# ISOLATION AND STRUCTURE ELUCIDATION OF POTENT α-GLUCOSIDASE INHIBITORY BIOACTIVE COMPOUND FROM *Stenochlaena palustris* BY SPE, HPLC AND NMR

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By

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## ABSTRACT

### ISOLATION AND STRUCTURE ELUCIDATION OF POTENT α-GLUCOSIDASE INHIBITORY BIOACTIVE COMPOUND FROM *Stenochlaena palustris* BY SPE, HPLC AND NMR

**VIVIAN KAH WAI LENG**

Diabetes mellitus, a warning disease is increasing and the symptoms are worrying. α-glucosidase inhibitors such as acarbose are well-known to treat diabetes by delaying carbohydrates digestion and decreases postprandial hyperglycaemia. Usage of synthetic α-glucosidase inhibitors causes unwanted adverse effects. Natural α-glucosidase inhibitors from medicinal plants are of interest. *Stenochlaena palustris* is edible and has been used in traditional medicine for disease treatment. This study is conducted to isolate and structure elucidate potent α-glucosidase inhibitory bioactive compound from *S. palustris* water fraction (SPWF) using Solid Phase Extraction (SPE), High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR). Reversed-phase SPE was used to fractionate SPWF and HPLC was used to check for fraction purity. The most potent antiglucosidase SPE fraction was determined for the mode of α-glucosidase inhibition and also characterized for concurrent antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and superoxide anion radical scavenging assays. Among SPE fractions obtained, 0% methanol (0% MeOH) fraction exhibited strongest

antiglucosidase activity with $EC_{50}$ value of 42.71 ± 0.95 μg/mL and it possessed non-competitive inhibition. This antiglucosidase activity was better than that of quercetin and acarbose. SPE has decreased the inhibition where 0% MeOH fraction has weaker activity than unfractionated SPWF ($EC_{50}$ = 39.03 ± 3.57 μg/mL). On the other hand, 0% MeOH fraction exhibited lowest DPPH radical and superoxide anion radical scavenging activity with $EC_{50}$ value of 78.27 ± 2.66 μg/mL and 1919.51 ± 36.32 μg/mL respectively. A pyridine alkaloid compound was predicted and proposed based on NMR analysis. This compound was believed to be the potent bioactive compound.

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## DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

(VIVIAN KAH WAI LENG)

**APPROVAL SHEET**

This project report entitled **"ISOLATION AND STRUCTURE ELUCIDATION OF POTENT α-GLUCOSIDASE INHIBITORY BIOACTIVE COMPOUND FROM *Stenochlaena palustris* BY SPE, HPLC AND NMR"** was prepared by VIVIAN KAH WAI LENG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biochemistry at Universiti Tunku Abdul Rahman.

Approved by:

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(Dr. Chai Tsun-Thai) Date:……..

Supervisor

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Date: _____

**PERMISSION SHEET**

It is hereby certified that **VIVIAN KAH WAI LENG** (ID No: **13ADB07353**) has completed this final year project entitled "**ISOLATION AND STRUCTURE ELUCIDATION OF POTENT α-GLUCOSIDASE INHIBITORY BIOACTIVE COMPOUND FROM *Stenochlaena palustris* BY SPE, HPLC AND NMR"** supervised by Dr. Chai Tsun-Thai (Supervisor) and Dr. Tong Kim Suan (Co-Supervisor) from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the university to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

_____

(VIVIAN KAH WAI LENG)

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α-amylase | Alpha-amylase |
| α-glucosidase | Alpha-glucosidase |
| C18 | Octadecyl carbon chain |
| DM | Diabetes mellitus |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| EC | Enzyme commission number |
| $EC_{50}$ | Half maximal effective concentration |
| FTIR | Fourier transform-infrared spectroscopy |
| FYP | Final year project |
| GD | Gestational diabetes |
| HPLC | High performance liquid chromatography |
| HPLC-MS/MS | HPLC-tandem mass spectrometry |
| IDF | International diabetes federation |
| $K_m$ | Michaelis-menten constant |
| LC-MS | Liquid chromatography-mass spectrometry |
| LSD | Least significant difference |
| mAU | Milliabsorbance units |
| ME | Methanol extract |
| MeOH | Methanol |
| mg | Milligram |
| MHz | Megahertz |
| µL | Microliter |

| | |
|---|---|
| Mm | Micrometer |
| mL | Milliliter |
| mM | millimolar |
| NADH | Nicotinamide adenine dinucleotide |
| NBT | Nitrotetrazolium blue chloride |
| Nd | Not determined |
| Nm | Nanometer |
| NMR | Nuclear magnetic resonance |
| NPGP | 4-nitrophenyl-α-D-glucopyranoside |
| PMS | Phenazine methosulfate |
| ppm | Part per million |
| RP-HPLC | Reversed-phase high performance liquid chromatography |
| RP-SPE | Reversed-phase solid phase extraction |
| [S] | Substrate concentration |
| SAS | Statistical analysis system |
| SPE | Solid phase extraction |
| SPWF | *S. palustris* water fraction |
| TMS | Trimethylsilane |
| U | Units |
| UHPLC-MS | Ultra high performance liquid chromatography-Mass spectrometry |
| UV-VIS | Ultraviolet-visible |
| v | velocity |

| | |
|---|---|
| v/v | volume per volume |
| $V_{max}$ | Maximum reaction velocity |
| WF | Water fraction |
| WP | Western pacific |

# CHAPTER 1

# INTRODUCTION

Plants have been used as a source of food, medicines and other life necessities in olden times. Pteridophytes (fern and fern allies), being the primitive vascular plants dispersed all over the globe. Unfortunately, medicinal values of pteridophytes have been ignored (Benniamin, 2011). Examples of pteridophytes are ferns, club-mosses and horsetails. Around 13,000 species of pteridophytes can be found (The Plant List, 2013). In Malaysia, compilation was done for 1165 pteridopytes species with 647 species in Peninsular Malaysia, 615 species in Sarawak and 750 species in Sabah (Ahmad Bedawi, Go and Mustafa, 2009).

The family Blechnaceae comprising the genus *Stenochlaena* is in the group of pteridophytes. *Stenochlaena palustris* is one of the pteridophytes which was edible and used in traditional medicine. With fronds having the property of antibacterial, it was used to treat fever, throat, gastric ulcers and skin diseases. On the other hand, its rhizomes and leaves were used as cooling agent in the treatment of burns and ulcers (Benniamin, 2011).

Solid Phase Extraction (SPE) is a technique of sample pretreatment used to extract and isolate the analytes of interest from complex interfering matrices. Recently, it has been rapidly developed and gives a good possibility for sample clean-up and preconcentration of analytes. Specific material introduced for

SPE initially was first hydrophobic bonded-silica sorbents. Different choices of sorbents ranging from polar, semi-polar, nonpolar, strong and weak ion exchange and steric exclusion with property of limited access are available (Wells, 2000).

Diabetes mellitus, a metabolic disorder resulted from defects in the production and action of insulin or both and is characterized by hyperglycemia (American Diabetes Association, 2013). With disturbance of carbohydrate, protein and fat metabolism, insufficient insulin leads to chronic hyperglycaemia. When diabetes develops damage to tissue or vascular, severe consequences such as nephropathy, neuropathy, retinopathy, ulceration and cardiovascular complications arises (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2002). Hence, a wide range of heterogeneous diseases happen simultaneously with diabetes (Bastaki, 2005).

α-glucosidase inhibitors with the role as antidiabetic were used to treat diabetic patients. It delayed digestion of complex carbohydrates and decreased the postprandial hyperglycaemia (van de Laar, 2008). In the gut, brush border enzymes which are necessary to hydrolyze oligosaccharides and polysaccharides into its monomers were inhibited by these inhibitors. Therefore, inhibition of these enzymes delayed carbohydrates digestion and absorption and diabetes can be managed (Bastaki, 2005). However, treatment using commercial antidiabetic drugs such as acarbose, voglibose and miglitol

can cause adverse effect and complications to the abdominal and gastrointestinal tract (Bastaki, 2005).

Due to the side effects and expensive cost of antidiabetic drugs, the search for potent α-glucosidase inhibitory bioactive compounds from natural sources such as medicinal ferns is of great interest (Kumar, et al., 2011). Discovery of antiglucosidase agent in traditional medicinal plants is important in the development of low cost management options with less adverse effect for diabetic patients (Tundis, Loizzo and Menichini, 2010).

Not much information regarding the antiglucosidase property of young fronds of *S. palustris* has been seen. Thus, the antiglucosidase activity was investigated in the collected SPE fractions prepared from the young fronds of *S. palustris* water fraction (SPWF). Upon identification of the most potent antiglucosidase fraction, the mode of inhibition on yeast α-glucosidase and its concurrent antioxidant activity was determined. Reversed-phase High Performance Liquid Chromatography (RP-HPLC) was used to compare the profile of SPWF (before SPE) and the most potent antiglucosidase fraction (after SPE). Nuclear Magnetic Resonance (NMR) was used to propose the structure of the bioactive compound that has the potent inhibitory effect on yeast α-glucosidase.

The objectives of this study are:

i. To isolate and structure elucidate a potent α-glucosidase inhibitory bioactive compound from SPWF by SPE, HPLC and NMR.
ii. To compare the antiglucosidase activity of SPWF before and after fractionation by using reversed-phase SPE (RP-SPE) cartridges
iii. To compare HPLC profile of SPWF before and after SPE fractionation.
iv. To determine the mode of inhibition of the most potent antiglucosidase SPE fraction.
v. To predict a structure for potential antiglucosidase compound based on NMR data.
vi. To characterize the most potent antiglucosidase SPE fraction for concurrent antioxidant activity.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Medicinal Ferns

On earth, pteridophytes have been contributed to the plant diversity, forming major and leading component of many plant communities. Since ancient times, their medicinal values are well-known to people for many years (Benniamin, 2011). In general, pteridophytes can be used as medicines, food, ornamentations and insecticides. Being a food source, many parts of fern such as leaves, rhizomes, stems, shoots and young fronds or the entire plants can be consumed. For instance, pteridophytes are used as medicines in Ayurvedic, Homoeopathic, Tribal and Unani. Playing a role in medicinal field, numerous plant parts of pteridophytes are used in the traditional treatment of diseases (Mannan, Maridass and Victor, 2008). Pteridophytes are taken orally for the treatment of diarrhea, skin problems and stomacheache (Benniamin, 2011).

There are findings on the presence of bioactive compounds in medicinal ferns that were potent sources of antioxidant and antidiabetic. Medicinal ferns such as *Cyathea latebrosa*, *Cibotium barometez* and *Drynaria quercifolia* exhibited very strong antioxidant activity due to high total phenolic content in the methanol extract (ME) of leaf (Lai and Lim, 2011). A study done by Chai, et al. (2015) found that medicinal fern such as *Blechnum orientale*, *Diplazium esculentum*, *Nephrolepis biserrata* and *Pteris vittata* experienced dose-dependent cytotoxicity against cells of K562 whereas *D. esculentum* contained

potent antiglucosidase inhibitor that makes it a potential source of antidiabetic agent.

Besides antioxidant and antidiabetic properties, medicinal ferns also possessed antibacterial effect. Ponnusamy, et al. (2013) reported that ME of *Dicranopteris linearis* and *S. palustris* has antibacterial effect against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. With the significant properties of antioxidant and antibacterial, *D. linearis* and *S. palustris* would be a choice of traditional medicine used to treat wound injuries and infection (Ponnusamy, 2013).

## 2.2 Edible fern *S. palustris*

In coastal condition, *S. palustris* is a highly adaptable species where it colonized edges of hot springs and mangroves. With tropical and subtropical climate, in the damp open places on forest margins on lowland regions, it grows permanently. It was found abundant in secondary forest and climbing high up trees and palms. *S. palustris* is widely occupied, distributed and found abundant throughout Asian countries, India, Malesia, Australia, south-west Pacific region, Papua New Guinea, and the ecological niches on Indian Ocean islands including Madagascar and East Africa (Chambers, 2013). *S. palustris* are edible and has medicinal values as some of its parts used as traditional medicine such as leaves were used to cure fever (Mannan, Maridass and Victor, 2008).

Liu, et al. (1998) reported that a few glycosides such as stenopaluside particularly (4S*,5R*)-4-[(9Z)-2,13-di-(O-β-D-glucopyranosyl)-5,9,10-trimethyl-8-oxo-9-tetradecene-5-yl]-3,3,5-trimethylcyclohexanone, new cerebroside, namely 1-O-β-D-glucopyranosyl-(2S*,3R*,4E,8Z)-2-N-[(2R)-hydroxytetracosanoyl]octadecasphinga-4,8-dienine, along with four known natural products, namely 3-oxo-4,5-dihydro-α-ionyl β-D-glucopyranoside, β-sitosterol-3-O-β-D-glucopyranoside, 3-formylindole and lutein were isolated from the leaves and their structures were elucidated through spectroscopic methods.

Previous study by Liu, et al. (1999) also revealed that acylated flavonol glycosides in leaves of *S. palustris* were isolated and identified by spectroscopic methods. They found five new O-acylated flavonol glycosides, known as stenopalustrosides A (kaempferol 3-O-(3’’,6’’-di-O-Z-p-coumaroyl)-β-glucopyranoside), B (kaempferol 3-O-(3’’-O-Z-p-coumaroyl)-(6’’-O-E-feruloyl)-β-glucopyranoside), C (kaempferol 3-O-(3’’-O-Z-p-coumaroyl)-(6’’-O-E-p-coumaroyl)-β-glucopyranoside), D (kaempferol 3-O-(3’’-O-E-p-coumaroyl)-(6’’-O-Z-p-coumaroyl)-β-glucopyranoside) and E (kaempferol 3-O-(3’’-O-E-pcoumaroyl)-[6’’-O-E-{4-O-[1-(4-hydroxy-3-methoxyphenyl)-1,3-dihydroxy-isopropyl]-feruloyl}]-β-glucopyranoside). These compounds were found with five known compounds, namely kaempferol 3-O-(3’’-O-E-p-coumaroyl)-(6’’-O-E-feruloyl)-β-D-glucopyranoside, kaempferol 3-O-(3’’,6’’-di-O-E-p-coumaroyl)-β-D-glucopyranoside, kaempferol 3-O-(3’’-O-E-p-coumaroyl)-β-D-glucopyranoside, kaempferol 3-O-(6’’-O-E-p-coumaroyl)-β-

D-glucopyranoside (tiliroside), and kaempferol 3-O-β-D-glucopyranoside. Compounds stenopalustrosides A to D were reported to have antibacterial activities due to the presence of coumaroyl-substituted flavonol glycosides with the configuration of double bond which were important in the property of antibacterial (Liu, et al., 1999).

*S. palustris* is also known as 'kelakai'. Its leaves possessed potent antioxidant effect which it has chelating effect on ferrous ion and was able to scavenge hydroxyl radical and hydrogen peroxide. The antioxidant effect was caused by the presence of flavonoids (Suhartono, et al., 2012). The effect of antioxidant in young sterile frond and mature sterile frond of *S. palustris* also reported by Chai, et al. (2012) due to the presence of high phenolic contents that causes it to have high specific metal chelating activity and effective as reductants respectively. Studies showed that leaf (ME) of *S. palustris* contributed to the inhibitory effect against fungal and bacterial strains. This indicated that it has antimicrobial property (Zuraini, et al., 2010).

A study revealed by Adenan and Suhartono (2010) stated that *S. palustris* contained alkaloids and steroids which possessed anti-inflammatory property has contributed to antipyretics effect. This was similar to that reported by Sudjarwo (2006) who revealed that alkaloid piperine in *S. palustris* plays a role in antipyretic effect via prostaglandin synthesis inhibition. Chai, et al. (2015) found that water fraction (WF) of mature frond of *S. palustris* is a potential antiglucosidase agent possibly due to the presence of phenolic compounds,

particularly hydroxycinnamic acids. This finding showed that WF of mature frond of *S. palustris* is more specific towards α-glucosidase (Chai, et al., 2015).

Since there is no studies on the antiglucosidase potential of young frond of *S. palustris*, this study has been conducted to isolate and structure elucidate the potent α-glucosidase inhibitory bioactive compound from young frond of *S. palustris* using SPE, HPLC and NMR. It has been a significance to search for natural derived plant compounds with potent antiglucosidase activity (Mayur et al., 2010). Figure 2.1 shows the young fronds of *S. palustris*.


**Figure 2.1:** Young fronds of *S. palustris.*
(Source: Japan International Research Center for Agricultural Sciences, 2010)

## 2.3 Diabetes Mellitus (DM)

In ancient times, DM was known and its treatment was known only in the Middle Ages. In 20$^{th}$ century, the clarification of its pathogenesis only occurred (Piero, Nzaro and Njagi, 2014). DM is the representative of heterogeneous group of disorders that commonly presented with glucose

intolerance and hyperglycaemia (International Diabetes Federation, 2006). Symptoms of noticeable hyperglycemia are characterized by excessive thirst, fatigue, polyuria, polydipsia, weight loss and blurred vision. In the presence of one or more related complications, DM may become manifest (American Diabetes Association, 2013).

DM can be categorized into several types. The two main types are type 1 and type 2. Type 1 DM, the insulin-dependent diabetes is characterized by an absolute insufficient of insulin production where individuals have an autoimmune pathogenesis in the pancreatic islets. Type 2 DM which also known as non-insulin dependent diabetes accounted for 90 to 95% of diabetic patients. The cause of type 2 DM is a combination of both insulin action resistance and insufficient compensatory of insulin secretory response (American Diabetes Association, 2013). In high-income countries, 85 to 95% are mostly type 2 DM and currently it is a severe and general health concerned (Piero, Nzaro and Njagi, 2014). By the year 2030, it is predicted that 439 million people would experienced type 2 DM (Olokoba, Obateru and Olokoba, 2012).

As insulin resistance causes insulin insensitivity in type 2 DM, a declined in the transportation of glucose to liver, fat and muscle cells leads to a rise in the breakdown of fat as well as hyperglycemia (Olokoba, Obateru and Olokoba, 2012). For type 2 DM, hyperglycemia is enough to cause functional and pathological changes in different target tissues without clinical symptoms

which may present for a long time period before diabetes is detected and chronic hyperglycemia is related to dysfunction, long term damage and organ failure such as in the heart, kidney, nerves, eyes and blood vessels (American Diabetes Association, 2013). Postabsorptive hepatic glucose generation which positively correlates with fasting plasma glucose concentration increased in type 2 DM (Piero, Nzaro and Njagi, 2014). Gestational Diabetes (GD) is the initial recognition or onset of glucose intolerance in the second or third trimester during pregnancy. About 4% of all pregnant women have GD. Individuals who have GD usually will have a chance of 30 to 50% to develop type 2 DM (Bastaki, 2005).

International Diabetes Federation (IDF) of Western Pacific (WP) region reported that 415 million of people in the world have diabetes and in the WP region, almost 153 million of people have diabetes and by the year 2040, this value is estimated to increase to 215 million. In Malaysia, there were 3.3 million of diabetes cases in 2015. In 2015, the prevalence of diabetes in adults was 16.6% which is 3303 cases from total adult population of 19887 (International Diabetes Federation, 2015)

Figure 2.2 shows the prevalence of diabetes in adults in year 2015 by age in Malaysia. In the population, age groups which have the highest proportions for diabetes can be known from the figure. In the figure, dotted line showed the distribution of diabetes prevalence by age for the world; black line for the WP region and red line for Malaysia. For middle- and low-income countries, when

compared to the world average, there are more people with diabetes under 60 years old whereas for high-income countries, the highest proportion of diabetes prevalence is over 60 years old (International Diabetes Federation, 2015).

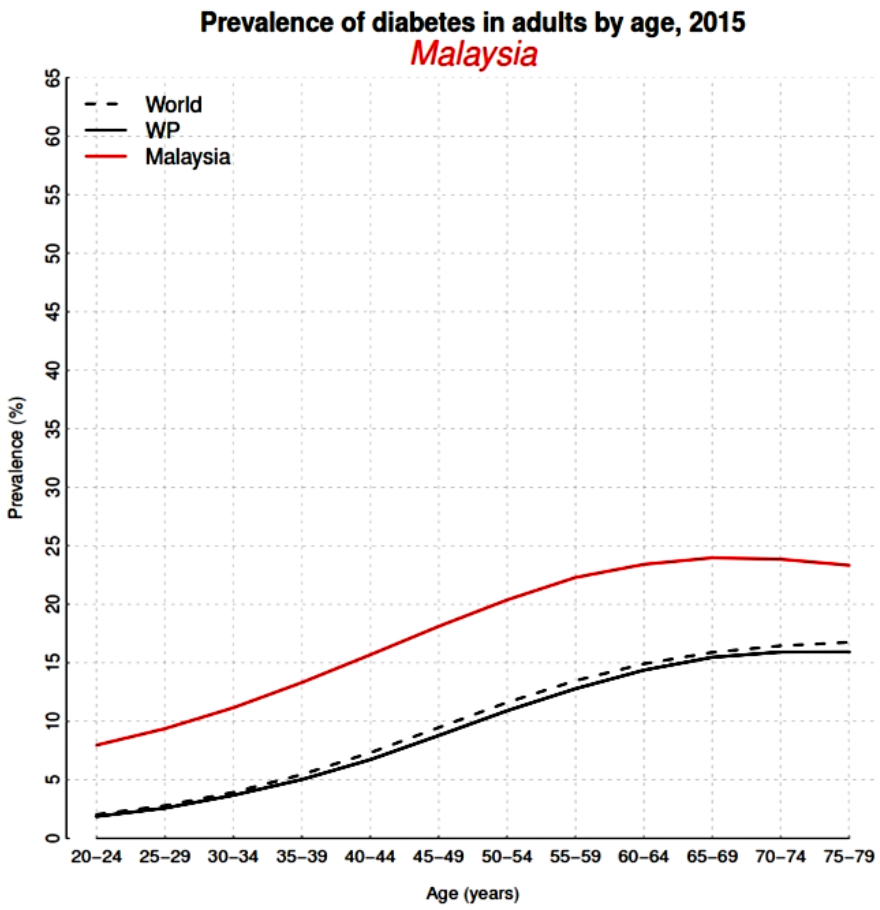



**Figure 2.2:** Malaysia versus World Prevalence of Diabetes.
(Source: International Diabetes Federation, 2015)

## 2.4 α-glucosidase Inhibitors

α-glucosidase (EC 3.2.1.20) is an exo-type of α-glucosidic-O-linkage hydrolases which dissociates D-glucose from substrate at the non-reducing end side. In microorganism, plant and animal tissues, various types of α-glucosidases can be found (Kimura, et al., 2004). Not only synthetic α-

glucosides and oligosaccharides are being broken down by most of the α-glucosidase, α-glucans such as glycogen and soluble starch can also be hydrolyzed. α-glucosidase has higher affinity to soluble starch as highest hydrolysis activity can be obtained (Chiba, 1997).

Initially, α-glucosidase inhibitors being the antidiabetic drugs were isolated from bacterial strains or their derivatives (Kalra, 2014). Acarbose, voglibose and miglitol is the examples of synthetic α-glucosidase inhibitors. Acarbose is isolated from Actinoplanes, voglibose derived from validamycin A (product of *Streptomyces hygroscopicus* var. limoneus) and miglitol is a semisynthetic derivative of 1-deoxynojirimycin from *Bacillus* and *Streptomyces sp.* (Hanefeld and Schaper, 2007). These α-glucosidase inhibitors which also known as oral hypoglycaemic agents can be used to maintain blood glucose level at the requisite threshold in diabetic patients (Piero, Nzaro and Njagi, 2014).

α-glucosidase inhibitors do not have a pancreato-centred mechanism of action. They help to manage diabetes by control postprandial hyperglycemia which delayed the absorption of carbohydrates in the gastrointestinal tract. The mechanism of inhibition possessed was competitive (Bastaki, 2005). Acarbose inhibits the action of α-amylase which breaks down starch into disaccharides and also inhibits other α-glucosidases, thereby blocking the absorption of starch and other carbohydrates from the intestine's brush border. On the other hand, voglibose and miglitol has no effect on the starch digesting α-amylase

enzyme but they inhibit the disaccharide digesting enzymes (Laube, 2002; Chen, Zheng and Shen, 2006).

The action of inhibition contributed to unwanted effects. Adverse effects such as abdominal pain, diarrhoea and flatulence may occur when undigested disaccharides remained in the lumen of intestine (Kalra, 2014). In the lower gastrointestinal tract, accumulation of the delayed carbohydrate digestion raised the amount of fermentable carbohydrates reaching the colon and leads to occurrence of the adverse effect (Bastaki, 2005). Due to the side effects caused by commercial α-glucosidase inhibitors, natural α-glucosidases inhibitory compounds derived from plants are of great interest to be used in diabetes management.

A study conducted by Chai, et al. (2013) revealed that highland fern such as *Phymatopteris triloba* was of potential therapeutic agents due to its potent antiglucosidase activity. Piero, et al. (2011) reported that plants which have been used in herbal medicine displayed significant antidiabetic activity by lowering the blood glucose level when feed to induced diabetic mice. There are many plant species have antidiabetic action due to the presence of natural hypoglycaemic compounds and they are of interesting alternatives to replace synthetic drugs and to be used in the treatment (Coman, Rugina and Socaciu, 2012).

## 2.5 Antioxidant Activity

In nature, antioxidant compounds are endogenous and exogenous (Talukdar, et al., 2011). Antioxidants, which present in food or body at very low concentration are compounds that control, delay or prevent oxidative processes. Oxidative processes causes initiation and multiplication of degenerative diseases in the body or leads to exacerbation of food quality (Shahidi and Zhong, 2015). In food, antioxidant compounds are important as health protecting factor as they decreases the risk for chronic diseases such as cancer and heart disease. Intake of dietary antioxidants enhances the defending against free radicals (Alam, Bristi and Rafiquzzaman, 2013). In view of the presumed safety, potent nutritional and therapeutic effects of natural antioxidants, their presence in food and other biological matters are of interest. Studies reported that natural antioxidants in medicinal plants act as radical scavengers by converting harmful radicals into less reactive species (Palvai, Mahalingu and Urooj, 2014).

Antioxidants such as tocopherols and polyphenols can be found abundantly in many plants. Upon isolation of these constituents from plant, they can be used in the industry of pharmaceutical (Shahidi and Zhong, 2015). There are many *in vivo* and *in vitro* methods for the assessment of antioxidant activity. The *in vitro* methods includes DPPH radical scavenging activity, ferric reducing-antioxidant power assay, nitric oxide scavenging activity, hydrogen peroxide scavenging activity, superoxide anion radical scavenging activity and etc. On the other hand, *in vivo* methods include superoxide dismutase method, catalase

method, lipid peroxidation assay and etc (Alam, Bristi and Rafiquzzaman, 2013).

## 2.6 SPE

SPE is an extraction method which combines non-linear chromatography for sample purification, separation, concentration and solvent exchange of analyte of interest. It involves analyte removal from a flowing liquid sample by retention on solid sorbent and analyte of interest are recovered by elution from the sorbent (Wells, 2000). SPE is a heterogeneous alternative which involves partition in between a solid phase and liquid phase (Zwir-Ferenc and Biziuk, 2006).

There are several types of SPE depending on the type of sorbent which selected based on contact between the sorbent and compound of interest (Zwir-Ferenc and Biziuk, 2006). Initially, the first materials introduced were hydrophobic bonded silica sorbents which known as normal-phase SPE. A variety of sorbents with different properties are available such as non-polar sorbents for RP-SPE, ion exchanging sorbents for ion-exchange SPE and sorbents with steric exclusion properties (Wells, 2000).

SPE is well-known to rapidly replace liquid-liquid extraction due to its low cost, ease to use and convenient. Utilization of SPE has advantages over liquid-liquid extraction. It ameliorated selectivity and specificity, enhanced removal of particulates from a sample and has greater reproducibility. It

coupled with other chromatographic techniques to optimize sample separations. (Phenomenex, n. d.). Zhang et al. (2010) have revealed that there was a need to remove interfering substances from extracts to determine enzyme inhibition activities. Pretreatment of sample by SPE in the study conducted by Pilipczuk, et al. (2015) was required to analyze indolic compounds in plant extracts.

In this study, RP-SPE was used as previous study done by previous FYP student, Miss Low Si Ling showed that SPWF has potent polar compounds which causes the antiglucosidase activity to be high. RP- SPE is a system with non-polar stationary phase and polar mobile phase. Carbon chain bonded silica such as butyl, octyl and octadecyl are universal extraction sorbents and they have been commonly used in research (Zwir-Ferenc and Biziuk, 2006). It is generally used to extract hydrophobic or polar compound from aqueous samples (Phenomenex, n. d.). Study showed that RP-SPE has been used to fractionate and isolate most phytochemicals from crude plant extracts (Lau, et al., 2015). According to Zhang, et al. (2015), involvement of SPE in the extraction and purification of plant hormone showed high efficiency.

## 2.7 HPLC

Chromatography is an analytical method that separates molecules due to differences in their composition and structure; it involves the movement of sample over a stationary phase through the mobile system leading to molecules separation as the molecules in the sample have different interactions with the stationary phase. Chromatographic separations through a column can occur

using various stationary supports (Kupiec, 2004). HPLC is typically an important tool derived from classical column chromatography and is capable to separate, identify and quantify compounds that present in sample dissolved in liquid (Bhardwaj, Dwivedi and Agarwal, 2015).

In the pharmaceutical field, HPLC is the choice to apply in drugs identification, purity evaluation and quantification due to its ease of automation, accuracy, specificity, precision and rapidity. HPLC helps in checking for peak purity of new chemical entities, controlling reaction changes in synthetic process and evaluating new formulations (Bhardwaj, Dwivedi and Agarwal, 2015). Generally, there are few types of HPLC which used in analysis including normal-phase, reversed-phase, size exclusion, ion exchange and bioaffinity chromatography. Each type has different phase system used in the process (Malviya, et al. 2010).In this study, RP-HPLC with gradient elution system was used. A non-polar stationary phase along with an aqueous, moderately polar eluent was applied in RP-HPLC. RP-HPLC separates analyte based on the resulted hydrophobic interactions from repulsive forces between the non-polar stationary support, non-polar analyte and polar eluent (Malviya, et al. 2010). Gradient elution is often employed in sample separation by HPLC. By combining the pressurized eluent flows from two pumps and changes their individual flow rates with a data system, a gradient is generated while maintaining the overall flow rate constant (Kupiec, 2004).

There are phytochemical study showed that established and validated HPLC which known as HPLC diode array detector method was used. The bioactive substances, polyphenols, in apple extracts have been demonstrated to have potential effect of antibacterial, antioxidant, antitumor, antiradiation, anti-inflammatory and antiobesity. This method was reliable, accurate and simple and can develop HPLC fingerprint for the determination (Bai, et al., 2016). Zhang, et al. (2015) revealed that combination in one step of column chromatography extraction and solid phase separation technologies completely extract and purify the peanut plant endogenous abscisic acid where the highly concentrated form is subjected for direct HPLC analysis. HPLC helps to detect and identify abscisic acid in the peanut plant (Zhang, et al., 2015).

HPLC can be coupled with mass spectrometry in biochemical analysis. A study conducted by Cai, et al. (2015) described the usage of HPLC-tandem mass spectrometry (HPLC-MS/MS) coupled with isotope-coded derivatization to determine the plant growth regulators in extracts of cucumber and tomato. HPLC aided in the separation of plant growth regulators and the determination of retention time between the derivatives (Cai, et al., 2015). According to de Melo and Sawaya (2015), UHPLC-MS allowed the evaluation of coumarin and chlorogenic acid concentrations in guaco samples (medicinal plants) while permitted the detection of other sample components. The complex chemical profile of the plant extracts were detected using the technique (de Melo and Sawaya, 2015).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Instruments and Chemicals

The instruments and chemicals used in this research were provided by Department of Chemical Science, Faculty of Science in Universiti Tunku Abdul Rahman. The instruments and chemicals with its respective manufacturers are listed down in Table 3.1 and 3.2, respectively.

**Table 3.1:** List of instruments used with respective manufacturer.

| **Instruments** | **Manufacturer** |
|---|---|
| HPLC | Shimadzu |
| Electronic balance | Sartorius |
| CoolSafe$^{TM}$ freeze-dryer | Chemopharm |
| Rotary Evaporator | Büchi R-200 |
| Digital water bath | Daniel |
| pH meter | Mettler Toledo |
| UV-VIS spectrophotometer | Biochrom libra S4 |
| Oven | Memmert |
| Refrigerator | Toshiba |
| Vortex mixer | Scientific Industries, Inc |
| StrataTM C18-E (55um, 70A) 500 mg/6 mL | Phenomenex® |
| Gemini 5u C18 110A (150 × 4.60 mm) | Phenomenex® |
| JEOL LA-400 MHz NMR spectrometer | JEOL Ltd. |

**Table 3.2:** List of chemicals used with respective manufacturer.

| **Chemicals** | **Manufacturer** |
|---|---|
| Potassium dihydrogen phosphate ($KH_2PO_4$) | QRëC™ |
| di-Potassium hydrogen phosphate anhydrous ($K_2HPO_4$) | QRëC™ |
| Sodium carbonate anhydrous ($Na_2CO_3$) | QRëC™ |
| 4-nitrophenyl-α-D-glucopyranoside (NPGP) | Acrōs Organics |
| p-nitrophenol | Bio Basic Inc. |
| Quercetin hydrate, 99% | Acrōs Organics |
| Phenazine methosulfate, 98% (PMS) | Acrōs Organics |
| Nitrotetrazolium blue chloride, approx, 98% (NBT) | Sigma® |
| β-Nicotinamide adenine dinucleotide (NADH), reduced disodium salt hydrate | Sigma® |
| 2,2-diphenyl-1-picrylhydrazyl (DPPH) | Sigma® |
| Methanol (MeOH) | Merck |
| Acarbose | Fluka |
| α-glucosidase from *Saccharomyces cerevisiae* | Sigma® |
| Sodium hydroxide (NaOH) | R&M chemicals |
| Acetonitrile | RCI Labscan |

## 3.2 Experimental Design

The overview of the experimental approaches for this research is summarized as follows:

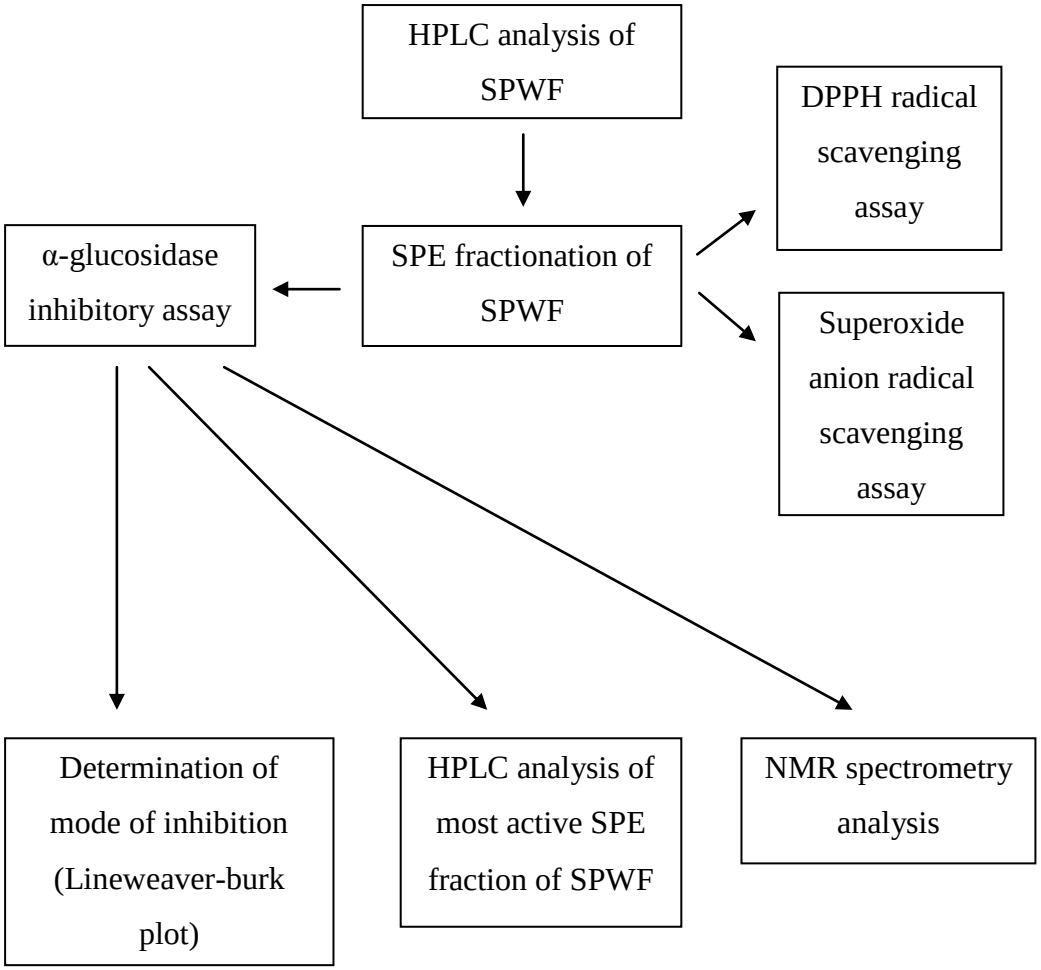



**Figure 3.1:** Summary of the experimental approaches for this study.

### 3.3 Sample Materials

SPWF was provided by previous FYP student, Miss Low Si Ling who has done sequential extraction and solvent partition on the young frond of *S. palustris*. In sequential extraction, the sequence of the solvents used was hexane, chloroform, ethyl acetate, methanol and deionized water. Then, the water extract was redissolved in deionized water and the resulting solution was partitioned sequentially with ethyl acetate and n-butanol yielded the water fraction which was used in this study.

### 3.4 RP-HPLC

Liquid chromatography technique was assessed according to Wang, et al. (2016) with modifications. A 5 mg/mL of sample was prepared and subjected to RP-HPLC equipped with a C18 packed column. Mobile phase containing (A) acetonitrile and (B) 0.1% (v/v) formic acid in deionized water was delivered at flow rate of 0.5 mL/min. The gradient elution program applied in this analysis was: equilibrate system with 20% A for 10 minutes, followed by running system with 25% A for 10 minutes and hold 25% A for 10 minutes. The wavelength of UV-VIS detector was measured at 260 nm, 320 nm and 460 nm.

### 3.5 RP-SPE

The RP-SPE was performed according to Chu, Wu and Hsieh (2014) with slight modifications. Eight cartridges were used in this study. Firstly, each of the cartridges was conditioned with 12 mL of MeOH, and then equilibrated with 12 mL of deionized water. A 200 mg of SPWF (filtered using 0.22 μm

membrane filter and resulting solution was freeze dried) was redissolved in 16 mL of deionized water and a volume of 2 mL of the SPWF (25 mg) was loaded into each cartridge. After that, SPWF was eluted with stepwise method using 6 mL of 0%, 10%, 20%, 30%, 40%, 50% and 100% MeOH respectively. Total of seven fractions with each fraction of 6 mL were collected. After that, the fractions were concentrated by freeze dryer and rotary evaporator. The 0% MeOH fraction was concentrated by freeze dryer while other fractions (10%, 20%, 30%, 40% and 50% MeOH) were concentrated by rotary evaporator and followed by freeze dryer. The 100% MeOH fraction was concentrated by rotary evaporator. All dried fractions were stored at -20°C for further used.

### 3.6 Yield Determination of All Fractions

By using the formula as shown below, the yield of all collected fractions was calculated and expressed in percentage (%).

$$\text{Yield of fraction (\%)} = \frac{\text{Mass of collected fraction (mg)}}{\text{Mass of SPWF used for fractionation (mg)}} \times 100\ \%$$

## 3.7 Antiglucosidase Activity

### 3.7.1 α-glucosidase Inhibitory Assay

The α-glucosidase inhibitory assay was carried out according to Wong, et al. (2014). An assay mixture of 250 μL of 100 mM potassium phosphate buffer (pH 7.0), 150 μL of 0.5 mM NPGP (dissolved in 100 mM potassium phosphate buffer of pH 7.0), 50 μL of sample and 150 μL of 0.1 U/mL α-glucosidase (dissolved in 10 mM potassium phosphate buffer of pH 7.0) was incubated at 37 °C for 30 minutes. After 30 minutes, 600 μL of 200 mM sodium carbonate, $Na_2CO_3$ was added to stop the reaction. The absorbance of the mixture was measured at 400 nm. For blank solution, the α-glucosidase was replaced with 10 mM potassium phosphate buffer (pH 7.0). Antiglucosidase activity was calculated using the formula:

$$\text{Antiglucosidase activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\ \%$$

$A_{control}$, the absorbance reading of control reaction with the absence of sample (deionized water was used instead) and $A_{sample}$, the absorbance reading of the reaction with sample. Quercetin and acarbose is the positive control for this assay. Results were interpreted in the form of half maximal effective concentration, $EC_{50}$ which is the sample concentration required to achieve 50% of α-glucosidase inhibitory activities in the assay mixture.

### 3.7.2 Mode of Inhibition of α-glucosidase

The mode of α-glucosidase inhibition by the most active fraction was determined by Lineweaver-Burk plot referring to the method described by Chai, et al. (2015). The fraction was used as inhibitor using $EC_{50}$ samples at 0 × $EC_{50}$ (control), 1 × $EC_{50}$ and 2 × $EC_{50}$. As described previously, α-glucosidase inhibitory assay was carried out by varying the concentration of NPGP from 0 to 1.6 mM. A reaction mixture of 250 µL of 100 mM potassium phosphate buffer at pH 7.0, 150 µL of NPGP (0 – 1.6 mM), 50 µL of sample and 150 µL of 0.1 U/mL α-glucosidase was incubated at 37 °C for 30 minutes. After 30 minutes, the reaction was stopped by adding 600 µL of 200 mM $Na_2CO_3$ and the absorbance was read at 400 nm. For the control, deionized water was used in replacement of sample. A p-nitrophenol standard curve ranging from the concentration of 0 to 0.5 mM was prepared. The amount of p-nitrophenol produced during the enzymatic reaction was determined from the standard curve. The reaction velocity (v) was calculated using the formula below.

$$\text{Reaction velocity, v } \left(\frac{\text{mM}}{\text{min}}\right) = \frac{\text{Amount of product formed (mM)}}{\text{Reaction time (min)}}$$

A double reciprocal plot, also known as Lineweaver-Burk plot (1/v versus 1/[S]) was plotted where 1/v is the reciprocal of reaction velocity and 1/[S] is the reciprocal of NPGP substrate concentration. The mode of inhibition by the most active fraction on α-glucosidase was determined by using Michaelis-Menten kinetics. Maximum reaction velocity ($V_{max}$) was determined from the

y-intercept value of the plot while Michaelis-Menten constant ($K_m$, substrate concentration that yields half-maximal velocity) was determined from the x-intercept value of the plot.

## 3.8 Antioxidant Activity

### 3.8.1 DPPH Radical Scavenging Assay

DPPH radical scavenging activity of sample was determined according to Wong, et al. (2014) with slight modifications. A volume of 500 μL of sample was mixed with 700 μL of 0.10 mM DPPH (in MeOH). The reaction mixture was allowed to stand at room temperature for 30 minutes in dark. Absorbance was measured at 517 nm. For blank solution, DPPH was replaced with MeOH. DPPH radical scavenging activity of the sample was calculated using the formula:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\ \%$$

$A_{control}$, the absorbance reading of control reaction in absence of sample (deionized water was used) whereas $A_{sample}$, the absorbance reading of the reaction with sample. The positive control for this assay is quercetin. Results were expressed as $EC_{50}$ which defined as the concentration of sample needed to scavenge 50% of DPPH radicals in the reaction mixture.

### 3.8.2 Superoxide Anion Radical Scavenging Assay

Superoxide anion radical scavenging activity of sample was accessed using the procedure according to Kumar, Kumar and Kaur (2011) with modifications. An assay mixture of 800 μL of 100 mM potassium phosphate buffer (pH 7.4), 100 μL of sample, 100 μL of 780 μM NBT (dissolved in 100 mM potassium phosphate buffer, pH 7.4), 100 μL of 2340 μM NADH (dissolved in 100 mM potassium phosphate buffer, pH 7.4) and 50 μL of PMS (dissolved in water) was mixed well. The mixture was incubated in dark for 20 minutes at room temperature. Absorbance was read at 560 nm using blank solution without NADH but contained 900 μL of 100 mM potassium phosphate buffer (pH 7.4). Superoxide anion radical scavenging activity of the sample was determined using the formula:

$$\text{Superoxide anion radical scavenging activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\ \%$$

$A_{control}$, the absorbance reading of control where sample was replaced with deionized water and $A_{sample}$, the absorbance reading of the reaction with sample. Quercetin was used as the positive control. Results were expressed as $EC_{50}$, the sample concentration required to scavenge 50% of superoxide anion radicals in the reaction mixture.

### 3.9 NMR analysis

JEOL LA-400 MHz NMR spectrometer was used for the analysis of the most active fraction. Approximate 40 mg of the most active fraction was dissolved in a sufficient amount of deuterium oxide which then filled into a NMR tube. The NMR tube was capped and wrapped with parafilm. The NMR spectra of $^{1}H$ (400 MHz) and $^{13}C$ (100 MHz) were obtained with trimethylsilane (TMS) as the internal standard.

### 3.10 Statistical Analysis

Assays were carried out in triplicates. Data were expressed as mean ± standard errors. Student's *t*-test was carried out to determine the significance of differences between means at significance level $p < 0.05$. Ranking of means value of $V_{max}$ determined from Lineweaver-Burk plot was performed with Fisher's LSD procedure using SAS 9.3 statistical software.

# CHAPTER 4

# RESULTS

## 4.1 RP-HPLC of Unfractionated SPWF (Before SPE)

Figure 4.1 shows the HPLC chromatograms of SPWF before SPE (unfractionated) at the three wavelengths of 260 nm, 320 nm and 460 nm, respectively. The peak at 0 to 5 minutes is belonged to the solvent peak. The intensity of the peaks in chromatogram at wavelengths of 260 nm, 320 nm and 460 nm was found to be 150 mAU, 100 mAU and 5 mAU after 5 minutes, respectively.

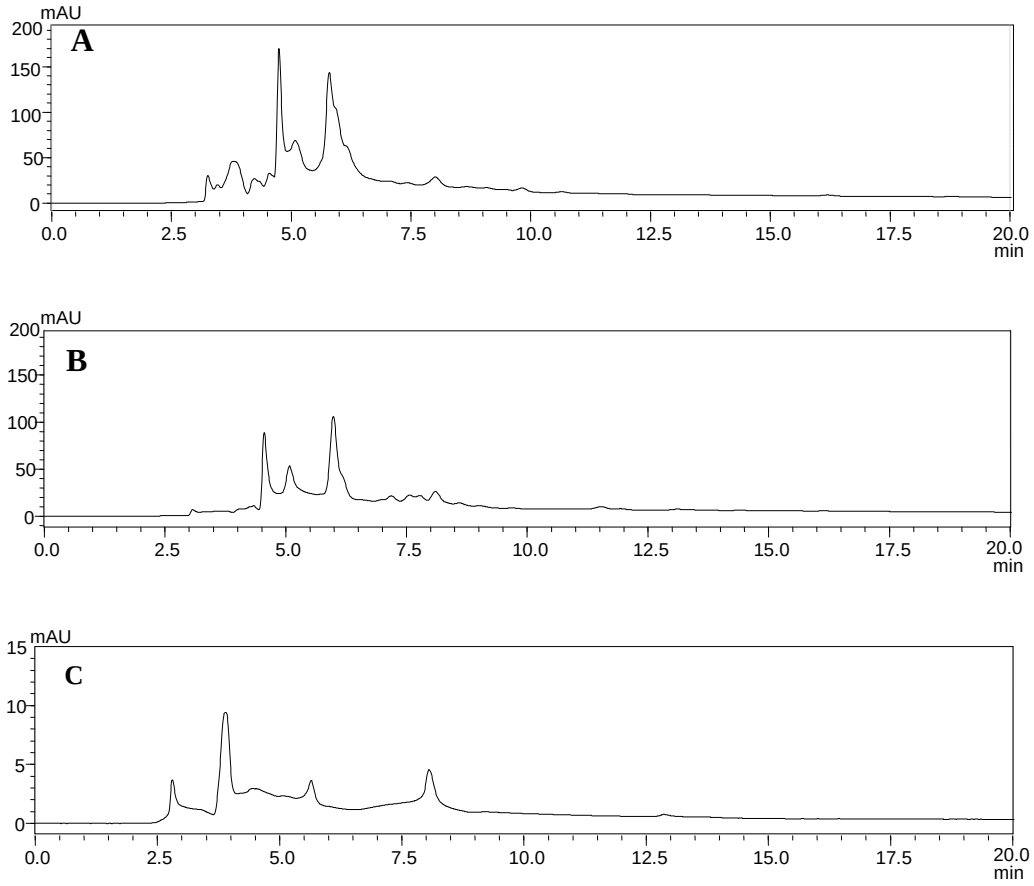



**Figure 4.1:** HPLC chromatograms of SPWF at the wavelengths of **(A)** 260 nm, **(B)** 320 nm and **(C)** 460 nm.

## 4.2 Fraction Yield

Table 4.1 shows the yield of fractions collected from RP-SPE of SPWF. The yield of eluted fractions was calculated based on milligram of SPWF. The fraction of 0% MeOH gave the highest yield while fraction of 100% MeOH gave the least yield. The 0% MeOH fraction showed 145.1-fold higher mass as compared to 100% MeOH fraction. Presented yield data were based on one replicate of fractionation using eight RP-SPE cartridges.

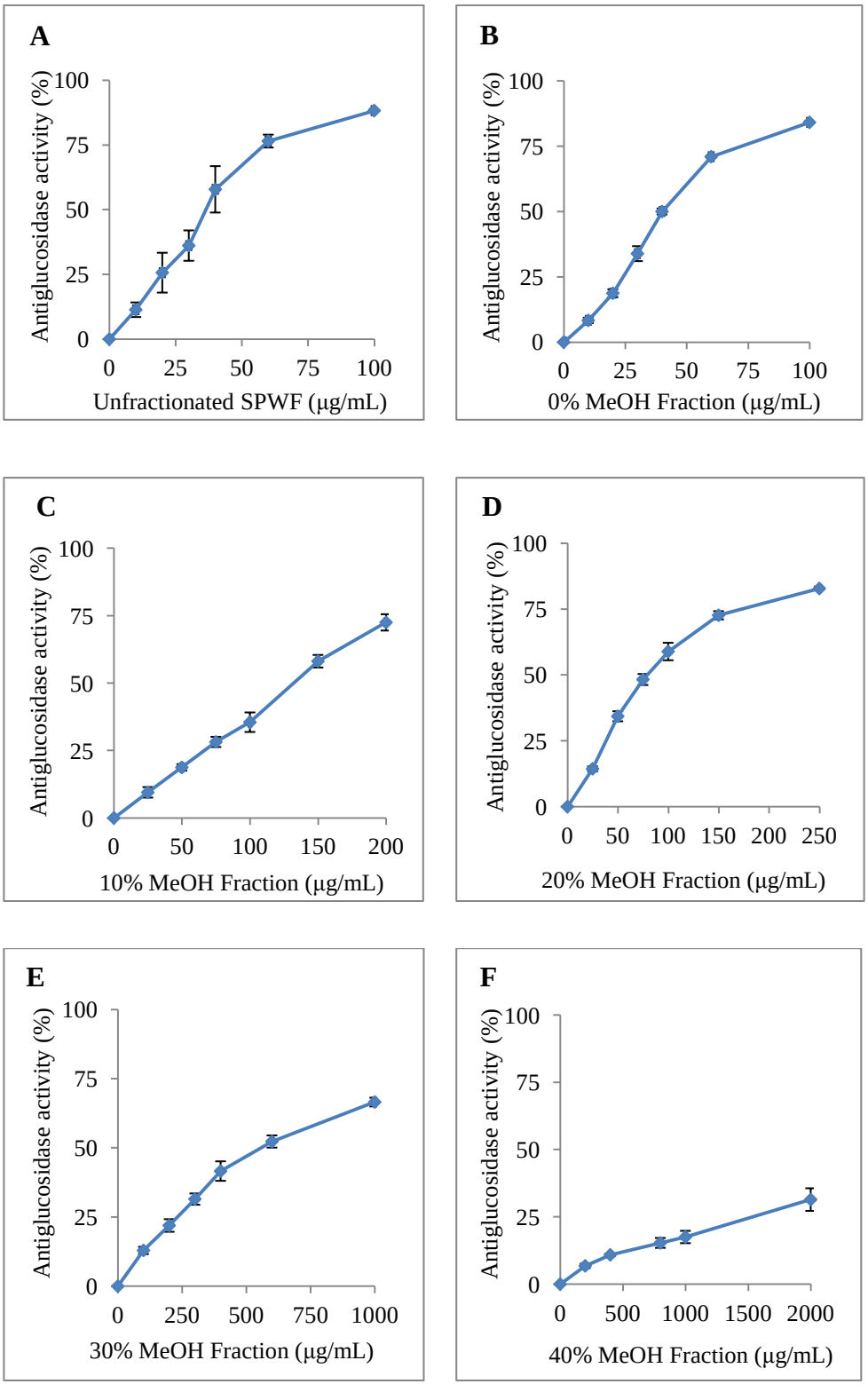
**Table 4.1:** Yield of fractions collected after RP-SPE of SPWF.

| Fractions | Mass (mg) | Yield (%) |
|---|---|---|
| 0% MeOH | 130.60 | 65.30 |
| 10% MeOH | 14.60 | 7.30 |
| 20% MeOH | 5.70 | 2.85 |
| 30% MeOH | 2.70 | 1.35 |
| 40% MeOH | 2.70 | 1.35 |
| 50% MeOH | 1.00 | 0.50 |
| 100% MeOH | 0.90 | 0.45 |

## 4.3 Antiglucosidase Activity

### 4.3.1 α-glucosidase Inhibitory Assay

Unfractionated SPWF and each eluted fractions from RP-SPE of SPWF was assayed for antiglucosidase activity. Figure 4.2 illustrates the individual graphs of antiglucosidase activity for unfractionated SPWF, each eluted fractions of fractionated SPWF using different percentage of MeOH and positive controls. Antiglucosidase activity plots for unfractionated SPWF, fractions of 0% MeOH, 10% MeOH, 20% MeOH, 30% MeOH, quercetin and acarbose (Figure 4.2A-E, I, J) showed antiglucosidase activities that were more than 50%. The activities were more than 50% at the concentration of 100 μg/mL for unfractionated SPWF and 0% MeOH fraction, 200 μg/mL for 10% MeOH fraction, 250 μg/mL for 20% MeOH fraction, 1000 μg/mL for 30% MeOH fraction, 400 μg/mL for quercetin and 3000 μg/mL for acarbose. On the other hand, antiglucosidase activity plots for fractions of 40% MeOH, 50% MeOH and 100% MeOH (Figure 4F-H) showed activities that were less than 50% even at the concentration of 2000 μg/mL for 40% MeOH fraction and 800 μg/mL for 50% and 100% MeOH fractions.


A
Antiglucosidase activity (%)
100
75
50
25
0
0
25
50
75
100
Unfractionated SPWF (µg/mL)
B
Antiglucosidase activity (%)
100
75
50
25
0
0
25
50
75
100
0% MeOH Fraction (µg/mL)
C
Antiglucosidase activity (%)
100
75
50
25
0
0
50
100
150
200
10% MeOH Fraction (µg/mL)
D
Antiglucosidase activity (%)
100
75
50
25
0
0
50
100
150
200
250
20% MeOH Fraction (µg/mL)
E
Antiglucosidase activity (%)
100
75
50
25
0
0
250
500
750
1000
30% MeOH Fraction (µg/mL)
F
Antiglucosidase activity (%)
100
75
50
25
0
0
500
1000
1500
2000
40% MeOH Fraction (µg/mL)

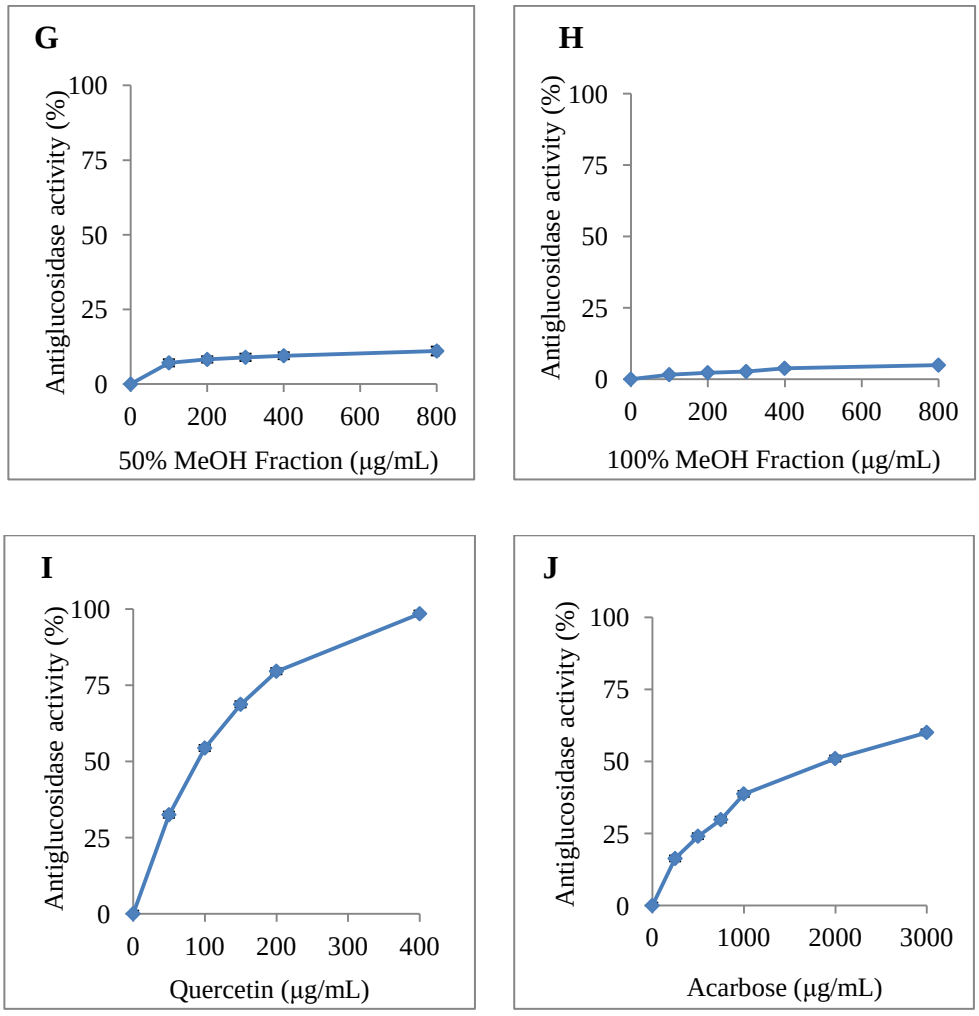



**Figure 4.2:** Antiglucosidase activity of **(A)** unfractionated SPWF, fractionated SPWF eluted at **(B)** 0% MeOH, **(C)** 10% MeOH, **(D)** 20% MeOH, **(E)** 30% MeOH, **(F)** 40% MeOH, **(G)** 50% MeOH, **(H)** 100% MeOH and positive control of **(I)** quercetin and **(J)** acarbose. Data are presented as mean ± standard error (n = 3).

Table 4.2 shows the values of $EC_{50}$ for antiglucosidase activity of each eluted fractions after SPE of SPWF with quercetin and acarbose as the positive control. Among the eluted fractions, 0% MeOH fraction showed the greatest antiglucosidase activity. $EC_{50}$ values were not determined for the fractions of 40% MeOH, 50% MeOH and 100% MeOH due to their negligible antiglucosidase activities. The fractions of unfractionated SPWF, 0% MeOH, 20% MeOH were 2.59-, 2.36- and 1.24-fold lower $EC_{50}$ values than quercetin, respectively. While the $EC_{50}$ values for 10% MeOH and 30% MeOH were 1.32- and 5.55-fold higher than quercetin, respectively. The $EC_{50}$ values for the fractions of unfractionated SPWF, 0% MeOH, 10% MeOH, 20% MeOH and 30% MeOH were 42.03-, 38.41-, 12.33-, 20.19- and 2.93-fold lower than acarbose, respectively. However, unfractionated SPWF exhibited greater antiglucosidase activity as compared to 0% MeOH fraction. Unfractionated SPWF showed 1.09-fold lower $EC_{50}$ value when compared to 0% MeOH fraction. The $EC_{50}$ values of the unfractioned SPWF and eluted fractions were significantly different ($p < 0.05$) from that of quercetin and acarbose, both the positive control, as determined by Student's *t*-test.

**Table 4.2:** $EC_{50}$ for antiglucosidase activity of unfractionated SPWF, eluted fractions and positive controls.

| **Fractions** | **$EC_{50}$ (µg/mL)** |
|---|---|
| Unfractionated SPWF | 39.03 ± 3.57* |
| 0% MeOH | 42.71 ± 0.95* |
| 10% MeOH | 133.10 ± 7.72* |
| 20% MeOH | 81.25 ± 3.88* |
| 30% MeOH | 560.70 ± 67.66* |
| 40% MeOH | nd |
| 50% MeOH | nd |
| 100% MeOH | nd |
| Quercetin | 100.95 ± 2.99 |
| Acarbose | 1640.57 ± 31.83 |

Data are presented as mean ± standard error (n = 3). Asterisks (*) denote the $EC_{50}$ values are significantly different ($p < 0.05$) from that of quercetin and acarbose, both the positive control, as determined by Student's *t*-test. nd, not detected.

### 4.3.2 Mode of Inhibition of α-glucosidase

Among the eluted fractions, 0% MeOH fraction with the strongest α-glucosidase inhibitory activity was assayed for the mode of α-glucosidase inhibition. The mode of inhibition was assessed using the $EC_{50}$ value at 0 × $EC_{50}$ (control), 1 × $EC_{50}$ and 2 × $EC_{50}$. The fraction of 0% MeOH has $EC_{50}$ value of 42.71 µg/mL. Figure 4.3 shows the Lineweaver-Burk plot of α-glucosidase activity in the presence and absence of 0% MeOH fraction acting as an inhibitor. The Lineweaver-Burk plot showed intersection at x-axis indicating that the inhibition was of non-competitive mode.

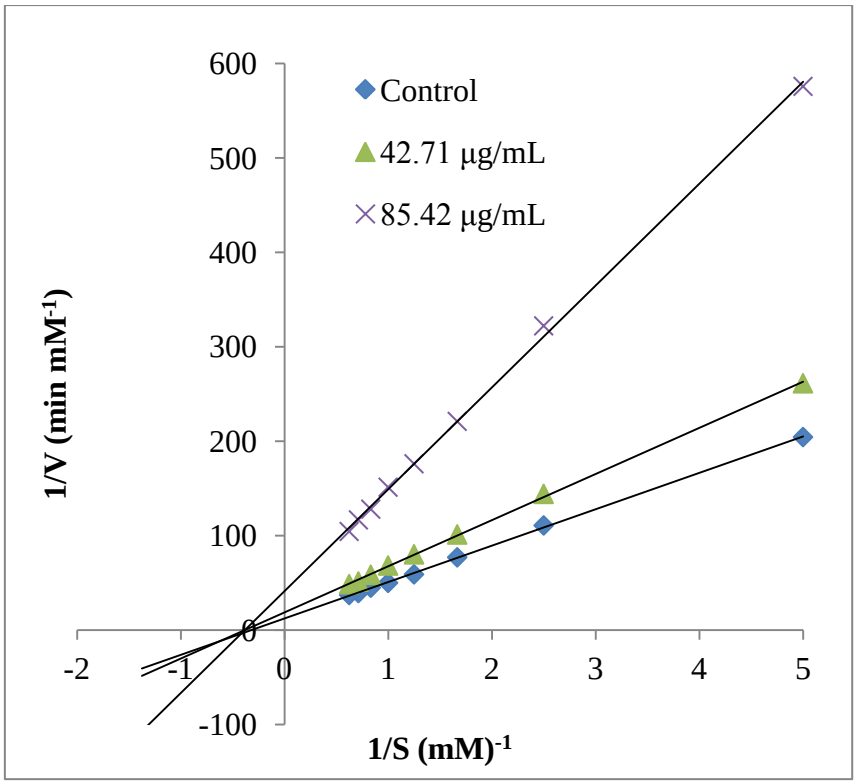



**Figure 4.3:** Lineweaver-Burk plot (1/v against 1/[S]) for the inhibition of α-glucosidase in the presence and absence of 0% MeOH fraction. Data are presented as mean (n = 3).

Table 4.3 indicates the values of $K_m$ and $V_{max}$. Among each other, $V_{max}$ values were significantly different ($p < 0.05$) and $K_m$ values were not significantly different ($p > 0.05$) as determined from Fisher's LSD test using SAS 9.3 statistical software. $K_m$ values remain unchanged and $V_{max}$ values decreased as the concentration of 0% MeOH fraction increased. This also indicated that the inhibition was of non-competitive mode.

**Table 4.3:** $K_m$ and $V_{max}$ values of α-glucosidase inhibitory activity in the presence and absence of 0% MeOH fraction.

| **Fraction** | **Concentration (µg/mL)** | **$K_m$ (µM)** | **$V_{max}$ (µM/min)** |
|---|---|---|---|
| 0% MeOH | 0.00 (control) | $3142.57 \pm 186.63^a$ | $81.57 \pm 2.79^a$ |
| | 42.71 | $2606.04 \pm 133.83^a$ | $53.45 \pm 1.86^b$ |
| | 85.42 | $2733.59 \pm 337.14^a$ | $25.58 \pm 3.77^c$ |

Data are presented as mean ± standard error (n = 3). Different superscript letters at the values within the same column indicates significant differences ($p < 0.05$), as determined by Fisher's LSD test using SAS 9.3 statistical software.

## 4.4 Antioxidant Activity

### 4.4.1 DPPH Radical Scavenging Assay

The most active fraction with greatest antiglucosidase activity among the eluted fractions was 0% MeOH fraction. Only 0% MeOH fraction was investigated for its DPPH radical scavenging activity. Figure 4.4 shows the individual graphs of DPPH radical scavenging activity for unfractionated SPWF, 0% MeOH fraction and the positive control, quercetin. DPPH radical scavenging activity plots of unfractionated SPWF, 0% MeOH fraction and quercetin (Figure 4.4A-C) showed more than 50% radical scavenging activities at the concentration of 100 μg/mL for unfractionated SPWF and 0% MeOH fraction and 20 μg/mL for quercetin.

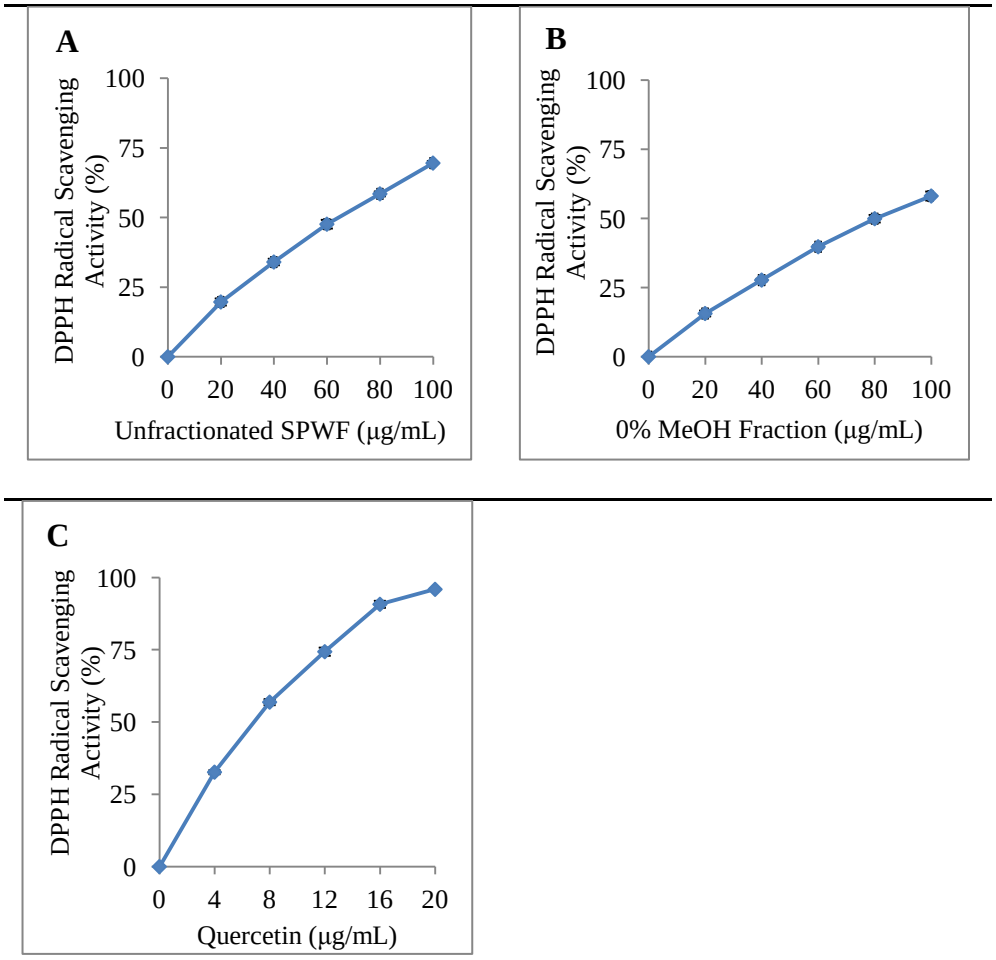



**Figure 4.4:** DPPH radical scavenging activity of **(A)** unfractionated SPWF, **(B)** 0% MeOH fraction and **(C)** quercetin. Data are presented as mean ± standard error (n = 3).

Figure 4.5 compares the $EC_{50}$ for DPPH radical scavenging activity of unfractionated SPWF and 0% MeOH fraction with quercetin. The fraction of 0% MeOH exhibited the lowest DPPH radical scavenging activity with high $EC_{50}$ whereas quercetin showed highest activity with low $EC_{50}$.

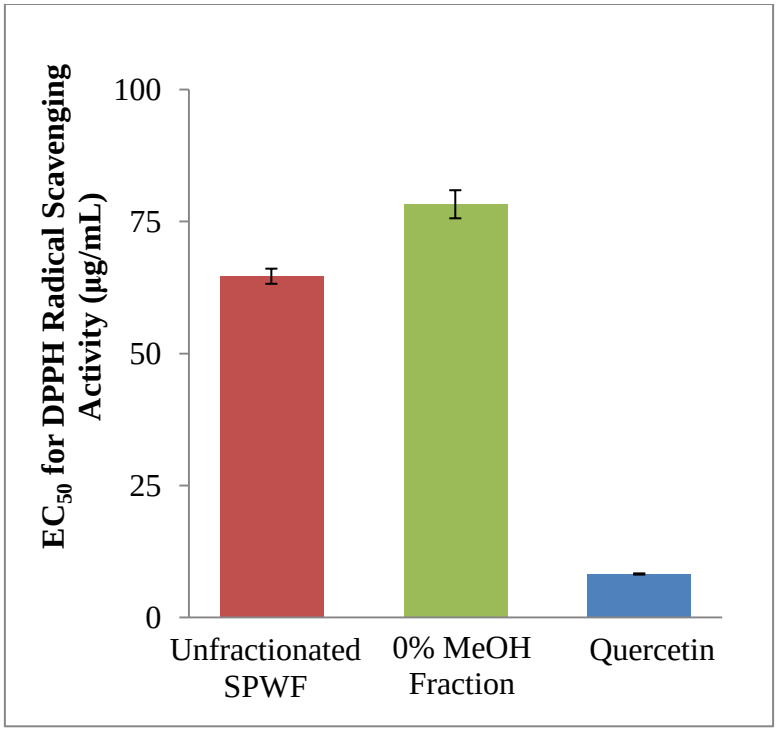



**Figure 4.5:** $EC_{50}$ for DPPH radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin. Data are presented as mean ± standard error (n = 3).

Table 4.4 summarizes $EC_{50}$ values for DPPH radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin. Quercetin has the greatest activity. As compared to quercetin, the $EC_{50}$ values of 0% MeOH fraction and unfractionated SPWF were 9.56-fold and 7.89-fold higher, respectively. The fraction of 0% MeOH exhibited weaker activity with 1.21-fold higher $EC_{50}$ value as compared to unfractionated SPWF. The $EC_{50}$ values of unfractionated SPWF and 0% MeOH fraction were significantly different ($p < 0.05$) from that of quercetin, as determined by Student's *t*-test.

**Table 4.4:** $EC_{50}$ for DPPH radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin.

| **Fractions** | **$EC_{50}$ (µg/mL)** |
|---|---|
| Unfractionated SPWF | 64.63± 1.44* |
| 0% MeOH | 78.27 ± 2.66* |
| Quercetin | 8.19 ± 0.12 |

Data are presented as mean ± standard error (n = 3). Asterisks (*) denote the $EC_{50}$ values are significantly different ($p < 0.05$) from that of quercetin, the positive control, as determined by Student's *t*-test.

### 4.4.2 Superoxide Anion Radical Scavenging Assay

The fraction of 0% MeOH with greatest antiglucosidase activity was tested for superoxide anion radical scavenging activity. Figure 4.6 illustrates the individual graphs of superoxide anion radical scavenging activity for unfractionated SPWF, 0% MeOH fraction and the positive control, quercetin. Superoxide anion radical scavenging activity plots of unfractionated SPWF (Figure 4.6A) and 0% MeOH fraction (Figure 4.6B) showed more than 50% activities at 3000 μg/mL whereas the plot for quercetin (Figure 4.6C) illustrated more than 50% activities at 300 μg/mL.

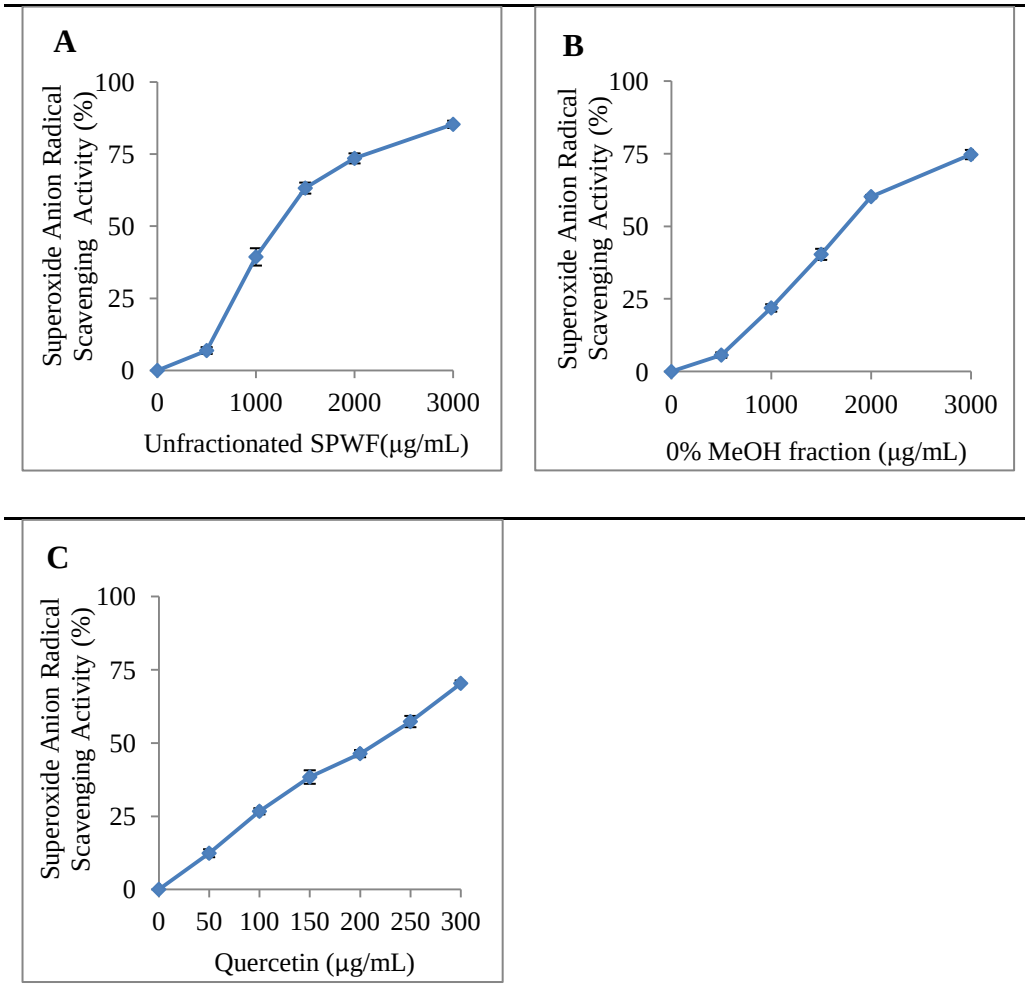



**Figure 4.6:** Superoxide anion radical scavenging activity of **(A)** unfractionated SPWF, **(B)** 0% MeOH fraction and **(C)** quercetin. Data are presented as mean ± standard error (n = 3).

Figure 4.7 compares the $EC_{50}$ values for superoxide anion radical scavenging activity of unfractionated SPWF and 0% MeOH fraction with quercetin. The 0% MeOH fraction exhibited the lowest superoxide anion radical scavenging activity with high $EC_{50}$ whereas quercetin exhibited highest activity with low $EC_{50}$.

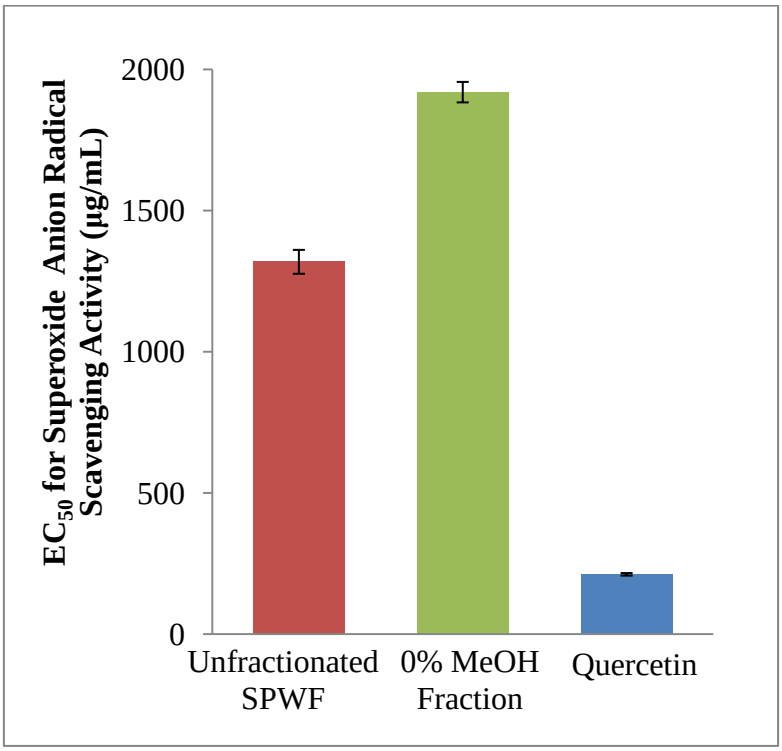



**Figure 4.7:** $EC_{50}$ for superoxide anion radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin. Data are presented as mean ± standard error (n = 3).

Table 4.5 summarizes $EC_{50}$ values for superoxide anion radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin. Quercetin has the greatest activity. The $EC_{50}$ values of 0% MeOH fraction and unfractionated SPWF were 9.06-fold and 6.22-fold lower, respectively in comparison to quercetin. As compared to unfractionated SPWF, the 0% MeOH fraction showed weaker activity with 1.46-fold lower $EC_{50}$ value. The $EC_{50}$ values of unfractionated SPWF and 0% MeOH fraction were significantly different ($p < 0.05$) from that of quercetin, as determined by Student's *t*-test.

**Table 4.5:** $EC_{50}$ for superoxide anion radical scavenging activity of unfractionated SPWF, 0% MeOH fraction and quercetin.

| **Fractions** | **$EC_{50}$ (µg/mL)** |
|---|---|
| Unfractionated SPWF | 1318.59 ± 42.35* |
| 0% MeOH | 1919.51 ± 36.32* |
| Quercetin | 211.84 ± 4.52 |

Data are presented as mean ± standard error (n = 3). Asterisks (*) denote the $EC_{50}$ values are significantly different ($p < 0.05$) from that of quercetin, the positive control, as determined by Student's *t*-test.

## 4.5 RP-HPLC of 0% MeOH Fraction (After SPE)

Figure 4.8 shows the HPLC chromatograms of 0% MeOH fraction at the wavelengths of 260 nm, 320 nm and 460 nm, respectively. The chromatogram illustrates that the intensity of the peaks after 5 minutes was around 100 mAU, 100 mAU and 0 mAU for wavelengths of 260 nm, 320 nm and 460 nm respectively. There were reduction in the intensities of peaks (at wavelength of 260 nm) and removed of some peaks (at wavelength of 460 nm) as compared to SPWF before SPE.

Chromatograms for before and after SPE displayed differences in the intensity and retention time of peaks. After SPE, there was splitting of peaks and reduction in intensity of peak seen at wavelength of 260 nm and removed of few peaks at wavelength of 460 nm. The chromatogram at wavelength 260 nm showed that the broad peak (before SPE) after 5 minutes have been separated in better resolution formed two peaks with lower intensity (after SPE) whereas the chromatogram at the wavelength 460 nm showed the peaks after 5 minutes have been removed after SPE.

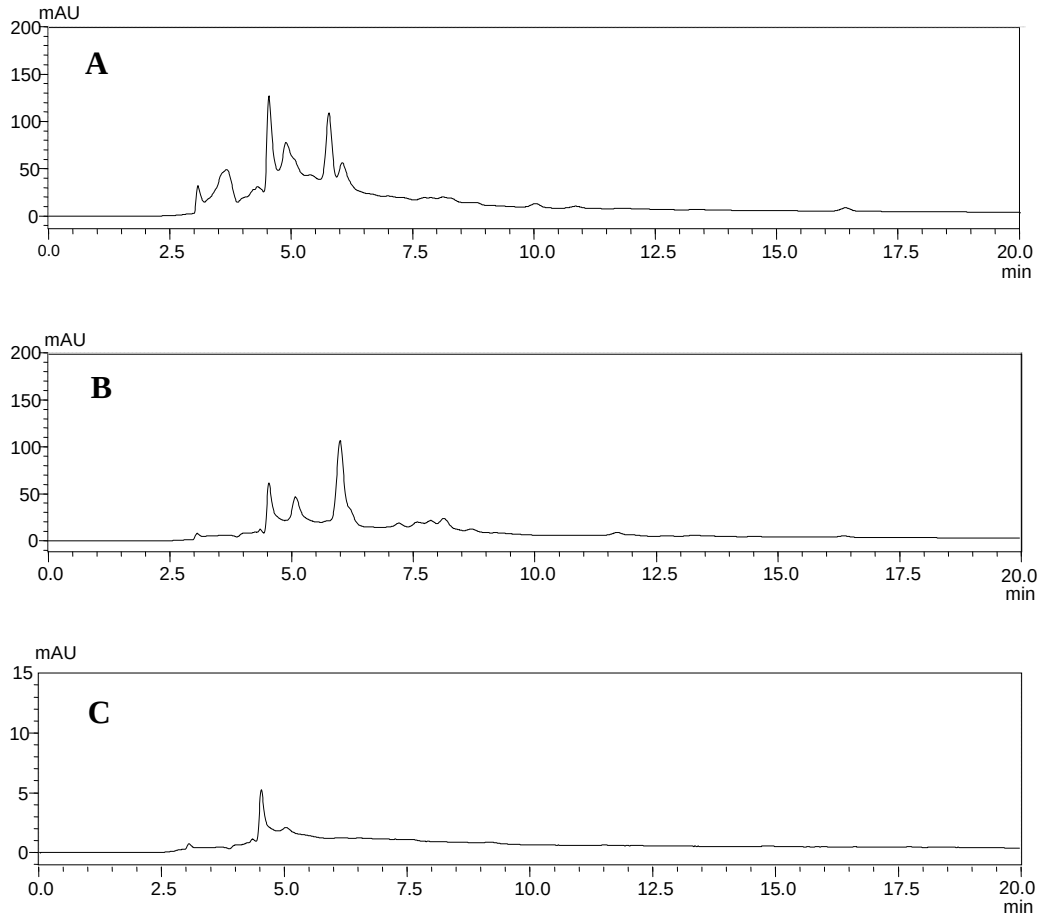



**Figure 4.8:** HPLC chromatograms of 0% MeOH fraction at wavelengths of **(A)** 260 nm, **(B)** 320 nm and **(C)** 460 nm.

## 4.6 NMR Analysis of Most Active Fraction (0% MeOH Fraction)

The most potent antiglucosidase SPE fraction (0% MeOH) was subjected to NMR analysis. The $^{1}H$ and $^{13}C$ NMR chemical shift spectrum are presented in Appendix B and C. Figure 4.9 shows the predicted compound, pyridine alkaloid ($C_{26}H_{39}O_5N_1$) with molecular weight of 445.

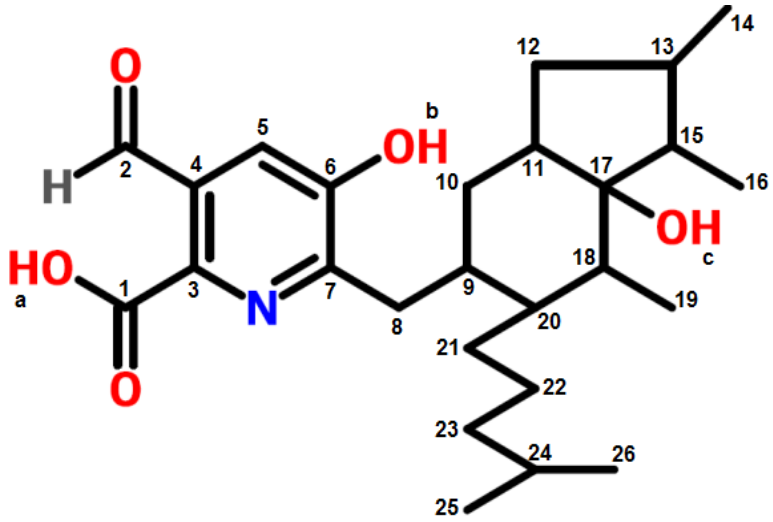



**Figure 4.9:** Pyridine alkaloid, the predicted compound, $C_{26}H_{39}O_5N_1$ (Molecular weight = 445).

Table 4.6 summarizes the $^{1}H$ and $^{13}C$ NMR chemical shift data from the observed spectra.

**Table 4.6:** $^{1}H$ and $^{13}C$ NMR chemical shift data for the predicted pyridine alkaloid.

| Position | $\delta_C$ (ppm) | $\delta_H$ (ppm) | Multiplicity |
|---|---|---|---|
| 1 | 183.96 | - | - |
| a | - | 2.36 | *s* |
| 2 | 182.57 | 8.30 | *s* |
| 3 | 136.05 | - | - |
| 4 | 130.53 | - | - |
| 5 | 76.48 | 4.53 | *s* |
| 6 | 181.32 | - | - |
| b | - | 2.32 | *s* |
| 7 | 171.14 | - | - |
| 8 | 72.96 | 2.67 – 2.68 | *d* |
| 9 | 75.65 | 2.49 – 2.59 | *m* (6) |
| 10 | 72.07 | 2.62 – 2.64 | *t* |
| 11 | 75.27 | 2.38 – 2.45 | *m* (5) |
| 12 | 69.33 | 2.85 – 2.87 | *t* |
| 13 | 69.78 | 3.79 – 3.85 | *m* (7) |
| 14 | 66.32 | 2.05 – 2.07 | *d* |
| 15 | 70.38 | 3.93 – 3.99 | *m* (5) |
| 16 | 66.86 | 2.25 – 2.26 | *d* |
| 17 | 70.47 | - | - |

| | | | |
|---|---|---|---|
| c | - | 2.30 | *s* |
| 18 | 69.17 | 2.38 – 2.45 | *m* (5) |
| 19 | 67.04 | 1.50 – 1.50 | *d* |
| 20 | 68.54 | 2.12 – 2.22 | *m* (5) |
| 21 | 54.69 | 3.38 – 3.41 | *m* (4) |
| 22 | 40.73 | 3.72 – 3.75 | *m* (5) |
| 23 | 37.43 | 3.25 – 3.28 | *m* (4) |
| 24 | 34.20 | 1.82 – 2.02 | *m* (9) |
| 25 | 26.99 | 1.43 – 1.43 | *d* |
| 26 | 20.11 | 1.31 – 1.33 | *d* |

*s*, singlet; *d*, duplet; *t*, triplet; *m*, multiplet; (), numbers of multiplet; -, no chemical shift or multiplicity.

# CHAPTER 5

# DISCUSSION

## 5.1 RP-HPLC (Before and After SPE)

From the observation (Figure 4.1 and 4.8), it indicates that SPE has pretreated the SPWF by retained interferences on the non-polar C18 sorbent and eluted out the compound of interest, polar compound (phenomenex, n. d.). As SPE plays an important role in sample concentration and impurities removal, some compounds in the unfractionated SPWF have been removed as well. Thus, clearer signals were produced and can be identified in the resulted most potent SPE fraction of cleaned-up sample (Simpson and Wells, 2000).

In this study, HPLC was used to determine the number of peaks in SPWF before SPE and of the most potent antiglucosidase fraction, 0% MeOH fraction which obtained after SPE. This showed that HPLC is able to determine the purity of peak based on intensity and retention time (Bhardwaj, Dwivedi and Agarwal, 2015). A study revealed by Chu, Wu and Hsieh (2014) stated that the eluted fractions after SPE was assayed for enzyme inhibition and the fraction that contained the most potent enzyme inhibitors was subjected to HPLC for isolation and characterization.

## 5.2 Yield of Eluted SPE Fractions of SPWF

The high mass and yield of 0% MeOH fraction (Table 4.1) indicated that the SPWF contained most of the polar compounds. RP-SPE cartridge contained carbon-based C18 sorbent which is a non-polar stationary phase (Zwir-Ferenc and Biziuk, 2006). When the SPWF was applied, non-polar compounds in SPWF will be retained by the sorbent. Due to the differential of polarity among the phytochemical compounds, the polar compounds in SPWF were eluted faster as compared to non-polar compounds and they are mainly distributed in the first fraction which is the polar eluent.

The main purpose of using MeOH as eluent is to disrupt the attractive forces between the non-polar stationary phase sorbent and the non-polar retented compounds (Zwir-Ferenc and Biziuk, 2006). Subsequently, when different percentages of MeOH are applied, compounds in its respective polarity index will be eluted out accordingly to the polarity of eluent (phenomenex, n. d.).

## 5.3 α-glucosidase Inhibitory Activity

In the antiglucosidase activity assay, 4-NPGP was hydrolyzed enzymatically by α-glucosidase and converted into α-D-glucopyranoside and 4-nitrophenol. Present of inhibitors in the reaction mixture will prevent the activity of α-glucosidase (Chu, Wu and Hsieh, 2014; Hossain, et al., 2009). The inhibitory activity of α-glucosidase was detected by measuring the released yellow coloured 4-nitrophenol at 400 nm (Kazeem, Ogunbiyi and Ashafa, 2013).

SPWF was subjected to RP-SPE cartridges and eluted with varying percentages of MeOH, thus yielding a few fractions which assayed for their antiglucosidase activities. Among the eluted fractions, 0% MeOH fraction appeared to have the greatest antiglucosidase activity ($EC_{50}$ value = 42.71 μg/mL) that has better inhibitory action on α-glucosidase than the positive control quercetin ($EC_{50}$ value = 100.95 μg/mL) and acarbose ($EC_{50}$ value = 1640.57 μg/mL) as shown in Table 4.2. The greatest antiglucosidase activity is due to the presence of potent polar compounds in the 0% MeOH fraction. However, since the glycosides and acylated flavonol glycosides which have been identified in the leaves of *S. palustris* by Liu, et al. (1998; 1999) did not showed much information on their water solubility, hence, their presence cannot be confirmed to be the compound that contribute to the antiglucosidase activity.

The antiglucosidase activity by 0% MeOH fraction was similar to unfractionated SPWF which also experienced better antiglucosidase activity than the two positive controls. However, the antiglucosidase activity of 0% MeOH fraction seem to be weaker than that of unfractionated SPWF ($EC_{50}$ value = 39.03 μg/mL) shown in Table 4.2. This indicated that SPE fractionation decreased the inhibitory activity of 0% MeOH fraction on α-glucosidase. The possible reason could be SPE has removed compounds that helped the polar compound to function optimally. Tumbas, et al. (2007) reported that there are multiple numbers of different compounds which have distinct polarity in extracts have the action of synergistic. There is finding

showed that the presence of both phenolic compounds and alkaloid are potent antidiabetic agents which act synergistically (Upadhyay and Dixit, 2015).

Chai, et al. (2015) revealed that the WF of ME of mature frond of *S. palustris* exhibited stronger antiglucosidase activity with an $EC_{50}$ value of 2.92 μg/mL as compared to quercetin ($EC_{50}$ = 114.05 μg/mL) used in their study. In comparison to the results obtained in this study, 0% MeOH fraction obtained after SPE of SPWF exhibited stronger antiglucosidase activity even the SPWF of young frond was fractionated. This showed that both mature and young fronds of *S. palustris* contained the potent α-glucosidase inhibitor.

In this study, acarbose was found to have weaker antiglucosidase activity with the $EC_{50}$ value of 1640.57 μg/mL (Table 4.2). Acarbose, being the synthetic antidiabetic drug has low inhibition activity on α-glucosidase from yeast *Saccharomyces cerevisiae* because it has weak or no inhibitory activity against the enzyme but has high inhibitory activity on mammalian α-glucosidase (Rey, Ospina and Aragon, 2015).

There is undetectable antiglucosidase activity by the fractions of 40% MeOH, 50% MeOH and 100% MeOH (Table 4.2). At the beginning of elution in SPE, most of the polar compounds have been eluted out as the first fraction which is the polar eluent. Eluent which is miscible with sample can be used because they will never contact directly. Since the SPWF was dissolved in water, the eluent which is of MeOH was miscible in all proportions with water (Simpson

and Wells, 2000). Different MeOH percentage and pure MeOH with moderate polarity breaks the non-polar bond between the stationary phase and non-polar compounds thus eluting the non-polar compounds with little or negligible antiglucosidase activity (Zwir-Ferenc and Biziuk, 2006).

The mode of inhibition of 0% MeOH fraction on α-glucosidase determined from the Lineweaver-Burk plot is non-competitive. The determination was based on the interception at x-axis, decreased $V_{max}$ and remained $K_m$ as shown in Figure 4.3 and Table 4.3, respectively (Berg, Tymoczko and Stryer, 2002). When the concentration of 0% MeOH fraction increased from 42.71 μg/mL to 85.42 μg/mL, the α-glucosidase inhibitory activity increased as well (Figure 4.3). This showed that the inhibition is dose-dependent (Kazeem, Ogunbiyi and Ashafa, 2013). Bioactive compounds with non-competitive mode possessed mechanism binding to a site other than active site in the free α-glucosidase enzyme or the α-glucosidase-NPGP enzyme-substrate complex and therefore blocking the action of α-glucosidase (Mayur, et al., 2010; Hossain, et al., 2009).

The study reported by Chai, et al. (2015) revealed that the mode of α-glucosidase inhibition by the WF of ME of mature frond of *S. palustris* was competitive. It was suggested that WF which acts as competitive inhibitor bound reversibly to α-glucosidase's catalytic site due to the presence of active compounds (Chai, et al., 2015). As compared to the results obtained from this study, the mode of inhibition on α-glucosidase by the 0% MeOH fraction

obtained after SPE fractionation of SPWF of young frond was non-competitive. This illustrated that different type of the fronds of *S. palustris* displayed different mode of inhibition on α-glucosidase.

According to Gurudeeban, Satyavani and Ramanathan (2012), commercial acarbose possessed a competitive inhibition on α-glucosidase where greater concentration was required to decrease the level of postprandial glucose. On the other hand, quercetin was found to be a non-competitive inhibitor on α-glucosidase (Iio, et al., 1984).

## 5.4 Antioxidant Activity

DPPH has a violet colour and is a stable chromogen radical. DPPH radical scavenging assay is based on the ability of antioxidants to neutralize DPPH radical by donating electron (Shahidi and Zhong, 2015). As shown in Figure 5.1, mixture of substrate (AH) which can donate a hydrogen atom with DPPH solution generates the reduced form along with violet discolouration (Alam, Bristi and Rafiquzzaman, 2013).

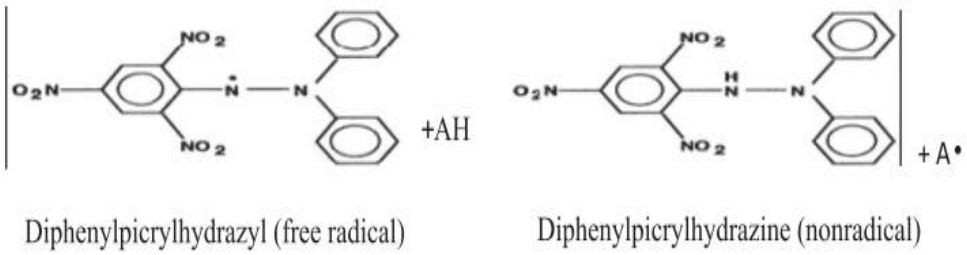



**Figure 5.1:** Reaction of DPPH and a substrate with hydrogen donating group. (Source: Alam, Bristi and Rafiquzzaman, 2013)

Superoxide anion radical is a highly reactive oxygen-derived species with short live. It is involved in the reduction of NBT together with NADH where the radical being produced in the reoxidation of PMS. Presence of antioxidant causes the blue decolourization (Nishikimi, Appaji Rao and Yagi, 1971).

In this study, the unfractionated SPWF and 0% MeOH fraction obtained after SPE exhibited DPPH radical scavenging activity with the $EC_{50}$ values of 64.63 μg/mL and 78.27 μg/mL respectively that was lower than quercetin with $EC_{50}$ value of 8.19 μg/mL (Table 4.4). The unfractionated SPWF and 0% MeOH fraction also have lower superoxide anion radical scavenging activity with the $EC_{50}$ values of 1318.59 μg/mL and 1919.51 μg/mL respectively as compared to quercetin with $EC_{50}$ of 211.84 μg/mL (Table 4.5). The weaker scavenging activity of unfractionated SPWF and 0% MeOH fraction on DPPH and superoxide anion radicals indicated that in the process of exerting an overall antidiabetic action, the antioxidant property acts synergistically with its hypoglycaemic activity (Coman, Rugina and Socaciu, 2012). This also can be explained as the fraction of 0% MeOH displayed a stronger antiglucosidase activity with better inhibitory action on α-glucosidase than the positive control and concurrently, it exhibited weaker antioxidant activity.

Similar to antiglucosidase activity assay, 0% MeOH fraction appeared to have lower antioxidant activity than the unfractionated SPWF. This could be also due to the effect of SPE that has removed compounds which acted synergistically to the eluted polar compound in the 0% MeOH fraction. A

variety of antioxidant substances in plant extracts possessed potential synergistic effects to enhance the antioxidant activity and thus the presence of synergistic compound is important to increase antioxidant activity (Shahidi and Zhong, 2015).

Usually, potential natural antioxidants are possibly found in plant sources (Talukdar, et al., 2011). For instance, quercetin is a powerful antioxidant. Study revealed that quercetin, a representative of flavonol subclass of flavonoids which is capable to scavenge free radicals (Bentz, 2009). Other phytochemical compounds found in plants such as polyphenols in group of phenolic compounds are also powerful antioxidants (Dai and Mumper, 2010).

### 5.5 NMR Analysis of Most Active Fraction

By analyzed the NMR chemical shift data, a compound ($C_{26}H_{39}O_6N_1$) with a molecular weight of 445 was predicted and proposed to be the bioactive compound for α-glucosidase inhibitor (Figure 4.9). In the $^{13}C$ NMR chemical shift spectrum, there was splitting of carbon signals for pyridine based structure at 130.53 ppm, 136.05 ppm, 171.14 ppm and 181.32 ppm suggested that the bioactive compound consists of a pyridine ring (Gottlieb, Kotlyar and Nudelman, 1997). The carbon signals at 182.57 ppm and 183.96 ppm together with proton signals at around downfield region (Table 4.6) suggested the presence of carbonyl functional groups in the bioactive compound (Starkey, n. d.). The chemical shift of proton signal at 2.30 ppm and 2.32 ppm is the indicator for the presence of hydrogen atom from the hydroxyl group. For a

carbon which is fully bonded and with no neighboring hydrogen atom, the carbon itself will have carbon signal but not proton signal.

Solvent that used to dissolve 0% MeOH fraction is deuterium oxide, thus the solvent peak for proton signals shown will be around 4.80 ppm. There were no carbon signals shown in the $^{13}C$ spectrum as the deuterated water does not contain carbon (Cambridge Isotope Laboratories, 2010). The structure shown in Figure 4.9 is the proposed compound which predicted based on the results from $^{1}H$ and $^{13}C$ NMR. The chemical structure of this proposed compound need to be further elucidated and confirmed by analyse using Liquid Chromatography-Mass Spectrometry (LC-MS) and Fourier Transform-Infrared spectroscopy (FTIR). In this study, the proposed polar compound in the 0% MeOH fraction is categorized in alkaloid group as it contained nitrogen atom and pyridine functional group. Alkaloid is a nitrogen-containing organic constituent (Woolley, 2001).

There is study reported that the natural active compounds found in nature resources which are in the alkaloid group have antidiabetic effect (Coman, Rugina and Socaciu, 2012). Upadhyay and Dixit (2015) also stated that alkaloids are associated with hypoglycaemic activity and resulted observable hypoglycaemic effect. Plant alkaloids are considered as the potent antidiabetic agent (Upadhyay and Dixit, 2015). Previous study revealed that alkaloids are known to have the property of antioxidant as well due to their ability to chelate metal, scavenge free radicals and donate electron or hydrogen (Kaur and Arora,

2015). On the other hand, according to Machado, et al. (2012), pyridine alkaloids have been found to display strong properties of antimicrobial.

## 5.6 Future Research Work

### 5.6.1 Purification using Sephadex LH-20

For very effective and complete purification, SPWF is suggested to be subjected to column chromatography using Sephadex LH-20 as packing materials. Sephadex LH-20 is a solid sorbent designed to separate and purify natural products such as terpenoids, steroids, lipids and low molecular weight peptides. It has both the characteristics of hydrophilic and lipophilic (Amersham Biosciences, n. d.). Sephadex LH-20 is very useful to separate highly polar and water soluble compound. It is able to fractionate highly polar compound with greater efficacy and improved reproducibility (Tsuda, 2004).

A study reported by Obead, Al-mzaein and Al-jumaily (2009) revealed that the purification of Sephadex-LH-20 possessed greater effect as compared to other packing material such as Sepharose CL-6B. Karioti, Vincieri and Bilia (2009) also revealed that the application of column chromatography over Sephadex LH-20 has achieved 98% purity of the isolated compound.

### 5.6.2 Other Instrumental Analysis

Bioactive compounds present in plant extracts can be analyzed using LC-MS and FTIR. FTIR helps to characterize the functional group present and its spectra are usually unique as a molecular fingerprint. Unknown compound can be identified by comparing the spectra to the library of known compound (Sasidharan, et al., 2011). LC-MS are often coupled with NMR in the elucidation of compounds together with FTIR which facilitate the identification of bioactive compounds. By summarizing the LC-MS, FTIR and NMR analyses, constituents from plant extracts with the known molecular weight (by LC-MS) can be identified and the structure can be elucidated (Zhang, 2015).

A study by Choudhary, et al. (2008) showed that NMR spectroscopic and mass spectrometry technique can be used to characterize isolated compound and the structure can be determined. According to Tan, et al. (2013), isolated compounds with potent antiglucosidase activity from *Gynura medica* leaf extract can be identified using NMR and mass spectral analyses. Also, FTIR analysis was conducted for the confirmation of the presence of active compound (Akshay, et al., 2016).

# CHAPTER 6

# CONCLUSIONS

In this study, SPE fractionation has been partially purified SPWF by removed impurities or compounds that act synergistic to the eluted compound of interest, polar compound. After SPE of SPWF, clearer identifiable signals were observed from the HPLC chromatograms. HPLC chromatograms showed peak splitting, reduction in the intensity and removal of a few peaks in the wavelength of 260 nm and 460 nm, respectively.

SPE fractionation of SPWF yielded fractions that were assayed for α-glucosidase inhibitory activity. Among the fractions, 0% MeOH fraction was found to have the greatest antiglucosidase activity with $EC_{50}$ of 42.71 μg/mL. Its antiglucosidase activity was found to be greater than that of the positive control quercetin and acarbose. However, it has weaker activity than unfractionated SPWF ($EC_{50}$ = 39.03 μg/mL). The fractions of 40% MeOH, 50% MeOH and 100% MeOH showed undetectable antiglucosidase activity due to their negligible activity. The mode of inhibition on α-glucosidase by 0% MeOH fraction was determined from Michaelis-Menten kinetics by plotting Lineweaver-Burk plot. The inhibition by 0% MeOH fraction on α-glucosidase was non-competitive.

Only the most potent antiglucosidase 0% MeOH fraction was investigated for its concurrent antioxidant activity by assayed on DPPH radical scavenging

activity and superoxide anion radical scavenging activity. The fraction of 0% MeOH exhibited weaker scavenging activities on DPPH radicals and superoxide anion radicals as compared to the unfractionated SPWF and positive control quercetin. Based on the $^{1}H$ and $^{13}C$ NMR chemical shift data, a predicted compound, pyridine alkaloid with the chemical formula of $C_{26}H_{39}O_5N_1$ and molecular weight of 445 was proposed.

In conclusion, SPE have decreased inhibition of SPWF on α-glucosidase as the antiglucosidase activity of 0% MeOH fraction obtained after SPE was weaker than that of the unfractionated SPWF before SPE. Among the fractions obtained, the 0% MeOH fraction showed greater antiglucosidase activity. When the 0% MeOH fraction was tested for its antioxidant activity, it exhibited weaker activity than the unfractionated SPWF. In future, further purification and isolation of compound from SPWF by Sephadex LH-20 are needed. Other instrumental analysis such as FTIR and LC-MS are also required to further confirm and structure elucidate the potent α-glucosidase inhibitory bioactive compound in *S. palustris*.

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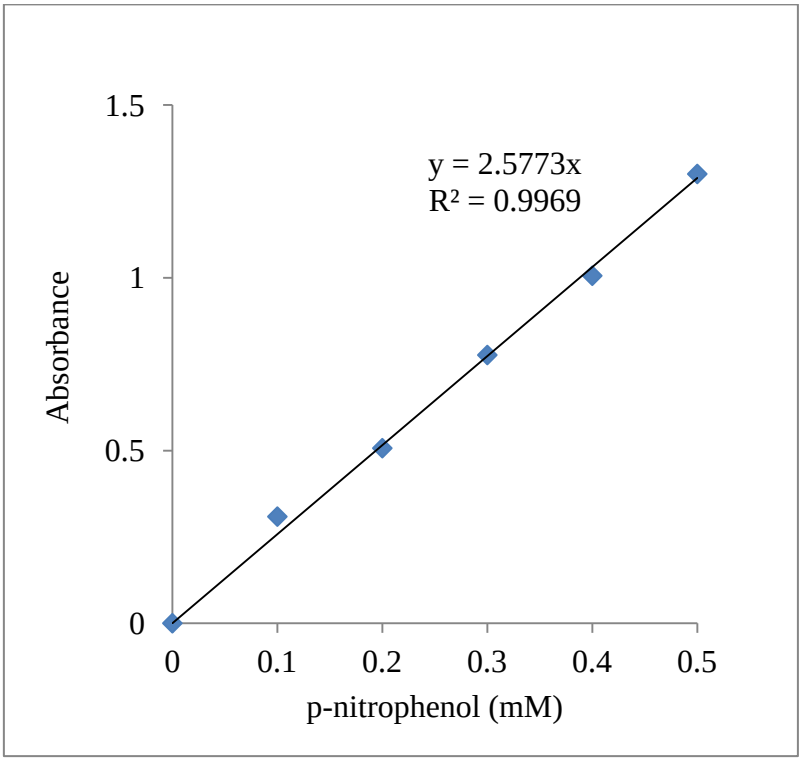
# APPENDICES

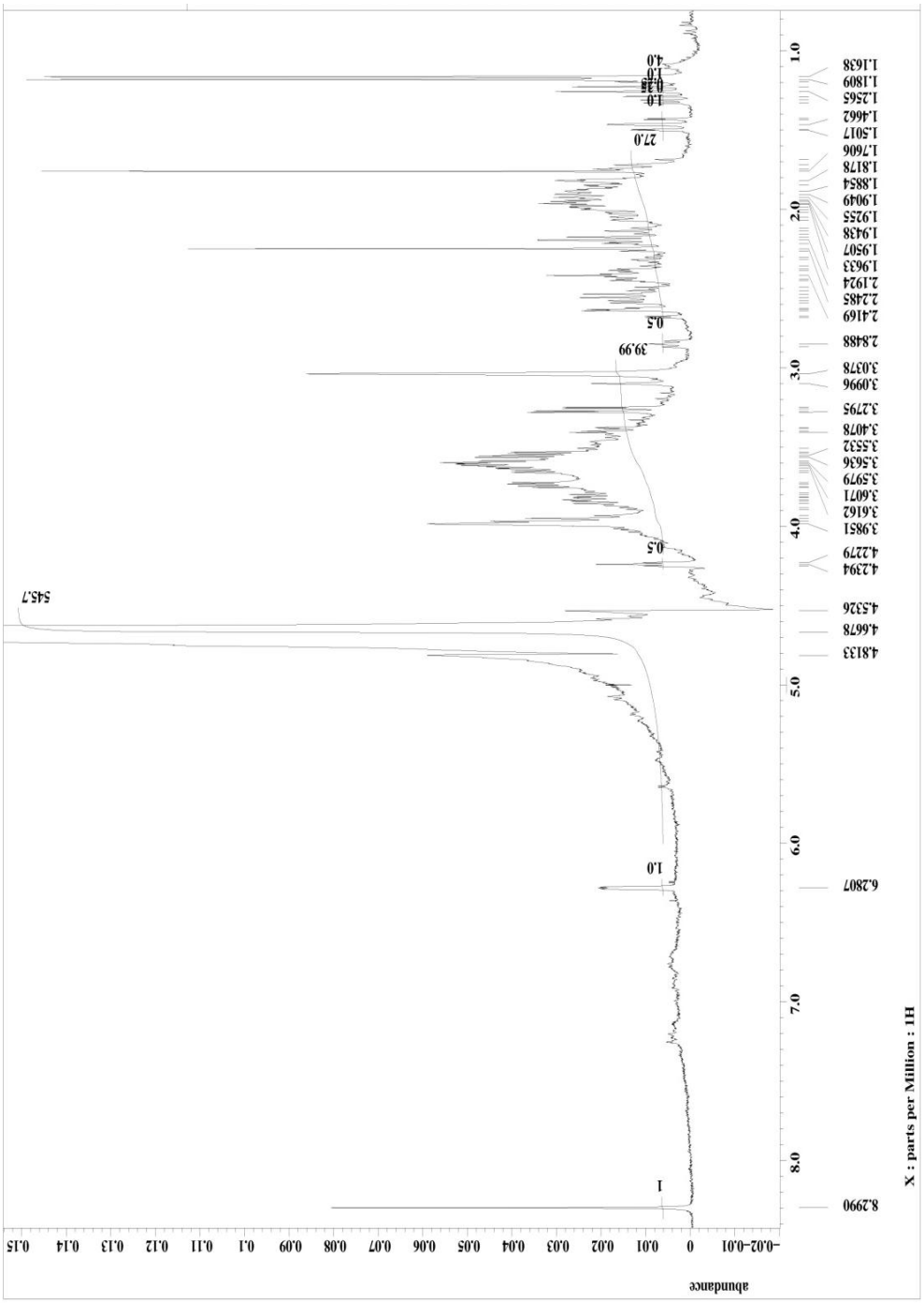
## Appendix A

**p-nitrophenol standard curve for α-glucosidase inhibition**



# Appendix B

## $^{1}$H NMR Spectrum



# Appendix C

## $^{13}$C NMR Spectrum