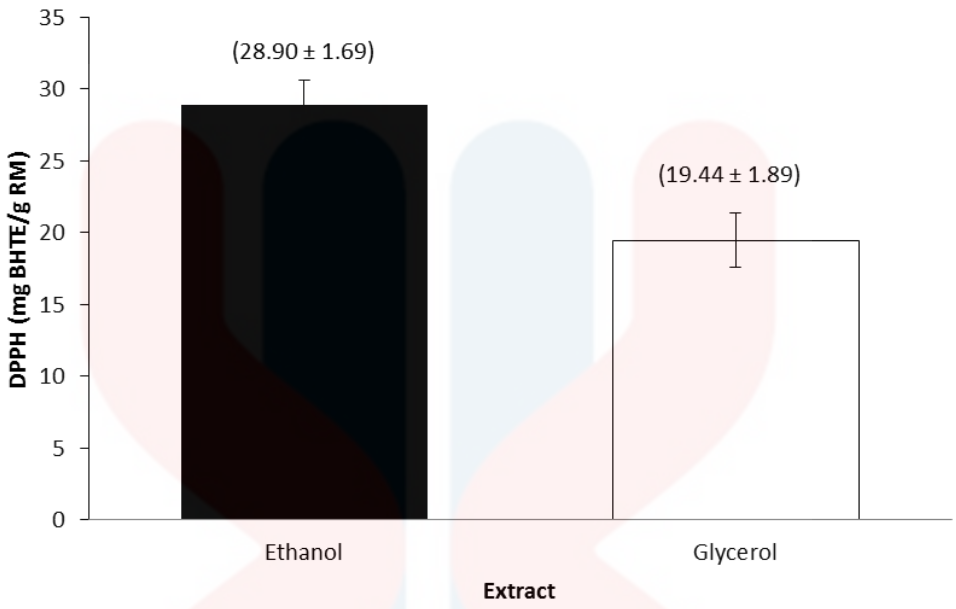

Figure 4.3 DPPH capacity against ethanol and glycerol extract of *F. macrophylla*.

Based on Figure 4.3, analysis of means (T-test) used to find the different of mean in antioxidant activity between samples. The antioxidant capacity between the ethanol and glycerol showed significant different at $P \leq 0.05$ level. Among many organic solvent such as DMSO and toluene were shown ineffective reaction as extraction solvent in which, only glycerol was found to be capable of extracting desirable compounds which obtained with 68% yield (Wolfson, Dlugy, Shotland, & Tavor, 2009). Based on previous publication by Sultana et al., (2009), the best extraction solvents that able to exhibit highest antioxidants compounds of plant root extract is aqueous solvent ethanol and methanol by using reflux and shaker extraction technique, which explain the polarity of the solvent play important factor in plant extraction.

According to Wang et al., (2012), the study of antioxidant properties of *Flemingia macrophylla* on the root part of the plant, it shown that the percentage of scavenging value

is (20.1 ± 0.3%). This shown that different parts of the plant, displayed different values of antioxidant properties. This may due to roots may have selected way to bypass certain chemical protection or toxin present, although roots have relatively low activity of antioxidant properties, roots have a strong structure and have the ability to defend temporary injury (McCune & Johns, 2007).

More antioxidant compound variety occur within the leaves compared to the root part of the plant. Flavonoids that can be found include phloretin, quercetin, catechin and myricetin (Hossain et al., 2013). Based on previous study by Shori (2015), *F. macrophylla* possess the highest radical scavenging activity followed by *F.lineata* and *F.strobilifera.* Different extracted part of *F.strobilifera* root and leaves contain polyphenolic compound such as flavonoids, tannins, phenolic compounds and steroid which act as free radical scavenging (Madan et al., 2010). *F.strobilifera* and *F.macrophylla* belong in the same family Paplionaceae which both plants share similar features therefore, phytochemical screening were generated and results found both plant share similar phytochemical compounds (Ghalot et al., 2012).

4.3 Determination of Total Phenolic Content

The total phenolic content of the *F. macrophylla* mature leaves were determined by using quantitative analysis based on Folin-Ciocalteu method. Phenolic compound is one of the categories of phytochemical found in plants. Phenolic compound have the ability to transfer electrons to Folin-Ciocalteu reagent in alkaline medium. Folin-Ciocalteu reagent

consist of sodium molybdate, phosphomolybdic, phosphotungstic acid, sodium tungstate and heteropoly acids (Iqbal et al., 2015). The reaction between the plant extracts and Folin-Ciocalteu reagent which is redox reagent will forms blue complex and observed by visible light spectrophotometry (Kannika Panyaphu, Panee Sirisa-ard, Preeyawis Na Ubol & Sunee Chansakaow, 2012). The results are expressed as gallic acid equivalents (GAE).

The relation between gallic acids and samples were showed in Figure 4.4, the value of gallic acids as a standard obtained based on the concentration 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL, under 765 nm absorbance of spectrophotometer detection. Figure 4.4 was plotted based on the concentration of gallic acids versus percentage of scavenging. The graph indicates the percentage of the scavenging increase as the concentration of BHT increase. Based on Figure 4.4 the value of absorbance are ranges from 0.09 to 3.27. The linear calibration curve of gallic acid with correlation coefficient (R^2) value obtained is 0.9945 and the equation of the regression linear graph is y = 0081x + 0.1069.

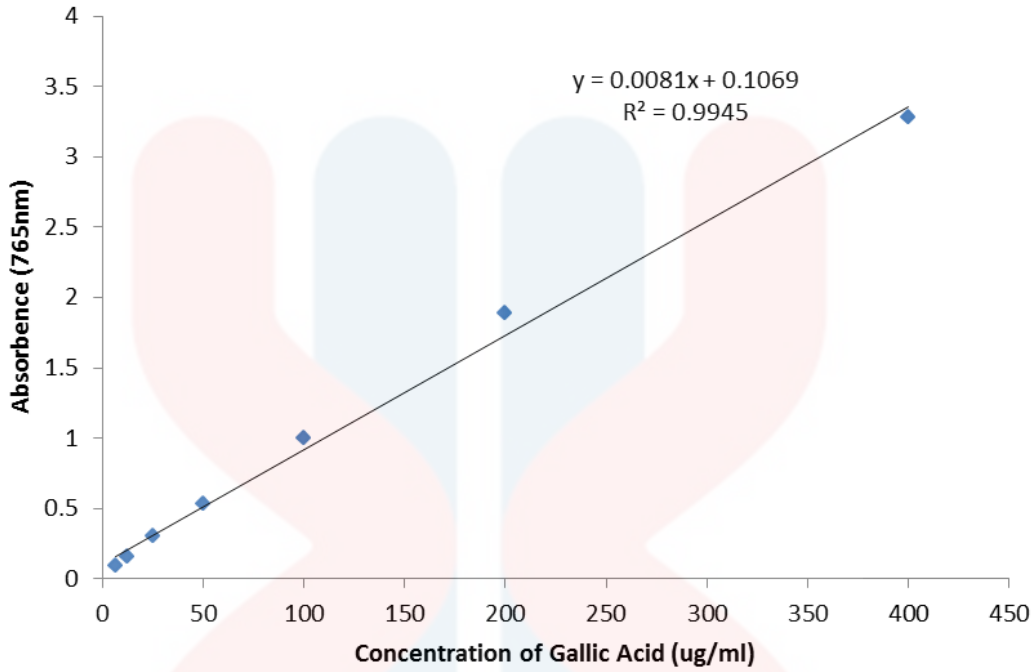

Figure 4.4 Gallic acids standard calibration curve at concentration 6.25 to 400 µg/mL.

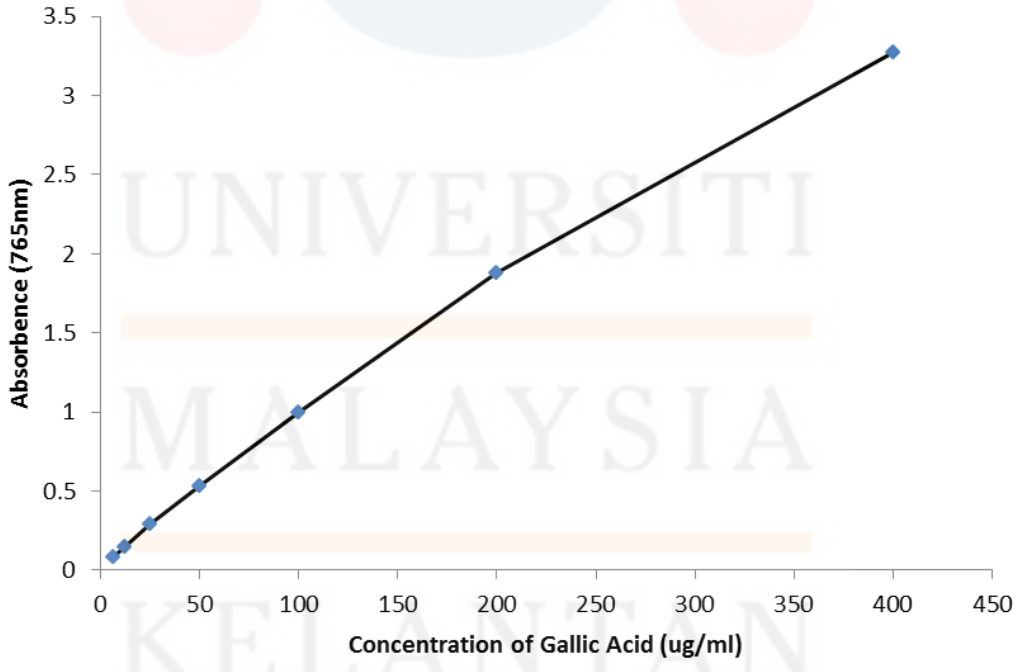

Figure 4.5 Graph of Gallic acid equivalent curves against ethanol and glycerol extract of *F. macrophylla.*

The antioxidant capacity for mg gallic acids equivalent per 1 g of raw material from sample was shown in Figure 4.6. The bar graph obtain was showed in appendix Figure A.2. The results obtain from samples are represented by mean ± standard deviation where ethanol extract (19.58 ± 0.08 mg GAE/ g raw material) and glycerol extract (40.21 ± 0.08 mg GAE/ g raw material). Analysis of means (T-test) used to find the different of mean in antioxidant activity between samples. The antioxidant capacity between the ethanol and glycerol showed significant different at $P \leq 0.05$ level.

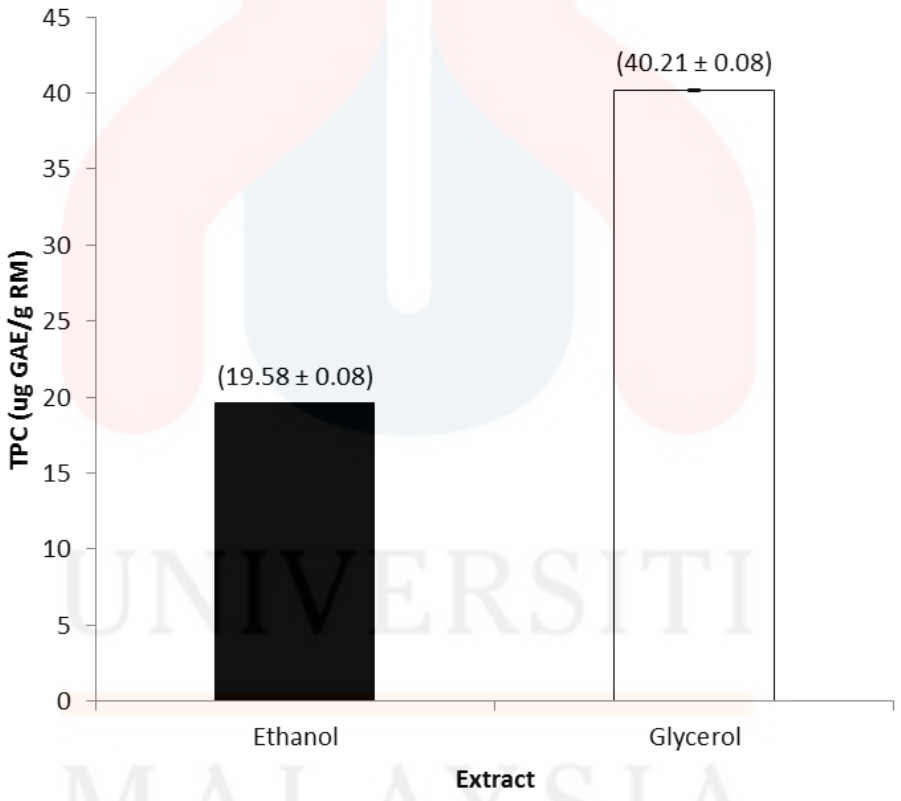

Figure 4.6 TPC capacity against ethanol and glycerol extract of *F. macrophylla.*

Glycerol show highest value compared to ethanol extract, this is due to, phenolics are often extracted in higher amounts of polar solvents such as glycerol as compared with

ethanol (Sultana et al., 2009). Glycerol is highly polar volatile organic compound with high boiling point thus it is more effective solvent than water in absorbing highly polar solutes (Scheepers & Muzenda, 2015). However, the efficiency of glycerol as extraction solvent based on polarity that able to extract phenolic compounds are been argued. Based on previous study that phenolic might be simply soluble in polar protic solvent yet other factors might restrict such as intermolecular forces between extract and solvents, the solubility of phenols in different solvent can be complicated (Shehata, Grigorakis, Loupassaki, & Makris, 2015).

4.4 Determination of Total Flavonoid Content

The total flavonoid content (TFC) of *F. macrophylla* mature leaves extracts were determined by using Aluminium Chloride Colorimetric method. The relation between quercetin and samples were showed in Figure 4.7, the values obtained are based on the concentration of quercetin as a standard at 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL, under 506 nm absorbance of spectrophotometer detection. The graph plotted based on the concentration of quercetin versus absorbance value. The graph indicates the value of the absorbance increase as the concentration of QE increase. Based on Figure 4.7 the absorbance value are ranges from 0.01 to 0.17. The linear calibration curve of quercetin with correlation coefficient (R^2) value obtained is 0.9991 and the equation of the regression linear graph is y = 0004x + 0.0056.

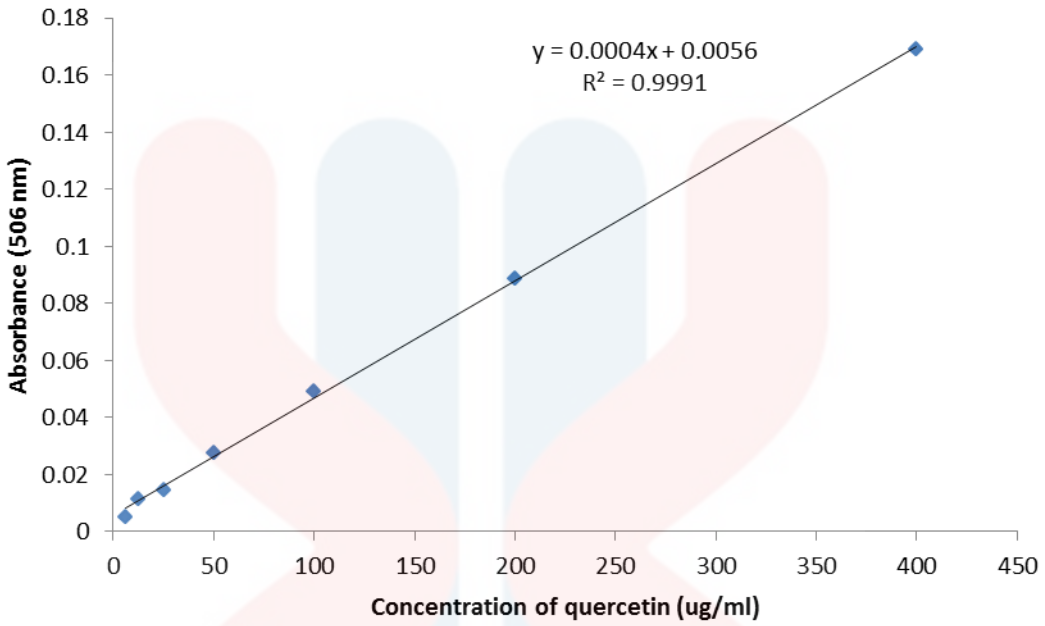

Figure 4.7 Quercetin standard calibration curve at concentration 6.25 to 400 µg/mL.

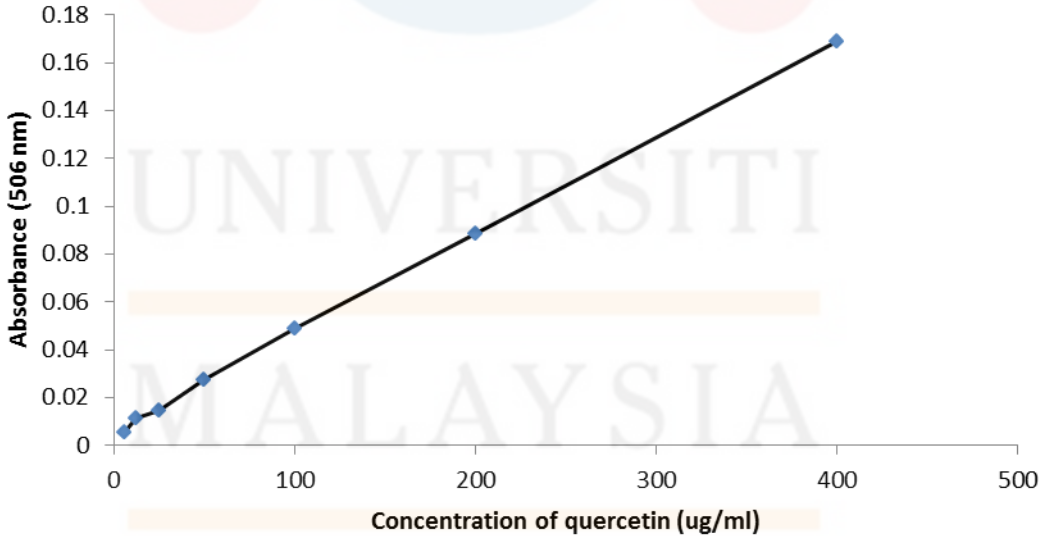

Figure 4.8 Graph of Quercetin equivalent curve against glycerol and ethanol extract of *F. macrophylla.*

The antioxidant capacity for mg quercetin equivalent per 1 g of raw material from sample was shown in Figure 4.9. The bar graph obtain was showed in appendix Figure A.3. The results obtain from samples are represented by mean ± standard deviation where ethanol extract and glycerol extract are (52.56 ± 0.72 mg QE/ g raw material) and (43.71 ± 0.63 mg QE/ g raw material) respectively. Based on Figure 4.9, analysis of means (T-test) used to find the different of mean in antioxidant activity between samples. The antioxidant capacity between the ethanol and glycerol showed significant different at $P \leq 0.05$ level.

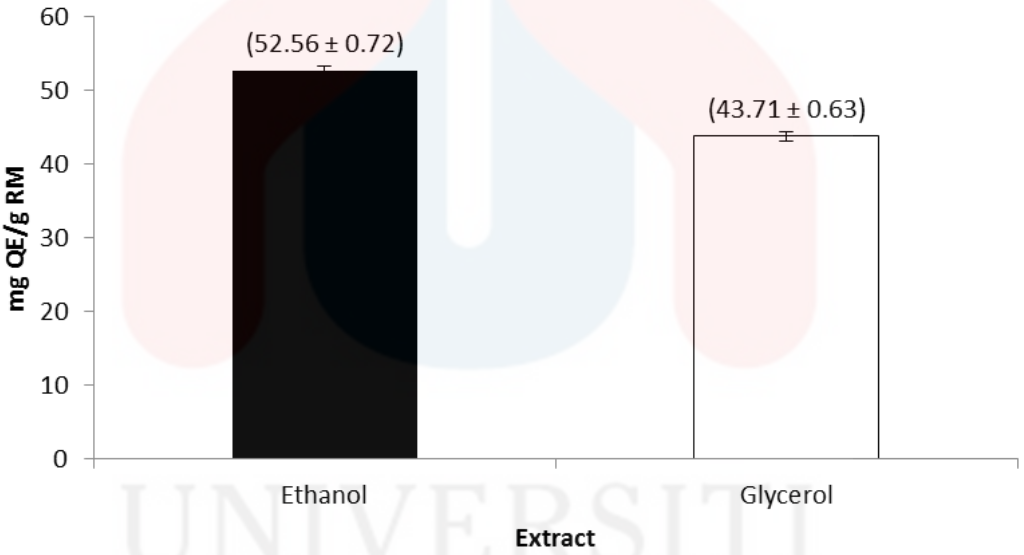

Figure 4.9 TFC capacity against ethanol and glycerol extract of *F. macrophylla.*

Based on Figure 4.9, it is shown that ethanol extracts is higher compared to glycerol extracts. Glycerol has the ability to dissolve in many organic and inorganic compounds which allow separation of desire compounds. However, glycerol is known for its high viscosity and low solubility of highly hydrophobic compounds which limits its usage (García, García-marín, & Pires, 2010). Based on Table 4.1 observation, qualitatively the

colour produce of glycerol extracts is yellowish green which indicates, less amount of chlorophyll being extracts compared to ethanol extract. Thus, compounds that are being extract by glycerol might be lesser compared to ethanol extracts which result in lower TFC.

Besides, the time of extraction is important in the extraction of bioactive compounds. In optimizing the extraction process, the time of extraction is crucial since the bioactive compounds will degrade in prolonged extraction time (Efthymiopoulos et al., 2018). However, the extraction time in this study, took 2 days during the filtration process and might cause the bioactive compounds of *F.macrophylla* degraded over time.

4.5 Phytochemical screening

The leaves of *F. macrophylla* are claimed able to treat several diseases such as fever and traditionally used in confinement process. The compound presence such as flavonoids, alkaloids, terpenoids, steroid and tannins inside the *F. macrophylla* leaves might have the ability to help in curing certain disease. Based on Table 4.2, phytochemical screening of the *F. macrophylla* leaves, it is shown presence of phytochemical compounds which are phenolic compounds, terpenoids, alkaloids and flavonoids for both ethanol and glycerol extracts.

Table 4.2 Phytochemical screening of *F.macrophylla* mature leaves extract.

Test	**Ethanol extract**	**Glycerol extract**
Phenolic compound	+	+
Terpenoids	+	+
Alkaloids	+	+
Flavonoids	+	+

*Note: + indicate presence; - indicate absence

Green-yellowish indicates the presence of phenols and tannin in mature *F. macrophylla* leaves extract. Tannins generally belong to a group of polymeric phenolic substances which capable precipitating gelatin from solutions Perumal Samy & Gopalakrishnakone (2010). Tannins can be found in almost every part of the plant. Tannins are water soluble and act as natural defense mechanism against microbial infections within a fruit. Other compound that presence is phenolics compound which is the simplest bioactive phytochemicals consist of single substituted phenolic ring. It is commonly used in traditional way as anti-bacterial and the properties can be used in functional food development for food preservatives (Perumal Samy & Gopalakrishnakone, 2010).

Presences of terpenoids compound indicate reddish brown colour was detected in mature *F. macrophylla* leaves. Terpenoids such as diterpenes, triterpenes and sesquiterpenes have been widely used in the pharmaceutical industry for antibiotics, insecticidal, antiseptic and anthelmintic (Khanam et al., 2015).

For Wagner's test, the appearance of brown precipitate indicate the presence of alkaloids that have been found in the *F. macrophylla* leaves which is one of the most common and largest group of secondary metabolites found in the plant (Igbinosa, Igbinosa, & Aiyegoro, 2009). Alkaloids have been developed into pain killer medications and have beneficial effect on human (Iqbal et al., 2015).

Another secondary metabolites that are found in mature *F. macrophylla* leaves extracts was flavonoid that belong to the group of polyphenolic compounds and usually helps in many ways such as antimicrobial, anti-angionic, anti-allergic, anti-cancer and antioxidant properties that helps in health promoting (Hossain et al., 2013). The development of red colour indicates the presence of flavonoid in this test (Rao et al., 2016).

CHAPTER 5

CONCLUSION & RECOMMENDATION

5.1 Conclusion

The ethanol extract of mature *Flemingia macrophylla* leaves showed high antioxidant activity by inhibiting the radical scavenging activity (DPPH) and Total Flavonoid Content. The glycerol extract of mature *Flemingia macrophylla* leaves showed high Total Phenolic Content. There were significant difference in antioxidant activity and antioxidant content. The phytochemical screening of mature *Flemingia macrophylla* leaves showed positive results in both ethanol and glycerol extracts. Overall, ethanol show better extraction solvent in antioxidants activity and phenolic qualification in *Flemingia macrophylla* leaves however, flavonoid qualification showed glycerol is good enough to substitute ethanol as extraction solvent thus, it support the usage of safer solvent extraction.

5.2 Recommendations

Further research is needed to identify individual compounds forming antioxidant system and development of their applications for pharmaceutical industries. Mature *Flemingia macrophylla* leaves able to commercialize as effective natural antioxidants. The limitation of this study is, one method of determining the antioxidants activity is not enough therefore, it is suggested to use other radical scavenging assay such as ABTS and FRAP. Further phytochemical analysis thru chromatography method can be use for further analysis. Before new glycerol derived solvents can be massively used, there are other issues needed to be addressed in terms of the solvent selectivity. Safety recommendation can be used before dealing with ethanol extracts in order to avoid any adverse effect to the health condition.

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APPENDIX A

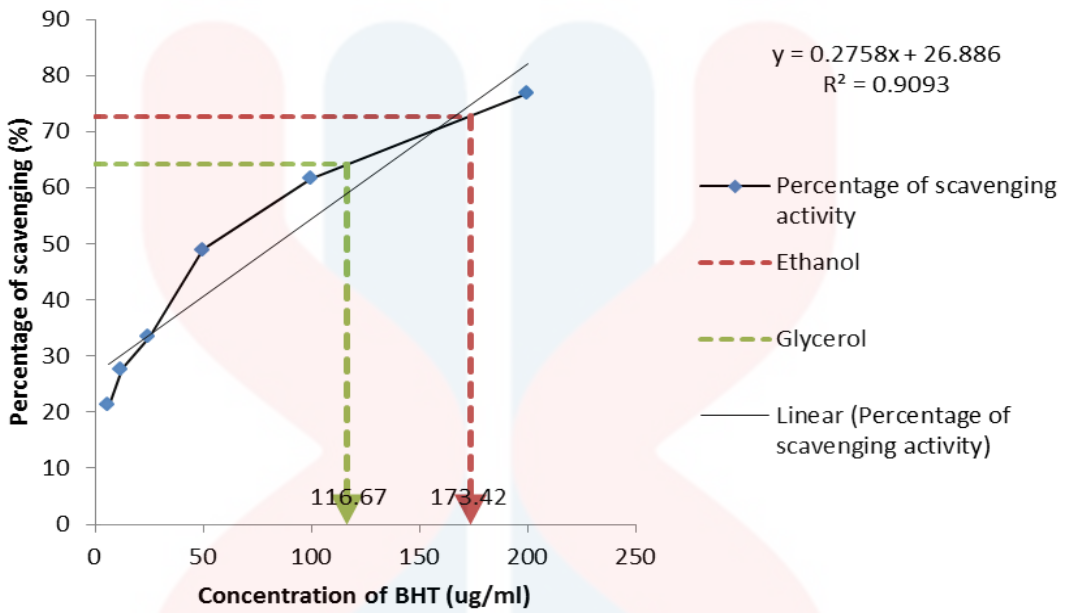

Figure A.1 Graph of concentration extract obtained based on mean value by using DPPH

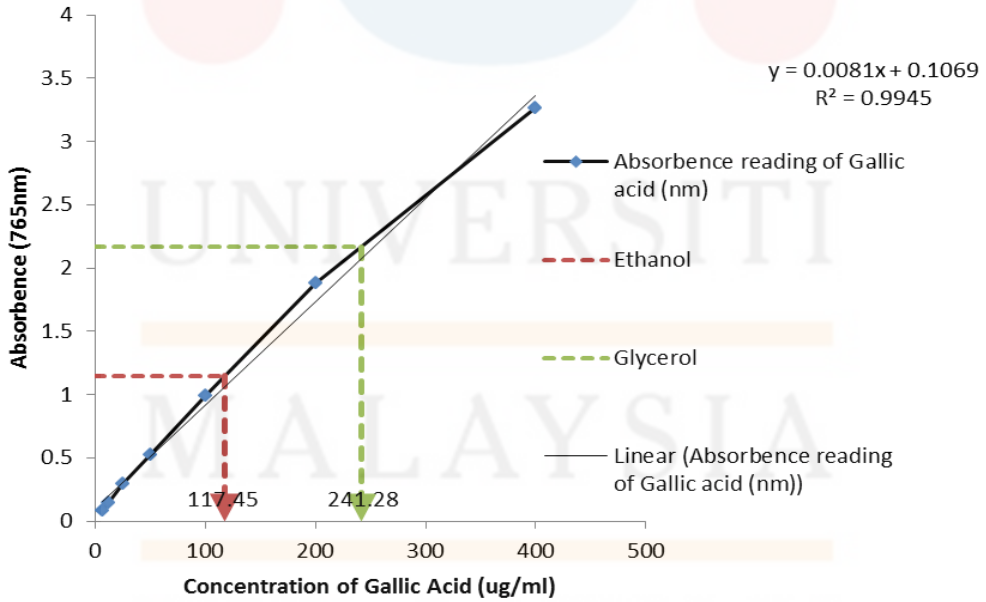

Figure A.2 Graph of concentration extract obtained based on mean value by using TPC

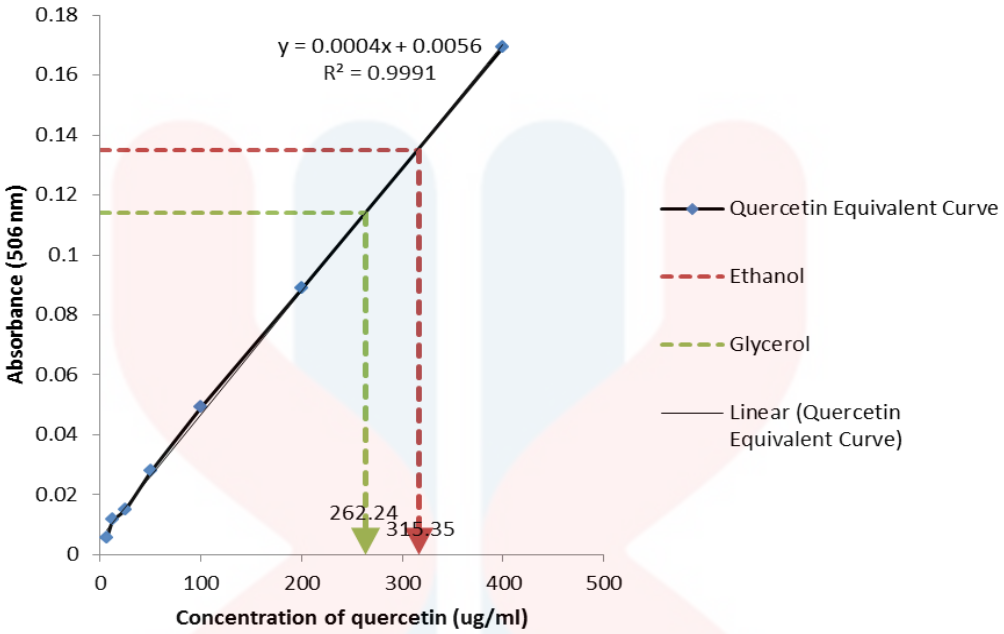

Figure A.3 Graph of concentration extract obtained based on mean value by using TFC

APPENDIX B

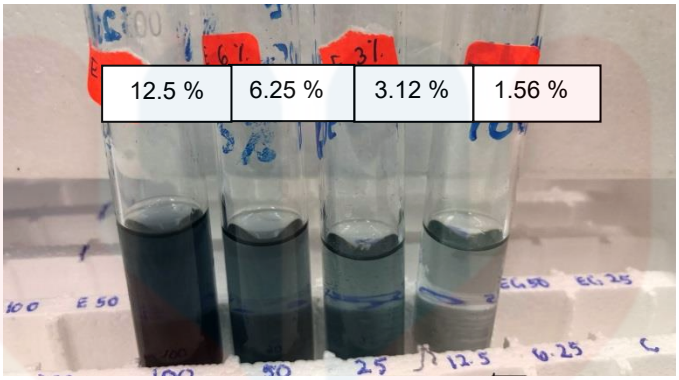

Figure B.1 Folin-Ciocalteu reagent with ethanol extract

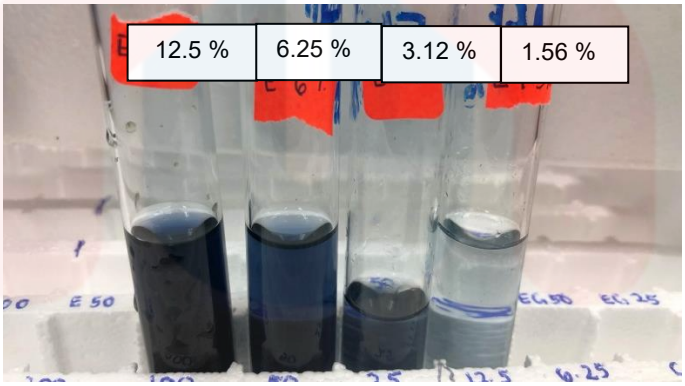

Figure B.2 Folin-Ciocalteu reagent with glycerol extract

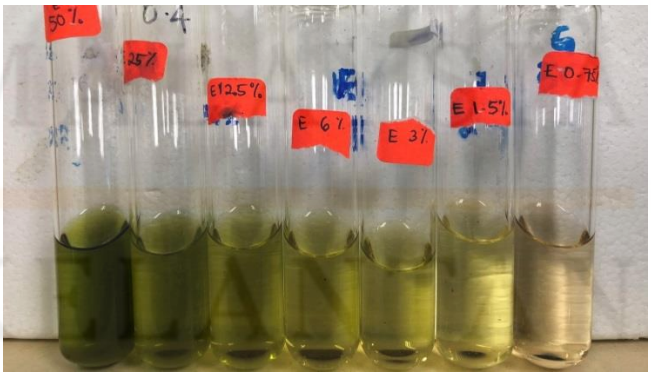

Figure B.3 Aluminium Chloride reagent with ethanol extract.

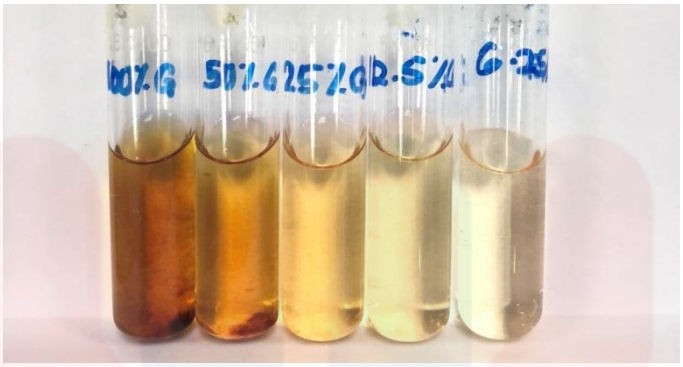

Figure B.4 Aluminium Chloride reagent with glycerol extract.

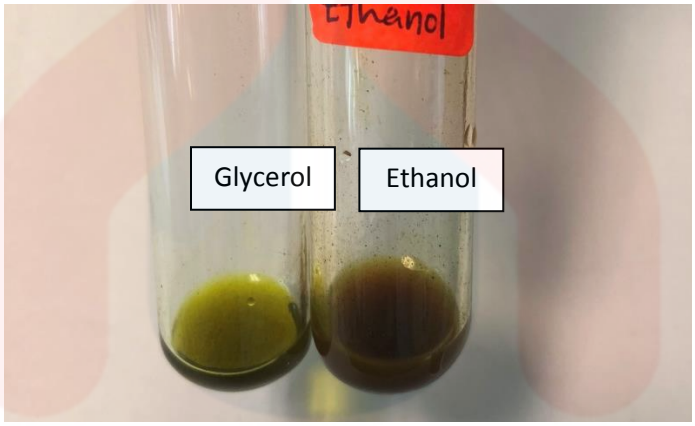

Figure B.5 Appearance of green-yellowish colour for test of phenols and tannins

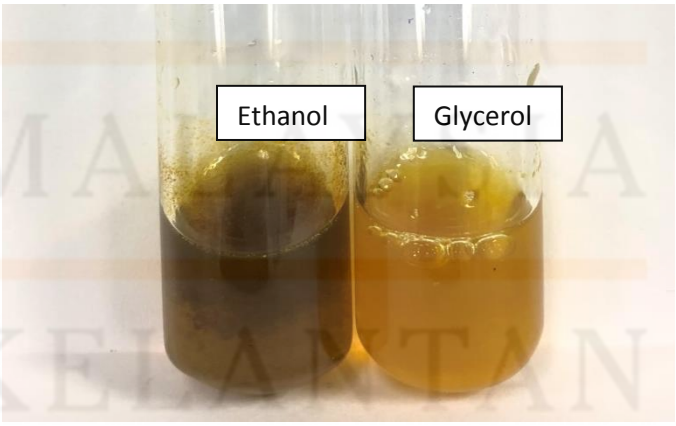

Figure B.6 Appearance of brown colour precipitate for flavonoids test.

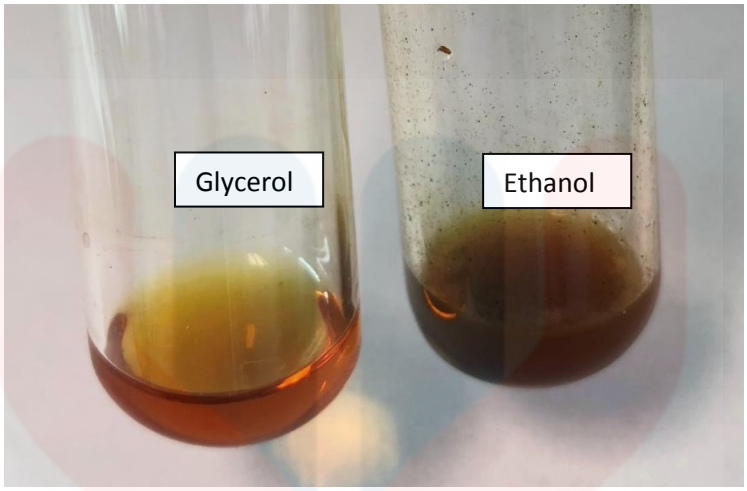

Figure B.7 Appearance of reddish brown colour for terpenoids test.

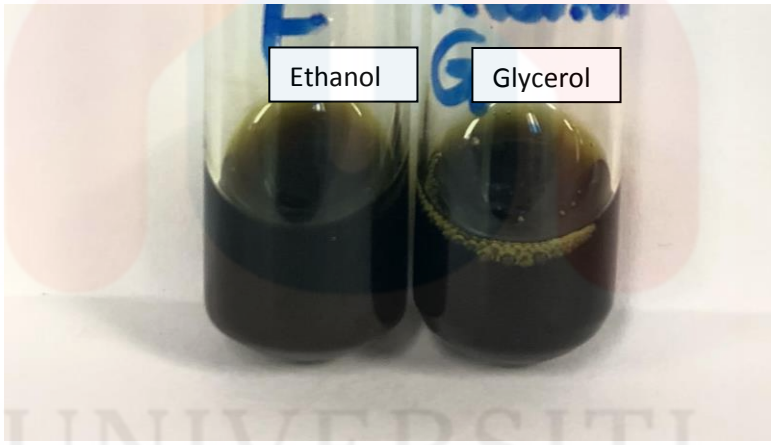

Figure B.8 Appearance of dark brown colour indicates that presence of alkaloids.