Determination of Antioxidant, Antidiabetic and Toxicity Capabilities of *Christia vespertilionis* (Pokok Rerama) Leaves

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A report submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Product Development Technology) with Honours

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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 and instituitons.

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Sudent

Name: Adibah Binti Bin Yamin

Date:

I certify that the report of this final year project entitled "Determination of Antioxidant, Antidiabetic and Toxicity Capabilities of *Christia vespertilionis* Leaves" by Adibah Binti Bin Yamin, matric number F15A0002, has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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ABSTRACT

Diabetes mellitus has been one of public health concern since years ago. The prevention and treatment of the metabolic disorder using medicinal plant has been done broadly in previous studies. *Christia vespertillionis* is known to have medicinal properties to treat various health problems because of its phytochemicals. This study characterizes total phenolic content, antioxidant and antidiabetic activities, and also toxicity evaluation in the leaves of *Christia vespertillionis*. Total phenolic content of *Christia vespertillionis* leaves was determined by improved Folin-Ciocalteu method. The antioxidant activity of the leaves sample was determined by DPPH assay with butylated hydroxytoluene (BHT) used as reference compound. Antidiabetic activity of *Christia vespertillionis* leaves was determined using α-amylase inhibition assay, and metformin was used as standard. Besides, the cytotoxicity of compounds in *Christia vespertillionis* leaves extract was investigated using brine shrimp lethality bioassay. The total phenolic content in the leaves extract was found to be 128.852 ± 3.90 mg gallic acid equivalent per g of dried sample. The percentage of free radical scavenging activity of *Christia vespertillionis* leaves varied from 34.72% to 2.01% from the highest (200 mg/ml) to the lowest (6.25 mg/ml) concentrations. The IC_{50} value calculated for its antioxidant activity was found to be 39.987 mg/ml. The percentage of α-amylase inhibition in concentration 250 mg/ml of *Christia vespertilionis* leaves extract was 23.33%, while 20.14% in concentration 500 mg/ml and followed by 15.34% in concentration 125 mg/ml. The IC_{50} value for α-amylase inhibitory activity in the extract was found to be 35.2 mg/ml. For toxicity evaluation of *Christia vespertilionis* crude extract, the percentage of mortality of brine shrimp varied from 21.59% to 10.87% from the highest (200 mg/ml) to the lowest (12.5 mg/ml) concentration. The level of toxicity of the extract was low, thus increase in potential therapeutic property. The study revealed that the extract of *Christia vespertilionis* has potential biological efficiencies.

Keywords: *Christia vespertilionis, diabetes mellitus, antidiabetic, antioxidant, toxicity*

Penentuan Keupayaan Antioxidant, Antidiabetik dan Ketoksikan dalam daun *Christia vespertilionis* (Pokok Rerama)

ABSTRAK

Diabetes mellitus telah menjadi kebimbangan kesihatan awam sejak bertahun-tahun yang lalu. Pencegahan dan rawatan untuk gangguan metabolik ini telah dilakukan secara meluas dalam kajian-kajian terdahulu. *Christia vespertillionis* diketahui mempunyai sifat perubatan untuk merawat pelbagai masalah kesihatan kerana kandungan fitokimianya. Tujuan kajian ini dilakukan adalah untuk mengkaji jumlah kandungan fenolik, antioksidan dan antidiabetik, dan juga penilaian toksik dalam daun *Christia vespertillionis.* Kandungan fenolik dalam daun *Christia vespertillionis* ditentukan melalui kaedah Folin-Ciocalteu yang telah ditambahbaik. Aktiviti antioksidan sampel daun ini ditentukan melalui assay DPPH dengan menggunakan butylated hydroxytoluene (BHT) sebagai sebatian rujukan. Aktiviti antidiabetik daun *Christia vespertillionis* ditentukan menggunakan ujian perencatan α-amilase. Dalam kajian ini, metformin digunakan sebagai kawalan positif. Selain itu, ketoksikan ekstrak daun *Christia vespertillionis* juga diuji menggunakan bioassay udang air garam. Kandungan fenolik dalam ekstrak daun *Christia vespertilionis* didapati mengandungi sebanyak 128.852 ± 3.90 mg setara asid gallic per g sampel kering. Peratusan aktiviti pelupusan radikal bebas dari daun *Christia vespertillionis* berbeza dari 34.72% hingga 2.01% daripada kepekatan tertinggi (200 mg/ml) hingga kepekatan terendah (6.25 mg/ml). Nilai IC_{50} yang dikira untuk aktiviti antioksidan ekstrak daun *Christia vespertilionis* adalah didapati sebanyak 39.987 mg/ml. Peratusan perencatan α-amilase dalam kepekatan 250 mg /ml ekstrak daun *Christia vespertilionis* adalah 23.33%, manakala 20.14% dalam kepekatan 500 mg/ml dan diikuti dengan 15.34% dalam kepekatan 125 mg/ml. Nilai IC_{50} untuk aktiviti perencatan α-amilase dalam ekstrak daun *Christia vespertilionis* didapati 35.2 mg/ml. Untuk penilaian ketoksikan ekstrak daun *Christia vespertilionis*, peratusan kematian udang air garam bervariasi dari 21.59% hingga 10.87%, dari kepekatan tertinggi (200 mg/ml) hingga kepekatan terendah (12.5 mg/ml). Tahap ketoksikan ekstrak tersebut adalah rendah, oleh itu ia mempunyai potensi sebagai bahan terapeutik. Kajian ini mendedahkan bahawa ekstrak daun *Christia vespertilionis* mempunyai potensi kecekapan biologi.

Kata kunci: *Christia vespertilionis, diabetes mellitus, antidiabetik, antioksida, ketoksikan*

CHAPTER 1

INTRODUCTION

1.1 Research Background

Currently, there are many research made regarding plants that contain antioxidant compounds and their potential to treat diseases. Since years ago, medicinal plants have been recognized and utilized broadly due to their tendency to treat diseases with less side effects to the consumer (Pan *et al.,* 2013) Phytochemicals in plant's extracts are being explored to recognise their goodness and benefits to human (Leitzmann, 2016). Hence, it can be used in the formulation of modern medicine to produce drugs to treat diseases. Some plants have been used traditionally since long time ago for health treatments even though the medicinal compounds are still unknown. Therefore, broad study related to phytochemicals of potential medicinal plant should be done to recognize the compounds that are beneficial to pharmaceutical industry. However, there are some plants containing chemicals that could bring harm to human health. For this reason, toxicity evaluation needs to be done on the plant's compounds to ensure that it is safe for human consumption (Bode & Dong, 2014).

Diabetes mellitus is one of the chronic metabolic disorder that paid attention by people around the world. The incline number of diabetic patients leads to the new discoveries on treatment of the disorder. Besides making research to treat diabetes using modern

method, studies are made on plants that said to have the potential to treat the metabolic disorder by traditional folk's practices. Based on the findings, some phytoconstituents present in certain plants are having insulin mimetic activity. Insulin is a hormone produced by the body to control glucose level in blood (Surkova, 2013). The example of plants containing these bioactive compounds are *Camellia sinensis, Citrullus colocynthis* and *Eugenia jambolana* (Patel *et al.*, 2012).

Christia vespertilionis, or also known as butterfly wing leaves in common name, is known to have medicinal properties among the traditional practitioners (Hofer *et al.*, 2013). They use the plant to treat certain illness. Before, the plant has been discovered scientifically to have medicinal properties such as having anticancer and anti-inflammatory activities (Azman, 2014). However, there is no studies made regarding the potential of the plant to have antidiabetic activity. Therefore, this study is made to determine the potential of the plant to treat diabetes and the evaluation of its toxicity for human consumption.

1.2 Problem Statement

Currently, there are a lot of discoveries made regarding plant's antioxidant activity and its potential to treat diabetes mellitus. The phenolic compounds present in certain herbal plants are proven by scientific research to have antioxidant and antidiabetic activity. The examples of plant that contain antidiabetic properties are *Diplazium esculentum*, *Ficus bengalensis* and *Cosmos caudatus.* A discovery has been done on the potential of *Christia vespertilionis* to treat cancer by researchers from Universiti Putra Malaysia (UPM) in 2014. However, there is no research has been done regarding the potential of the plant to have antidiabetic properties. Moreover, there is lack of

information regarding antioxidant activity and toxicity of the plant. This study is made to determine the potential of antidiabetic properties, antioxidant activity and evaluation of toxicity of *Christia vespertilionis* leaves.

1.3 Hypotheses

H_o: There are no significant biological activities such as antioxidant, antidiabetic activity, and cytotoxic effects of leaves extract of *Christia vespertilionis*.

H_1: There are significant biological activities such as antioxidant, antidiabetic activity, and cytotoxicity effects of leaves extract of *Christia vespertilionis*

1.4 Objectives

i. To determine the phytochemical compounds, total phenolic content, and antioxidant activity present in the leaves extract of *Christia vespertilionis*
ii. To determine the antidiabetic activity and toxicity evaluation of the leaves extract of *Christia vespertilionis*

1.5 Scope of the Study

The study is focussed on determining the antioxidant activity, antidiabetic properties and cytotoxicity of *Christia vespertilionis* leaves. The leaf part of the plant was extracted in an alcoholic aqueous solvent with maceration technique before the

determination of phytochemical compounds and total phenolic content are made. The antioxidant activity in the leaves extract was determined by the ability of the extract scavenging free radical molecules in DPPH solution. The antidiabetic activity of the leaves extract was determined by its ability to inhibit the α-amylase enzyme. As for cytotoxicity evaluation, the rate of mortality of brine shrimp was calculated when leaves extract of *Christia vespertilionis* was introduced to the organism.

1.6 Significance of the Study

The research regarding antidiabetic activity in leaves of *Christia vespertilionis* able to give contributions to pharmaceutical industry to treat diabetic problems. In addition, the research also could contributes to the additional facts and knowledge about the plant, such as in terms of antioxidant activity and toxicity, since it is not broadly discovered yet. On the other hand, the methodology applied in the research also may enhance the experimental skill of the researcher.

CHAPTER 2

LITERATURE REVIEW

2.1 *Christia vespertilionis*

Christia vespertilionis or commonly known as ‘green butterfly wing’ (daun rerama hijau) is a flowering plant that found in tropical South East Asia, Malaysia and Australia. It is in the phylum Tracheophyta and Fabaceae family that consists of 13 species (Azman, 2014). The plant is a non-climbing perennial herb that is used as an ornamental plant as in the stated countries. This is because of the uniqueness of the leaves that resembles butterfly wings.

Figure 2.1 *Christia vespertilionis*

Table 2.1 The phylum, class, order, family and genus of butterfly wing plant

Phylum	Tracheophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	Christia

The leaves have dark green or red stripes along the prominent veins. Based on the literature source, the plant has not been discovered broadly on its pharmacological and phytochemical aspects. However the plant has been used traditionally by the folks to treat tuberculosis, healing bone fractures and snake bites since long time ago (Hofer *et al.*, 2013). The pharmacological aspect of the plant should be proven by scientific research to identify the components that able to treat the stated illness. In 2013, researchers from Universiti Putra Malaysia (UPM) has made a research regarding the potential of the plant to have anticancer properties.

Based on the scientific finding, the plant showed that it has anti-cancerous and anti-inflammatory characteristics. This is because it contains alkaloids, triterpenes, fatty acids, phenols, alkanes, long chained alcohols, polyphenols, cytotoxicity and epigenetics in the plant (Azman, 2014). Due to the finding, UPM plans to conduct further research on the effectiveness of the plant foliage for its second stage of research, which is through screenings and pre-clinical trials (Azman, 2014).

Nowadays, the popularity of butterfly wing leaves in the local's market increases as people begin to acknowledge the goodness of the herb. The leaves of butterfly wing plant is being dried and infused with hot water before it is served as tea drink. Some feedbacks from the consumer claimed that the tea capable to improve their health.

Figure 2.2 The example of *Christia vespertilionis* product

2.2 Diabetes Mellitus

The word 'diabetes' is derived from a Greek word "Diah" which means urine and 'mellitus' is a Latin word for "sweetened for honey". Diabetes mellitus is a disease that caused by metabolic disorder of carbohydrate, fat and protein, where the body of the patient tends to have high blood sugar level or hyperglycemia. The condition happens due to two factors which related to the production of hormone called insulin that produced by beta cell of pancreas (Chrvala *et al.*, 2016). Hyperglycemia happens because of defect in insulin secretion, insulin action or both. This is because insulin is responsible to control sugar level in blood. Hyperglycemia generates reactive oxygen species (ROS) causes lipid peroxidation and membrane damage (Wickramaratne *et al.*, 2016). People with diabetes mellitus commonly have symptoms such as increasing in urinary output, feeling thirsty frequently, feeling tired, having ketonuria and ketonemia. Ketonuria is a

condition where ketone body is found in urine while ketonemia occurs because of the presence of ketone body in blood (Wickramaratne *et al.,* 2016).

Diabetes mellitus is divided into two types. Type 1 diabetes occurs when the beta cell of pancreas is unable to produce insulin or producing only a little amount of insulin (Wickramaratne *et al.,* 2016). Insulin is needed to control glucose level in the blood. Type 1 diabetes can develop at any age, but usually begins in childhood or young adulthood. The disease develops when the immune system destroys the beta cells. This is called autoimmune response. The symptoms of having diabetes by the patients of Type 1 diabetes usually not been seen until more than 90 percent of the beta cells that produce insulin have been destroyed (Chrvala *et al.,* 2016). The disease can be detected when the patients do a medical blood test. It requires regular blood sugar monitoring and treatment with insulin to treat the disease. Treatment, lifestyle adjustments, and self-care can control blood sugar levels and minimize the risk of disease-related complications (Wickramaratne *et al.,* 2016).

In second condition, a person tends to have Type 2 diabetes because the insulin produced by the pancreas is not working efficiently to control the sugar level in the blood (Praveen *et al.,* 2017). This type of diabetes is a disorder that disrupts the way the body uses glucose. This condition is also known as insulin resistance. Moreover, Type 2 diabetes is often accompanied by other conditions, including hypertension, high serum-low-density lipoprotein (LDL) cholesterol concentrations, and low serum high-density lipoprotein (HDL) (Sangeetha & Vedasree, 2012). All these condition higher the chance of the patient to have cardiovascular problems. In addition, diabetic patient also tend to have their wound or cut to heal slowly, and can worsen rapidly. The factors that influence wound healing in a diabetic patient are blood glucose level, poor circulation of blood, immune system deficiency and diabetic neuropathy. The complications arising with

diabetes also are closely related to the production of free radicals enhancing the oxidative stress. Hence, the use of antioxidants has been effective in reducing the severity of diabetic complications (Ruhe & McDonald, 2001).

Diabetes mellitus can be treated with insulin. Insulin will be used to treat diabetes patients by injecting it into the body or via inhalation (Dansinger, 2017). The insulin will be given to patients with specific dosage according to the patients' condition and the severity of the disorder. Other than insulin, diabetes also can be treated using therapeutic remedies (Ruhe & McDonald, 2001).

2.3 Antioxidants and Diabetes

The major risk factors that cause Type 2 diabetes are obesity and physical inactivity. However, evidence from a research done by Montonen *et al.* in 2004 suggests that pathogenesis of Type 2 diabetes may contributed by oxidative stress due to increment of insulin resistance or impairing insulin secretion.

Oxidative stress may contribute in the pathophysiology of diabetes and cardiovascular disease due to overproduction of ROS. Overproduction of ROS is related to hyperglycemia and metabolic disorder, such as impaired antioxidant function in conjunction with impaired antioxidant activity (Ruhe & McDonald, 2001). When diabetes mellitus patients are exposed to oxidative stress for a period of time, it will induce a chronic inflammation and fibrosis in a range of tissues, which lead to formation and progression of disease states in these tissues. Therefore, oxidative stress are overexpressed in patients with diabetes mellitus, where the increment of ROS may be

primarily responsible for the development of diabetic complications (Ruhe & McDonald, 2001).

Hyperglycemia, which is the inevitable consequence for Type 2 diabetes, is the cause of most deleterious effects that usually related to this metabolism disorder. Auto-oxidation of glucose can be promoted by hyperglycemia to form free radicals. The generation of excessive free radicals could make the scavenging abilities of endogenous antioxidant defenses incompetent and results in micro- and macrovascular dysfunction and polyneuropathy (Ruhe & McDonald, 2001). Therefore Bajaj & Khan stated in their journal called Antioxidant and Diabetes (2012) that diabetic complications can effectively be reduced by antioxidants such as vitamin C, N-acetylcysteine and α-lipoic acid. Reducing the diabetic complications through antioxidants may be beneficial either by dietary supplement or ingestion of natural antioxidants.

2.4 Insulin

Insulin is a hormone that produced by beta-cell of pancreas. It helps the body to absorb and use glucose and other nutrients from food, store fat, and build up protein to treat people with diabetes (Praveen *et al.,* 2017). People with lack of insulin are facing the problem where sugar remains in their blood and unable to enter the cell to do the metabolism activities. The excess sugar presence in the blood results in higher than normal blood sugar level (Praveen *et al.*, 2017).

In between 1990-1915, variety of treatments were proposed to cure the metabolic disorder through foods. The treatments included were the consumption of oat, the milk diet, the rice cure and potato therapy (Henderson, 2016). In 1921, Sir Fredick Grant

Banting & Charles Herbert Best had come out with the proposal of treating diabetes with insulin. This came after they found out that the lack of insulin is the cause of diabetes. Since insulin is produced naturally by human's cells, Banting and Best proposed to get the insulin from animal to treat diabetic patients. Insulin from cow was extracted and purified before it is used for diabetes treatment (Barnett, 2018).

Nowadays, insulin is commercially made because the demand is increasing rapidly from time to time. Peptidomimetics or insulin-like chemicals are designed to circumvent the problems related to the native peptide or protein on which they are based. According to Vodnik *et al.* in 2016, the design process of mimics starts from developing structure-activity relationship (SAR) of the native ligand-target pair that identify the key residues that are responsible for the biological effect of the native peptide or protein. Then minimization of the structure and introduction of constraints (e.g. cyclization through hydrocarbon stapling, lactam, disulphide isostere) are applied to create the core active site that can interact with the receptor or enzyme with high affinity and selectivity. Developing peptidomimetics is not easy and very challenging, especially when peptides' interaction mechanism with their target is complex (Vodnik *et al.*, 2016).

According to American Diabetes Association in an article titled 'Insulin Basics' in 2015, there are four types of insulin manufactured in laboratory, which are rapid-acting insulin, regular or short-acting insulin, intermediate-acting insulin and long-acting insulin. These insulin are differ from each other on how quickly they work, when they peak and how long they last. All insulin produced come in the form of dissolved or suspended in liquids. Insulin is injected into the body of diabetes patients since consumption via mouth may cause the enzyme to break down during digestion process just like the protein in food.

2.5 Extraction

According to Altemimi *et al.* (2017), extraction is a method used to separate the composition of plant from the tissues. It is the first crucial step in preparing the plant for formulations. Plant extracts are widely used in various sectors such as food sector, industrial sector and pharmaceutical. Plants are also known for their constitution of chemical compounds which are potential to medicate certain diseases. Phytochemical extraction is applied to extract out secondary metabolites from cell sap of the plant before it being used to produce a drug (Delfanian *et al.*, 2015).

Materials and equipment needed for extraction process are depended on the method of extraction used. There are several methods of extraction that can be used to obtain plant's bioactive compound which include infusion, digestion, Soxhlet, maceration, decoction, aqueous alcoholic extraction, ultrasonication and others. The method used will influence the outcomes of the extraction. To extract the metabolites present in the plant, process of damaging the plant cell will be applied via these techniques. The yield of extraction obtained is depends on the damage occurred to the plant cell. The extraction yield improves when more damage is applied to the plant cell. However, this process may disrupt or altered the metabolites present in the plant and cause the yield of extraction of desired compounds lesser (Delfanian *et al.*, 2015).

2.5.1 Aqueous alcoholic extraction

Aqueous alcoholic extraction involves soaking of the crude plant, in the form of either a powdered or decoction for a specified period of time. Alcohol is used to facilitate

the active constituents contained in the plant to be extracted out. On the other hand, solvent used for this technique also influence the extraction result. The extraction yield of phytochemicals is depends on conditions of extraction and the solvent polarity. Solvent with higher polarity able to extract more polar compounds (Delfanian *et al.*, 2015). This method of extraction is used extensively since it is reported to cause less damage to the plant's constituents. Solvents that usually used in aqueous alcoholic extraction are methanol and ethanol. Methanol is more polar compared to ethanol, thus it is more effective to be used in extraction. However, due to the safety concern ethanol is more preferable since methanol is known to be a toxic substance (Altemimi *et al.*, 2017).

2.5.2 Maceration

Maceration is the extraction technique of plant's active compounds in a solvent with shaking or stirring. In this process, the whole or coarsely powdered crude plant extract is placed in a stoppered container with solvent and allowed to stand at room temperature for a period of time with frequent agitation until the soluble matter has dissolved (Azwanida, 2015). The process is done to soften and break plant's cell wall to obtain the phytochemicals. After the dissolving process, the mixture is filtered and the filtrate is evaporated in a closed environment. The advantages of using this technique for plant extraction is it is less costly compared to other techniques. Other than that, the possibility for the phytochemicals extracted to be altered is lower than other techniques such as ultrasonication since the sample is not intentionally being exposed to harmful rays (Azwanida, 2015).

2.6 Phytochemical Analysis

Phytochemicals are the compounds that produced by plants as metabolites. There are two types of metabolites produced by plants which are primary and secondary metabolites. Primary metabolites are the compounds that directly involved in the growth, development and reproduction of the plant. It used to perform physiological function in the plant. Meanwhile, secondary metabolites are compounds that specifically produced by a particular plant, which does not involve in physiological function (Jandera *et al.*, 2009) (Jandera *et al.*, 2009).

Phytochemical analysis is a process to identify and analyse the qualitative and quantitative of compounds present in plant sample via specific techniques. These techniques are used to separate the compounds in the plant sample before identification process is made (Jandera *et al.*, 2009).

Usually qualitative and quantitative analysis will be done using Gas Chromatography-Mass Spectroscopy (GCMS) or High Performance Liquid Chromatography (HPLC). This is because the techniques are using special instrument that able to quantify and qualify the sample for separation method. Both techniques are differ from each other by their stationary phase. GCMS applied gas as the stationary phase while HPLC is using liquid as the instrument (Bhanot, 2014). Column that used in GCMS tends to be long and narrow or capillary column, while HPLC has a short and wide packed column. Apart from that, volatiles and non-volatiles materials can be analysed by using HPLC when GCMS can only analyse for volatile matter (Bhanot, 2014).

2.6.1 Thin Layer Chromatography (TLC)

Chromatography is a technique that is used to separate mixtures of substances into their components. TLC is performed using an aluminium plate that been coated with silica get. The silica gel acts as stationary phase, where it is used to mobilized the eluent containing sample from the starting point to end point of the TLC plate using adsorption principle. Mobile phase is the solvent that aids the mobilization of eluent through the stationary phase and carries compounds of the mixture along. All compounds present in the sample travel at different rates and separated throughout the process. Compounds which are more polar travel the longest distance on the stationary phase due to the properties of the phase that adsorb the eluent. During the process, compounds with higher affinity to stationary phase travel at lower rate compared to the less ones (Jandera *et al.*, 2009). Based on these principles, separation of the compounds is achieved. The separation causes the individual compounds being visualized as spots on the stationary phase (Jandera *et al.*, 2009)..

According to the article titled *"Thin Layer Chromatography (TLC): Principle and Procedure"* that published in 2015, the author stated that there are three steps used to analyse the compounds that present in the sample through TLC method, which are spotting, development and visualization. Spotting is a technique to place the sample on the TLC plate. The solvent used for spotting will evaporate and leave behind a spot that contains a mixture that has not been separated yet.

For development technique, the TLC plate will be placed in a shallow pool of development solvent, which then will travels up the plate by capillary action. The spot on the original point moves along with the solvent (mobile phase) and will stop at a certain distance on the stationary phase. The distance travels by the compounds depend upon a balance among three polarities, which are of the TLC plate, the mobile phase and the spot material. Compounds that present in the sample have different polarities, and this will influence the distance travel on the stationary phase.

Lastly is visualization technique. The separated compound can directly being observed after the development step. The silica gel coated on the TLC plate is impregnated with fluorescent, which allow the visualization of colourless compound under ultraviolet UV light. The spot will interfere with fluorescent and cause it to appear as a dark spot on the glowing background. Thus, it can be marked using a pencil to record the distance of the compound travel. For second visualization, the TLC plate will be sprayed with sulphuric acid in ethanol to determine the presence of phenolic compounds, flavonoids and terpenes in the sample. The presence of these compounds is indicated by the formation of coloured spots after heating process. The presence of alkaloids can be detected by using Dragendorff reagent. Formation of orange spot indicates the positive presence of alkaloid when the reagent is sprayed upon the TLC plate.

2.7 Phenolic Compounds

Metabolites are the chemical compounds that produced from metabolic reactions, which catalysed by various enzymes that naturally occur within cells (Saikat et al., 2014). Primary and secondary metabolites in plants are differ by their function and sources.

Primary metabolites are usually referred as central metabolites because the compounds are directly involved in "primary" development of the plant such as growth, reproduction, photosynthesis and other basic functions (Mandal et al., 2010). Secondary metabolites are derivatives from primary metabolites which perform specific functions in the plant. The compounds play an important role for ecological function and found in a small quantity in the plant.

One of the examples of secondary metabolite is phenolic compound. Phenolic compounds possess a wide spectrum of biochemical activities such as antioxidant, antimutagenic, anticarcinogenic as well as ability to modify the gene expression (Gholivand & Piryaei, 2012). Phenolic compounds are the largest group of phytochemicals that account for most of the antioxidant activity in plants or plant products (Sulaiman et al., 2013). The compounds are responsible for antioxidant activities and act as signaling molecules in the initiation of symbioses in plant and agents in plant defense (Mandal et al., 2010).

2.8 Alpha-amylase Inhibition Assay

As stated by Smith and Morton (2011), alpha-amylase is an endoenzyme that hydrolyses the α-1,4 glycosidic linkages in amylose, to yield maltose and glucose. The enzyme presents in the pancreatic juice and saliva degrades amylose solution to maltose, and release glucose from ends of the chain (Smith & Morton, 2011). The enzyme is synthesized as part of multigene family, which is regulated in different tissues to provide different isozymes (McDowall, 2006).

Alpha-amylase inhibition is an action done by the enzyme's inhibitors to inhibit the activity of the enzyme. The inhibitors of alpha-amylase usually are extracted from medicinal plants to serve as an alternative way to cure diabetes problems with high potency and lower the adverse effects of existing synthetic drugs available to treat the disease. Alpha-amylase inhibitors inhibits alpha-amylase enzyme that cause the delay in absorption of glucose into the bloodstream (Dansinger, 2017). This method is used as an alternative treatment to treat patients with Type 2 diabetes instead of using insulin. Apart from that, alpha-amylase inhibitors also able to treat obesity problem by reducing digestion and absorption of starch taken from everyday meals. The examples of alpha-amylase inhibitor that being used clinically in treating patient with hyperglycemia are Acarbose, metformin and miglitol.

2.9 Toxicity

According to Chen *et al.* in 2017, toxicity is the degree to which a chemical substance or a particular mixture of substances can damage an organism. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity) (Silva *et al.,* 2017).

There are generally four types of toxic entities, which are chemical, biological, physical and radiation (Pavela, 2015). Chemical toxicants include inorganic substances such as, lead, mercury, hydrofluoric acid and organic compounds such as methyl alcohol. Other than that, some chemicals that found in plants also could be dangerous to human. For example of plant that contain toxin is *Actaea pachypoda.* The toxin that produced by

the plant able to attack a specific body part such as cardiac muscles and cause sudden arrest to the consumer (Edmuth, 2015). Disease-causing microorganisms and parasites are able to secrete toxin through their metabolism activity. Some of the substance secreted can cause health problems to human as microorganisms used them as a self-defense mechanism (Benhassaini *et al.*, 2007). The biological toxicity of pathogens can be difficult to measure because the "threshold dose" may be a single organism. Physical toxicants are substances that interfere with biological processes due to their physical nature (Pavela, 2015). The examples of physical toxicant include coal dust, asbestos fibers or finely divided silicon dioxide. These toxicant are highly toxic, which they can cause severe health problems or death to the inhaler (Pavela, 2015).

Toxicity can be measured by its effects on the target, which are organism, organ, tissue or cell (Silva *et al.,* 2017). Every individuals typically have different levels of response to the same dose of a toxic substance, therefore a population-level measure of toxicity is often used which relates the probabilities of an outcome for a given individual in a population. One such measure is the LD_{50}. When that data does not exist, estimates are made by comparison to known similar toxic things, or to similar exposures in similar organisms (Silva *et al.*, 2017).

CHAPTER 3

METHODOLOGY

3.1 Chemicals and Equipment

3.1.1 Chemicals and Reagent

Ethanol, Whatman filter paper no. 1, hexane, methyl-acetate, silica gel 60 F_{254} pre-coated plates, sulphuric acid, basic bismuth nitrate, glacial acetic acid, potassium iodide, ferric chloride, hydrochloric acid, gallic acid, Folin-Ciocalteu phenol reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH powder), butylated hydroxytoluene (BHT salt), α-amylase enzyme, starch, trisodium phosphate, potassium sodium tartrate tetrahydrate, sodium chloride, 3,5-dinitrosalicylic acid (DNSA powder), sodium hydroxide, metformin.

3.1.2 Equipment

Electrical grinder, rotary evaporator, vortex shaker, UV-spectrophotometer, UV-Vis spectrophotometer, brine shrimp hatchery incubator set, air pump.

3.2 Methods

3.2.1 Preparation of Sample

The *Christia vespertilionis* leaves was bought online from SP Models Nature website. The leaves was ordered from the seller on 1st August and received on 7th August 2018. The location of the nursery is at Platinum Homes, Jalan Platinum 7/43C, Seksyen 7, Shah Alam, Selangor. 7 packets of C*hristia vespertilionis* leaves were bought, which weighed approximately 30 g per packet. The leaves was dried for 40 hours under the sunlight before being packed and sent through postal delivery. The sample preparation required separation process to separate leaves part from the other parts of the dried plant. The total weight of whole packets of the sample purchased was 210 g and the net weighed 75 g excluding the residues such as stems and roots.

3.2.2 Extraction

Aqueous alcoholic extraction with maceration technique was applied to extract the leaves of *Christia vespertilionis*. The sample was grinded using an electrical grinder until powder form is achieved. Then, 500 ml of ethanol was added into a container containing 70 g of the powdered sample and macerated for 20 minutes. The mixture was left in a fume hood for 5 days long before it being filtered. The mixture was macerated for 20 minutes for each day during the period.

3.2.3 Preparation of Crude Extract

The leaves extract was filtered and dried in order to get a crude extract with lowest moisture content. Filtration process of the sample required the mixture to pass through a Whatman filter paper no. 1. The residue was discarded while the filtrate was kept for the next process, which is drying process. The evaporation of the solvent from liquid extract was performed by means of reduced pressure using a rotary evaporator vacuum at temperature of 60 ^{o}C and 30 rpm. Then, the sample was kept in a fume hood to remove the remaining moisture and ready to be used for the next step of the experiment. The yield of extraction was calculated using the following formula:

$$Extraction\ Yield = \frac{Weight\ of\ the\ dried\ extract}{Weight\ of\ the\ original\ sample} \times 100$$

3.2.4 Phytochemical Screening

The chromatographic analysis by thin layer chromatography (TLC) was performed using silica gel 60 F_{254} pre-coated plates over aluminium, 2 cm x 7 cm plates on layer thickness 0.25 mm. This experiment consisted of three steps which are spotting, development and visualization to screen the phytochemical in the sample.

3.2.4.1.1 Spotting

A small amount of the *Christia vespertilionis* leaves crude extract was diluted with 1 ml ethanol. The diluted solution was transferred to the TLC plate, which marked as initial point, using the tip of a disposable plastic pipette. The spotting solvent was quickly evaporated and left behind a spot of the material.

3.2.4.1.2 Development

The phytochemical in the sample was separated on the TLC plate by capillary action of development solvent. The development solvent (eluent), was prepared by mixing hexane and methyl-acetate with the ratio 8:2 in concentration volume/volume. As the solvent travelled up the plate, it moved over the original spot.

3.2.4.1.3 Visualization

a) Coloured compounds

The coloured compounds present in the leaves extract of *Christia vespertilionis* were observed directly after development. Observation was made under UV light with wavelength 254 nm and 365 nm. Spots that interfered with the fluorescent appeared as dark spot on a glowing background. The dark spots were outlined with pencil to mark their locations

b) Detection the presence of terpenes using 10% sulphuric acid

The spray reagent was prepared by adding 10 ml of concentrated sulphuric acid into 90 ml of ethanol. The TLC plate was sprayed with the reagent and heated on a hot plate of 80 ^{o}C for 10 minutes. The formation of reddish brown spot was observed and recorded into data.

c) Detection the presence of alkaloid compounds using Dragendorff reagent

0.85 g of basic bismuth nitrate was dissolved in 10 ml of acetic acid and 40 ml of distilled water. The solution was labelled as 'stock 1'. 8 g of potassium iodide was dissolved in 30 ml of distilled water to produce a solution which was labelled as 'stock 2'. Stock solution was prepared by mixing 'stock 1' and stock 2' with ratio 1:1. The spray reagent was prepared by mixing 1 ml of stock solution with 2 ml of

glacial acetic acid and 10 ml of distilled water. The formation of orange coloured spot or precipitate was observed and recorded into data.

d) The presence of phenols and phenolic acids using ferric chloride

The sprayed reagent was prepared by dissolving 2.7 g of iron (III) chloride in 100 ml of 0.1 M HCL. The formation of bluish-green coloured spot was observed and recorded into data.

3.2.5 Identification of R_f Value

The R_f value was determined by dividing the distance travelled by substance and the distance travelled by the solvent.

$$Rf\ Value = \frac{Distance\ travelled\ by\ substance}{Distance\ travelled\ by\ solvent}$$

3.2.6 Determination of Total Phenolic Content

Total phenolic content was determined by Folin-Ciocalteu method by Wickramaratne *et al.,* (2016) with slight modification. Sample solution (*Christia vespertilionis* leaves extract) and standard (gallic acid) with six different concentrations, which are 6.25, 12.5, 25, 50, 100 and 200 mg/ml, were prepared. 1 ml of sample with different concentrations were added into 9 ml of distilled water in a volumetric flask respectively. Then, 1 ml of Folin-Ciocalteu phenol reagent was added into each tube containing the mixture and shaken. After 3 minutes, 10 ml of 7% Na_2CO_3 was added

into the mixture before they were kept in dark for 90 minutes at room temperature. The procedure was repeated by replacing the sample with the standard solution. The blank was prepared by adding 1 ml of Folin-Ciocalteu phenol reagent into 9 ml of distilled water, followed by 10 ml of 7% Na_2CO_3 into the mixture after 3 minutes. The reaction was carried out in triplicate and the decrease in absorbance was measured at 550 nm using UV spectrophotometer. The total phenolic content was calculated from calibration curve, and the results were expressed as mg of gallic acid equivalent per g dry weight. The following formula is used to calculate the total phenolic content in leaves extract of *Christia vespertilionis:*

$$Total\ Phenolic\ Content = \frac{C \times V}{m}$$

Where;

C = Concentration of gallic acid from calibrium curve

V = Volume of extract in ml

m = Mass of extract in g

3.2.7 Determination of DPPH Free Radical Scavenging Activity

Free radical scavenging activity of *Christia vespertilionis* leaves was measured using the DPPH assay according to Devarajan *et al.,* (2012) with slight modification. For DPPH solution, 4 mg of DPPH powder was dissolved in 100 ml of ethanol. The solution was kept in a container which wrapped with aluminium foil before it being used for the next step of the experiment. Vary concentrations of *Christia vespertilionis* leaves extract

and standard solution (BHT salt) were prepared using ethanol, which are 6.25, 12.5, 25, 50, 100 and 200 mg/ml. 2 ml of DPPH solution was added into each tube containing different concentration of the leaves extract and standard. Corresponding blank was prepared by adding 2 ml of DPPH solution into 2 ml of ethanol. The reaction was carried out in triplicate and the decrease in absorbance was measured at 517 nm after 30 minutes in dark using UV-Vis spectrophotometer. The percentage of free radical scavenging activity was calculated using following formula:

$$\%\ Free\ Radical\ Scavenging\ Activity = \frac{Absorbance\ control - Absorbance\ test\ sample}{Absorbance\ control} \times 100$$

3.2.8 Determination the Presence of Antidiabetic Activity using α-Amylase Inhibition Assay

The α-amylase inhibition assay was carried out according to 3,5-dinitrosalicylic acid (DNSA) method by Wickramaratne *et al.,* (2016) with slight modification. Three concentrations of *Christia vespertilionis* leaves extract and standard solution (metformin) were prepared, which are 500, 250 and 125 mg/ml. 200 µl of phosphate buffer with pH 6.9 was added into 200 µl of the sample or standard. Next, 200 µl of α-amylase solution was added into the mixture and was shaken using vortex mixer before being incubated for 10 minutes in water bath at 30 °C. Thereafter, 200 µl of 1% starch solution was added into the mixture and incubated for 5 minutes in the same water bath. DNSA reagent was prepared by dissolving 12 g of potassium sodium tartrate tetrahydrate (Rochelle salt) in 8 ml of 2 M NaOH. Then, 12 g of 3,5-dinitrosalicylic acid was dissolved in the mixture to produce the reagent. 200 µl of DNSA reagent was added into the sample or standard mixture and incubated in water bath at 81 °C for 10 minutes. The mixture was cooled to

room temperature and diluted with 5 ml of distilled water. The absorbance value was measured using UV spectrophotometer at 540 nm wavelength. The blank was prepared similarly to the procedure without including the sample or standard solution. The α-amylase inhibitory activity was expressed as percent inhibition and calculated using the following formula:

$$\%\ \alpha - amylase\ inhibition = \frac{Absorbance\ control - Absorbance\ test\ sample}{Absorbance\ control} \times 100$$

3.2.9 Brine Shrimp Cytotoxicity Bioassay

The toxicity evaluation of *Christia vespertilionis* leaves extract was done according to brine shrimp cytotoxicity bioassay by Ndossi & Kilonzo (2018) with slight modification.

3.2.9.1 Preparation of Brine Shrimp

38 g of sea salt was dissolved into 1000 ml of distilled water and the mixture was filled in an incubator. Then, the air pump was connected to the inlet at the bottom of the incubator. 5 g of brine shrimp was added into the solution before the aeration was switched on. The mixture was incubated for 48 hours and surrounded with fluorescent lamps to warm it. The aeration was stopped after 48 hours and kept for 15 minutes before being used for next step of the experiment.

3.2.9.2 Preparation of Dilution

The sample was prepared by diluting the crude extract of *Christia vespertilionis* leaves using sea salt water. 10 mg of *Christia vespertilionis* leaves crude extract was dissolved into 10 ml sea salt water to produce 10 mg/ml of sample stock solution. From the stock solution, sample with vary concentrations, 6.25, 12.5, 25, 50, 100 and 200 mg/ml were prepared. Sea salt water was used as blank in this experiment.

3.2.9.3 Preparation of Treatment

1 ml of sea salt water containing 10-20 hatched brine shrimp was taken and transferred into the sample. The actual number of hatched brine shrimp transferred into the sample was counted and recorded. After 24 hours, the number of brine shrimp alive was counted and recorded into data.

3.2.10 Statistical Analysis

Analyses for all experiments were carried out in triplicate. The data were expressed as mean ± standard error (SE). The standard error were calculated using analysis of variance (ANOVA) in SPSS software.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction Yield

The extraction yield of *Christia vespertilionis* leaves was calculated and it is found to be 6.43%.

4.2 Phytochemical Screening

The separation of *Christia vespertilionis* leaf's constituents on the TLC plate occurred when the eluent travels up the plate. There are 8 separations were observed and outlined under the UV light with wavelength 254 nm and 365 nm.

The separation of *Christia vespertilionis* leaves mixture on the TLC plate happened due to the capillary action of the eluent on mobile and stationary phase. Compounds with different affinities of the analyte towards the mobile and stationary phase resulting in different separation of constituents. Affinity is the dictation of two properties of the molecule, which are adsorption and solubility (Sherma & Fried, 2003). According to the principle of TLC, compound with higher affinity to stationary phase

travels at lower rate compared to the less ones. The polarity of the eluent used as the mobile phase also influence the rate of separation. Solvent with higher polarity offers more efficient separation in thin layer chromatography. This is because more polar compounds have higher affinity due to hydrogen bonding. The separation of the compounds enable the identification of the individuals. As the compounds separated, they can be recognised to which group they belong to through R_f value and visualization process.

As reported in Table 4.1, no coloured spot appeared after the plate was sprayed with spraying reagents, which are 10% sulphuric acid, Dragendorff reagent and ferric chloride. This indicates that the present of terpenes, phenolic compounds, flavonoids, alkaloids and heterocyclic nitrogen compounds were not detected. The R_f value of the separated compounds on the TLC plate was calculated and presented in the Table 4.2. Separated compounds with R_f value 0.38, 0.46, 0.72 are recognised as pure compound, organic compound and phenolic compound (Abbas & Al-Maliki, 2011).

In *Christia vespertilionis*, there is significant indication about the presence of secondary metabolites such as phenolic acids, flavonoids, terpenes, alkaloids and heterocyclic nitrogen compounds in the leaves extract. The recognition of the compounds were made based on their R_f value. Comparing to the previous study by Dash in 2016, it is reported that *Christia vespertilionis* contains alkaloids, triterpenes, fatty acids, phenols, alkanes and long chained alcohols. The present of palmitine and corynoxidine was reported in the aerial parts.

However, the study failed to recognise the compounds by visualization process. This occurrence might happened due to some errors in handling the experiment. One of the possible errors done that influence the outcome of the study is the concentration or

quantity of the sample dotted on the TLC plate. When the sample is too dilute or low in quantity, the detection of the compounds become harder as their amount is insufficient to react with the chemical which introduced to them for detection process. Other possibility for the failure of detecting phytochemical compounds in *Chistia vespertilionis* leaves extract is the condition of the chemical reagent used. Some of the chemicals, such as ferric chloride, was exposed to the air for a period of time that cause the physical properties changed slightly. Thus, it may affecting the result of the study since the chemical is oxidised.

Figure 4.1 Silica gel TLC plate with separated compounds from *Christia vespertilionis* leaves extract

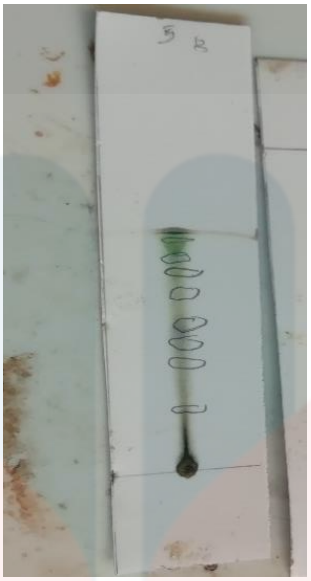

Figure 4.2 Silica gel TLC plate with separated compounds from *Christia vespertilionis* has been outlined and heated. The present of coloured spot was not detected

Table 4.1 Preliminary qualitative analysis results of phenolic compounds in *Christia vespertilionis* leaves extract

Spray Reagents	**Detection results**	**Indications**	**Conclusion**
10% sulphuric acid	-	No formation of reddish brown colour	No terpenes
Dragendorff	-	No orange precipitate or spot	No alkaloids
Ferric chloride	-	No formation of bluish-green colour	No phenols and phenolic acid compounds

Table 4.2 R_f value of separated compounds of ethanolic extract of *Christia vespertilionis*

Separations	R_f Value
1	0.21
2	0.31
3	0.47
4	0.53
5	0.65
6	0.79
7	0.88
8	0.97

4.3 Determination of Total Phenolic Content and Antioxidant Activity

The total phenolic content (TPC) presents in the extract of *Christria vespertilionis* leaves was evaluated using Folin-Ciocalteu assay in terms of the Gallic acid equivalent (GAE) in mg/g of the extract. The TPC was determined by the reduction of the reagent by phenolic compounds present in the sample, which is measured at 550 nm wavelength with gallic acid as the standard. The TPC was reported as gallic acid equivalent by the extrapolation of the standard calibration curve $y = 0.0018x + 0.0172$ [$R^2 = 0.9216$]. The TPC of *Christia vespertilionis* leaves estimated was found to be 128.852 ± 3.90 mg gallic acid equivalent per g of dried sample.

Total phenolic content in a plant influences its phytochemical activity. This is because phenolic compounds possess a wide spectrum of biochemical activities such as antioxidant and antidiabetic activities (Gholivand & Piryaei, 2012).

The study of total phenolic content in leaves extract of *Christia vespertilionis* showed a small differ from the previous study. In an article titled '*An Appraisal of Christia vespertilionis: A Promising Medicinal'* by Dash in 2016, the total phenolic content of *Christia vespertilionis* is reaching 216 mg gallic acid equivalent per g of dried sample. However based on the study, the total of phenolic content is lower than the predicted value. The significant difference between the total phenolic content may happened due to the source of plant originated.

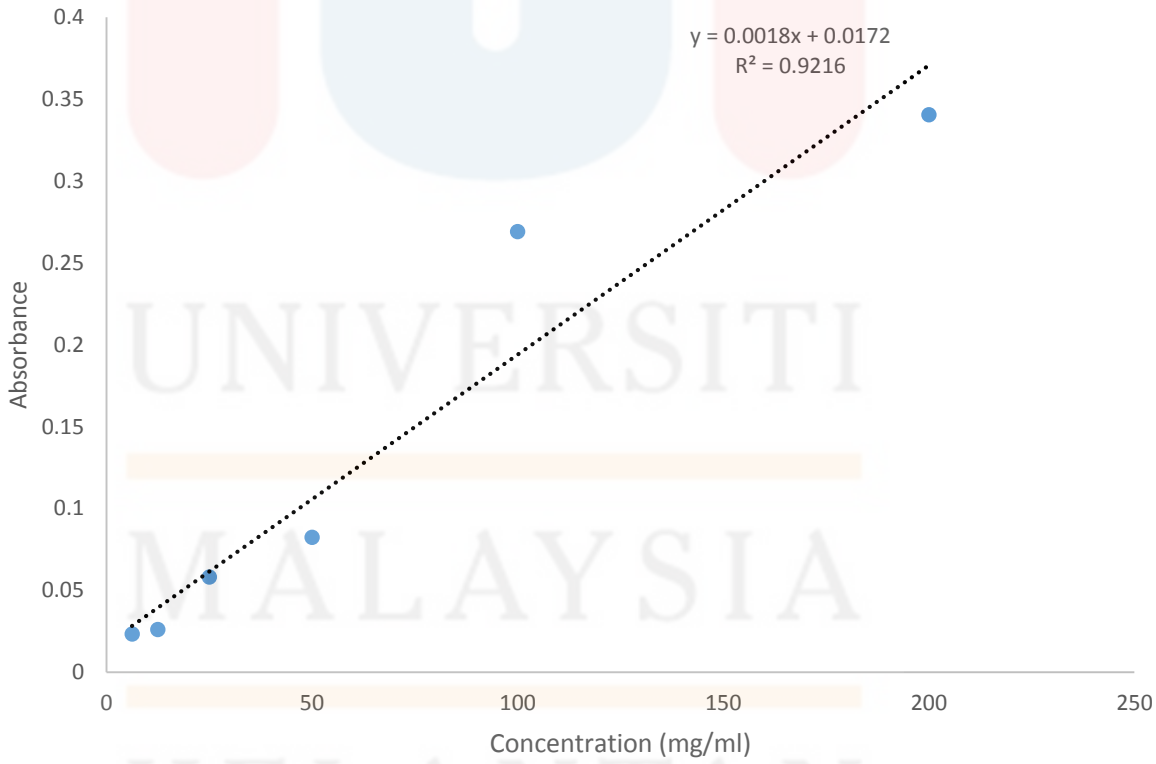

Figure 4.3 Standard calibration curve for total phenolic content in gallic acid

4.4 Determination of Free Radical Scavenging Activity using DPPH Assay

The DPPH antioxidant assay of *Christia vespertilionis* leaves extract was tested using ethanolic solution of DPPH free radical, which appeared dark purple colour with maximum absorption at 517 nm. The discolouration of DPPH solution was resulted from the scavenging activity of antioxidant molecules present in the sample. The more the absorbance decreases, the more potent the antioxidant activity of the *Chistia vespertilionis* leaves extract is. Different concentrations (6.25, 12.5, 25, 50, 100 and 200 mg/ml) of ethanolic *Chistia vespertilionis* leaves extract and standard BHT were examined. Figure 4.4 showed the results of percentage of free radical scavenging activity for both leaves extract and standard.

As the concentration increases, the scavenging effect of *Christia vespertilionis* leaves extract on DPPH radicals also increase. At concentration 200 mg/ml of the leaves extract and BHT, the scavenging effect was 34.72% and 80.38%, whereas 18.55% and 59.81% at 100 mg/ml, 11.57% and 37.48% at 50 mg/ml, 3.02% and 17.86% at 25 mg/ml, 2.39% and 10.44% at 12.5 mg/ml, 2.01% and 8.05% at 6.25 mg/ml. The IC_{50} value calculated for antioxidant activity in *Christia vepertilionis* leaves was found to be 39.987 mg/ml and 32.734 mg/ml for BHT. Based on the statistical analysis using one-way ANOVA, followed by Post-Hoc (Tukey), it showed that there is antioxidant activity in *Christia vespertilionis* leaves extract at significant level ($p < 0.05$).

Phenolic compounds exhibit considerable free-radical scavenging activity, which is determined by their reactivity as hydrogen- or electron- donating agents, their reactivity with other antioxidants and their metal chelating properties (Ruhe & McDonald, 2001). The more the phenolic content in a plant, the more its antioxidant activity. Antioxidant plays an important role in preventing oxidative stress by scavenging

the free radical that can cause the stress. In *Christia verpertilionis* leaves extract, the scavenging activity of free radical is directly proportional to the concentration of the extract. Therefore, the intake of the herbal plant in a large quantity could reduce the mean of oxidation activity in the body of consumer. Other than that, the consumption of the leaves extract also may reduce the risk of having hyperglycemia that caused by oxidative stress. The reduction of risk for having hyperglycemia by means also reducing the risk of having diabetes. As for the reason, *Christia vespertilionis* is a herbal plant that have antidiabetic potential.

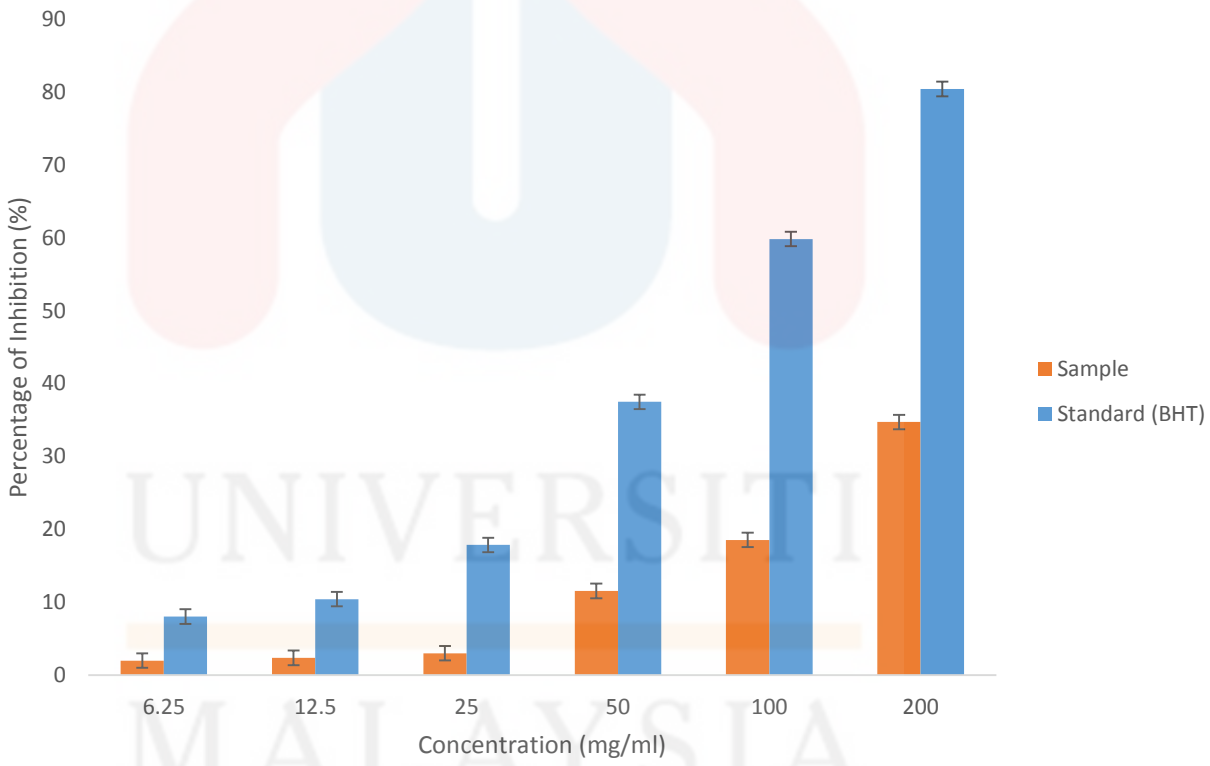

Figure 4.4 Free radical scavenging activity of *Christia vespertilionis* leaves ethanolic extract with reference to butylatade hydroxytuluene (BHT) as standard.

4.5 Determination the Presence of Antidiabetic Activity using α-Amylase Inhibition Assay

The presence of antidiabetic compounds in the *Christia vespertilionis* leaves extract was determined by α-amylase inhibitory assay. The results are expressed as percentage of sample absorbance decrease relative to absorbance of control solution at 540 nm wavelength. Metformin was used as standard reference drug in α-amylase inhibitory activity.

Based on the results, the percentage of α-amylase inhibition for concentration 250 mg/ml was the highest, which is 23.33%. Sample with the highest concentration of *Christia vespertilionis* leaves extract, 500 mg/ml, had 20.14% inhibition of α-amylase percentage and followed by 125 mg/ml with 15.34% α-amylase inhibitory activity. Whereas, the percentage of α-amylase inhibition in 500 mg/ml metformin was 37.69%, 32.62% for 250 mg/ml and 24.85% for 125 mg/ml. The IC_{50} value calculated for α-amylase inhibitory activity for metformin was found to be 31.325 mg/ml while 35.201 mg/ml for *Christia vespertilionis* leaves. Based on the statistical analysis using one-way ANOVA, there is α-amylase inhibitory activity in the leaves extract of *Christia vespertilionis* plant at significant level of ($p < 0.05$)

The trend of screening medicinal plant for diabetic therapeutic remedies has been widely done by researchers, as it is important to discover novel effective drugs for the metabolic disorder. Although the WHO has recommended to live a healthy lifestyle and do daily exercise as the method to control Type 2 diabetes problem, the intake of additional food such as herbs also might help in decreasing the risk of having chronic condition of the metabolic disorder.

The α-amylase inhibitory study performed demonstrated that the leaves extract of *Christia vespertilionis* had significant inhibitory potentials. The IC_{50} value of ethanolic extract of *Christia vespertilionis* leaves is almost similar to a commercial antidiabetic drug that is widely used and sell in the market (Yilmazer-Musa *et al.,* 2012). The present of phenolic compounds lead to the activity of antidiabetic in the leaves extract of *Christia vespertilionis.* This is because some of the compounds have α-amylase inhibition properties. The examples phenolic compound that have α-amylase inhibition properties are bakuchiol, cinnamaldehyde and isoorientin (Missoun *et al.,* 2018). These α-amylase inhibitors are also known as starch blocker, where they prevent or slower the absorption of starch into the body. The mechanism of action by these inhibitors block the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose and other simple sugars (Wickramaratne *et al.,* 2016).

In a study done by Ruhe & McDonald (2011), diabetic complications occur consequently to the oxidative stress caused by the formation of free radicals with glucose oxidation and subsequently oxidative degeneration of glycated protein. Therefore, the use of antidiabetic drugs along with the consumption of antioxidant would give a better effect in treating and avoiding such diabetic complications.

Even though the study showed that *Christia vespertilionis* leaves have a positive effect in inhibiting α-amylase enzyme, there is a discourage argument regarding the trend on inhibition by different concentration of *Christia vespertilionis* leaves extract. This is because the second concentrated extract had the highest percentage of α-amylase inhibition instead of the most concentrated one. The circumstance might happened due to the error while handling the study such as migration of small particles into the sample, which lead to the changing of colour to more intense before the absorbance reading was measured.

Table 4.3 Percentage of α-amylase inhibition of ethanolic extract of *Christia vespertilionis* leaves

Concentration (mg/ml)	**Percentage of α-Amylase Inhibition (%)**	
	Metformin	*Christia vespertilionis* leaves extract
125	37.69	15.34
250	32.62	23.33
500	24.85	20.14

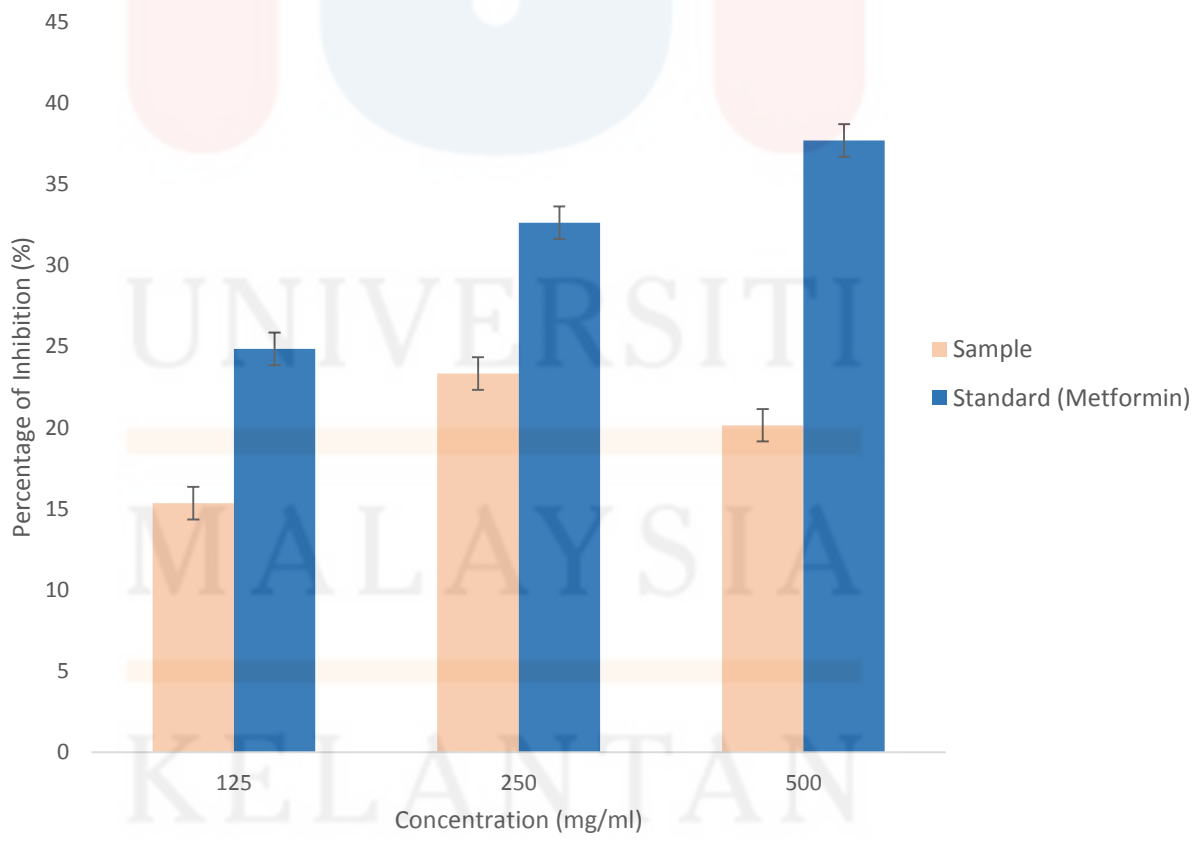

Figure 4.5 Percentage of inhibition α-amylase by metformin and ethanolic extract of *Christia vespertilionis* leaves

4.6 Brine Shrimp Cytotoxicity Bioassay

The percentage of mortality of the brine shrimp determined the cytotoxicity of the sample extract. The result showed that blank has the lowest percentage of brine shrimp mortality, which was 8.47%. Sample with concentration 12.5 mg/ml had percentage of mortality 10.87%, whereas 25 mg/ml had 11.44%, 50 mg/ml had 12.5%, 100 mg/ml had 18.84% and 200 mg/ml had 21.59% of mortality percentage. The percentage increased as the concentration of *Christia vespertilionis* leaves extract increases. The LD_{50} value calculated for the lethality of brine shrimp based on the graph was found to be 45.1092 mg/ml. Statistical analysis made using one-way ANOVA showed that the percentage of brine shrimp mortality in the leaves extract of *Christia vespertilionis* was not significant at level ($p > 0.05$).

Brine shrimp lethality bioassay was done to the leaves extract of *Christia vespertilionis* to evaluate the toxicity of the substance present in the leaves. It is important to acknowledge the cytotoxicity of a medicinal plant to determine whether it is safe for human consumption.

The leaves extract of *Christia vespertilionis* assessed that the herbal contained toxin. The slight increment of percentage of brine shrimp mortality in the increasing concentration of the leaves extract indicates that the plant is toxic to the brine shrimp. However the LD_{50} value calculated was higher than 1 mg/ml, which means that the leaves extract of *Christia vespertilionis* is safe for consumption.

Though the toxicity level of *Christia vespertilionis* leaves extract is low, the consumption of the plant in a large quantity could affect the consumer's health in the future. This is because the more the intake of the herbal, the more toxin is consumed by

the consumer. Toxin from the medicinal plant could disturb the system in the body and cause severe health effect to the consumer. It becomes worse if the consumption of toxin produced by the plant (such as in *Actaea pachypoda*) use to attack a specific body part such as cardiac muscles and cause sudden arrest to the consumer. Therefore, it is important to avoid the consumption of herbal plant that contains high level of toxin since it may affect the health of the consumer. Since *Christia vespertilionis* leaves extract has little amount of toxin, the plant is suitable for human consumption to reduce the risk of having Type 2 diabetes. The consumption of the herbal also impotent to give severe side effect to human health in the near future.

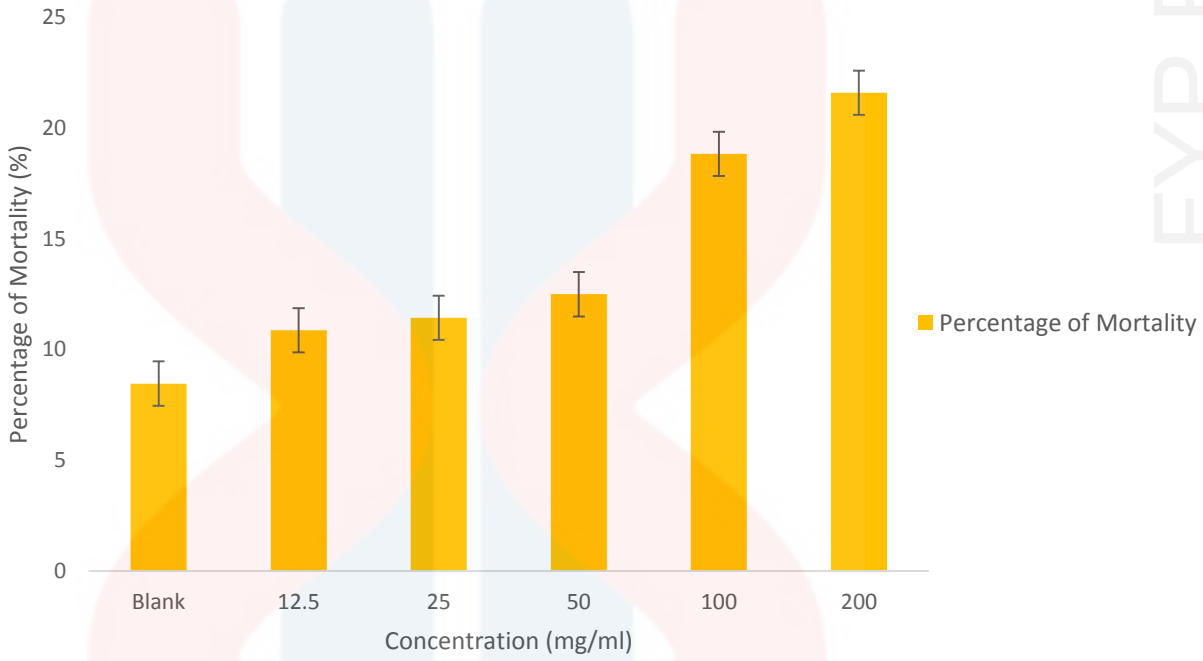

Figure 4.6 Percentage of mortality of brine shrimp in different concentration of ethanolic extract of *Christia vespertilionis* leaves

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Conclusion

According to the result of the study on leaves extract of *Christia vespertilionis*, it showed that the herb has antioxidant activity which potentially to help preventing the risk of having Type 2 diabetes. A part of that, the leaves extract also exhibit α-amylase inhibitory activity remarkable in the crude ethanolic extract, which could lower rate of hydrolysis of 1,4-glucosidic linkages of starch and cause high sugar level in body. However, *Christia vespertilionis* extract was found to be non-toxic for consumption.

Recommendations

The consumption of *Chistia vespertilionis* leaves for diabetic treatment is encouraged as it is able to reduce the risk of having Type 2 diabetes. Furthermore, this study has opened opportunities for further research in searching for novel effective drugs for antidiabetics that possess both antioxidant activity and antidiabetic activity. Lastly, future studies on mechanism of action should be made as it will give added advantage to herbal study.

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APPENDICES

APPENDIX A

Table A.1 The R_f value for separated compound on TLC plate

Plate	1	2	3	Average
Separation 1	0.24	0.21	0.19	0.21
Separation 2	0.4	0.28	0.26	0.31
Separation 3	0.46	0.49	0.47	0.47
Separation 4	0.52	0.53	0.53	0.53
Separation 5	0.62	0.68	0.66	0.65
Separation 6	0.78	0.79	0.79	0.79
Separation 7	0.84	0.91	0.89	0.88
Separation 8	0.96	0.98	0.98	0.97

Table A.2 Absorbance value of gallic acid for TPC assay

Concentration	R1	R2	R3	Mean	Std
6.25	0.023	0.023	0.024	0.023333	0.000577
12.5	0.025	0.026	0.027	0.026	0.001
25	0.055	0.06	0.059	0.058	0.002646
50	0.079	0.082	0.086	0.082333	0.003512
100	0.268	0.269	0.27	0.269	0.001
200	0.339	0.341	0.341	0.340333	0.001155

Table A.3 Absorbance value of sample (*Christia vespertilionis* leaves extract) for TPC assay

Concentration	R1	R2	R3	Mean	Std
6.25	0.021	0.02	0.022	0.021	0.001
12.5	0.023	0.023	0.025	0.023667	0.001155
25	0.027	0.029	0.024	0.026667	0.002517
50	0.033	0.034	0.032	0.033	0.001
100	0.04	0.043	0.041	0.041333	0.001528
200	0.058	0.06	0.06	0.059333	0.001155

Table A.4 Concentration calibration curve for gallic acid

Concentration	R1	R2	R3	CC R1	CC R2	CC R3	Mean	Std
6.25	0.023	0.023	0.024	3.222222	3.222222	3.777778	3.407407	0.6415
12.5	0.025	0.026	0.027	4.333333	4.888889	5.444444	4.888889	1.111111
25	0.055	0.06	0.059	21	23.77778	23.22222	22.66667	2.939724
50	0.079	0.082	0.086	34.33333	36	38.22222	36.18519	3.902094
100	0.268	0.269	0.27	139.3333	139.8889	140.4444	139.8889	1.111111
200	0.339	0.341	0.341	178.7778	179.8889	179.8889	179.5185	1.283001

Table A.5 Absorbance value of BHT for DPPH assay

Concerntration	R1	R2	R3	Mean	Std
6.25	0.487	0.487	0.488	0.487333	0.000577
12.5	0.468	0.474	0.482	0.474667	0.007024
25	0.432	0.441	0.433	0.435333	0.004933
50	0.324	0.334	0.336	0.331333	0.006429
100	0.221	0.209	0.209	0.213	0.006928
200	0.106	0.099	0.107	0.104	0.004359

Table A.6 Absorbance value of sample (*Christia vespertilionis* leaves extract) for DPPH assay

Concerntration	R1	R2	R3	Mean	Std
6.25	0.518	0.519	0.521	0.519333	0.001528
12.5	0.517	0.514	0.512	0.514333	0.002517
25	0.512	0.516	0.514	0.514	0.002
50	0.471	0.468	0.467	0.468667	0.002082
100	0.437	0.429	0.429	0.431667	0.004619
200	0.338	0.348	0.352	0.346	0.007211

Table A.7 Percentage scavenging activity of BHT

Concerntration	% Scavenging Activity R1	% Scavenging Activity R2	% Scavenging Activity R3	Mean % Scavenging Activity	Std deviation
6.25	8.113208	8.113208	7.924528	8.050314	0.108934
12.5	11.69811	10.56604	9.056604	10.44025	1.325239
25	18.49057	16.79245	18.30189	17.86164	0.930733
50	38.86792	36.98113	36.60377	37.48428	1.213038
100	58.30189	60.56604	60.56604	59.81132	1.307208
200	80	81.32075	79.81132	80.37736	0.822434

Table A.8 Percentage scavenging activity of sample (*Christia vespertilionis* leaves extract)

Concerntration	% Scavenging Activity R2	% Scavenging Activity R3	Mean % Scavenging Activity	Std Dev
6.25	2.075472	1.698113	2.012579	0.288212
12.5	3.018868	1.698113	2.389937	0.66262
25	2.641509	3.018868	3.018868	0.377358
50	11.69811	11.88679	11.57233	0.392767
100	19.0566	19.0566	18.55346	0.871472
200	34.33962	33.58491	34.71698	1.360585

Table A.9 Absorbance value of metformin for α-amylase inhibition assay

Concentration	R1	R2	R3	Mean	Std
125	1.217	1.227	1.231	1.225	0.007211
250	1.096	1.094	1.105	1.098333	0.005859
500	0.994	1.024	1.029	1.015667	0.01893

Table A.10 Absorbance value of sample (*Christia vespertilionis* leaves extract) for α-amylase inhibition assay

Concentration	R1	R2	R3	Mean	Std
125	1.373	1.379	1.388	1.38	0.00755
250	1.257	1.242	1.25	1.249667	0.007506
500	1.333	1.286	1.286	1.301667	0.027135

Table A.11 Percentage of α-amylase inhibition of metformin

Concentration	R1	R2	R3	Mean	Std
125	25.33742	24.72393	24.47853	24.84663	0.442399
250	32.76074	32.88344	32.20859	32.61759	0.359476
500	39.0184	37.17791	36.87117	37.68916	1.161331

Table A.12 Percentage of α-amylase inhibition of sample (*Christia vespertilionis* leaves extract)

Concentration	R1	R2	R3	Mean	Std
125	15.76687	15.39877	14.84663	15.33742	0.46318
250	22.88344	23.80368	23.31288	23.33333	0.460463
500	18.22086	21.10429	21.10429	20.14315	1.664752

Table A.13 Percentage of lethality of brine shrimp

Concentration	Percentage of lethality (%)	Std Mortality percentage
Blank	8.465608	7.501295
12.5	10.87302	3.070721
25	11.43791	9.917641
50	12.5	12.5
100	18.83838	10.84825
200	21.59605	2.965455

APPENDIX B

Table B.1 One-way ANOVA result for DPPH Assay

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
BHT	Between Groups	12904.104	5	2580.821	2385.584	.000
	Within Groups	12.982	12	1.082		
	Total	12917.086	17			
DPPH_Sample	Between Groups	678.011	5	135.602	11.760	.000
	Within Groups	138.365	12	11.530		
	Total	816.376	17			

Table B.2 One-Way ANOVA (Tukey HSD) result for DPPH Assay

DPPH_Sample

Tukey HSD

Sample	N	Subset for alpha = 0.05		
		1	2	3
[6.25]	3	2.01258		
[12.5]	3	2.38994	2.38994	
[25]	3	3.01887	3.01887	
[200]	3	11.13208	11.13208	11.13208
[50]	3		11.57233	11.57233
[100]	3			18.55346
Sig.		.056	.054	.152

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table B.3 One-way ANOVA result for percentage α-amylase inhibition

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
AA_Sample	Between Groups	97.207	2	48.603	45.595	.000
	Within Groups	6.396	6	1.066		
	Total	103.603	8			
AA_Positive_Control	Between Groups	251.039	2	125.520	224.996	.000
	Within Groups	3.347	6	.558		
	Total	254.387	8			

Table B.4 One-way ANOVA result for mortality percentage of brine shrimp in *Christia vespertilionis* leaves extract

ANOVA

Percentage_Mortality

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	390.965	5	78.193	1.050	.433
Within Groups	893.563	12	74.464		
Total	1284.529	17			

APPENDIX C

Figure C.1 *Christia vespertilionis* purchased from seller

Figure C.2 Grinding process for the sample (*Christia vespertilionis* leaves)

Figure C.3 Maceration technique in extracting the leaves of *Christia vespertilionis*

Figure C.4 Process of preparing Dragendorff reagent

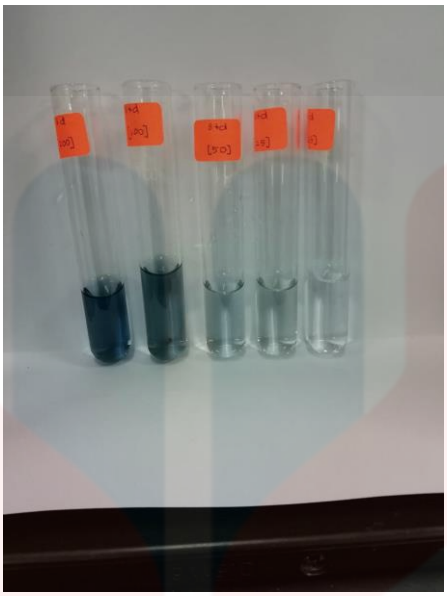

Figure C.5 Samples (*Christia vespertilionis* leaves extract) condition after being incubated for TPC assay

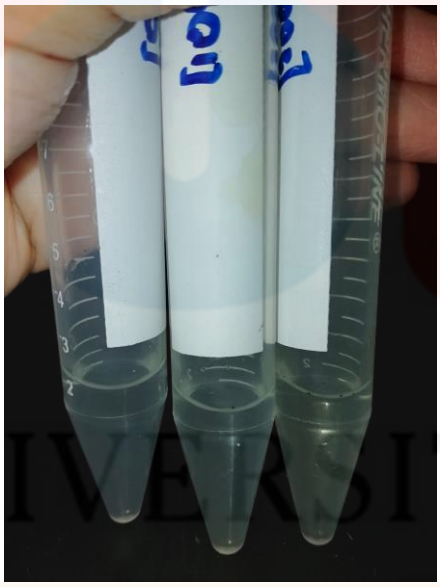

Figure C.6 Brine shrimp in different concentration of *Christia vespertilionis* leaves extract