PHYTOCHEMICAL PROFILING, ANTIOXIDANT PROPERTY, ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF MEDICINAL FERN *Christella dentata*

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MAY 2014

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By

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A project report submitted to the Department of Biological Science

Faculty of Science

Universiti Tunku Abdul Rahman

in partial fulfilment of the requirements for the degree of

Bachelor of Science (Hons) Biotechnology

May 2014

ABSTRACT

PHYTOCHEMICAL PROFILING, ANTIOXIDANT PROPERTY, ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF MEDICINAL FERN *Christella dentata*

Lam Weng Yee

Modern investigations have reported various promising bioactivities of ferns such as antioxidant, antitumor, antimicrobial, antiviral, enzyme modulation, hormonal action, physical action, interference on deoxyribonucleic acid replication and so on. The bioactive phenolic compounds are believed to be responsible for the medicinal value of ferns. A perennial fern, *Christella dentata*, was investigated in this study. Total phenolic (TP), total flavonoid (TF), total proanthocyanidin (TPro), total coumarin (TCou), total hydroxycinnamic acid (THC) and total saponin (TSap) contents were determined through phytochemical assays. Based on the findings, ethyl acetate fraction (EAF) of *C. dentata* contained the highest TP, TCou, THC and TPro contents whereas *n*-butanol fraction (BF) contained the highest TSap and equal amount of TF contents as in EAF. In addition, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay revealed that the EAF of *C.*

dentata exhibited the greatest antioxidant property. Besides that, water fraction (WF) had the greatest inhibitory activities on both α-amlyase and α-glucosidase inhibitory assay. Correlation analyses suggested that (i) antioxidant property of EAF could be attributed to TP, TCou, THC and TPro contents (ii) α-amylase and α-glucosidase inhibitory activities of WF were not directly attributed to TP, TF, TCou, THC, TSap and TPro contents. In short, high TP, TCou, THC and TPro contents may serve as an indicator of high antioxidant activity but TP, TF, TCou, THC, TSap and TPro contents may not directly associate with the α-amylase and α-glucosidase inhibitory activities in ferns.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my deepest gratitude to my project supervisor, Dr. Nor Ismaliza Binti Mohd Ismail for her enthusiastic encouragement and supervision throughout the three months of my final year project. Without her, I certainly would not have been in a position of completing my project successfully and this thesis would not have reached its present form. Also, I would like to thank my co-supervisor, Dr. Chai Tsun Thai, for his enlightening guidance, explanation and compassionate support in everything regarding my project.

I would also like to express my appreciation to my beloved parents for their abiding love, unconditional support and unfailing understanding. Last but not least, I am deeply indebted to all my beloved friends and laboratory partners, especially Khoo Chee Siong, Chiam Mei Jee, Liew Ying Hui, Lim Jing Wen, Tan Kien Hwa, Phai Yee Xian, Yee Yong Mei and Jonie Toh Kwei Jun, for their wholehearted supports and positive inspiration which enabled me to progress confidently and courageously.

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.

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Lam Weng Yee

APPROVAL SHEET

This project report entitled **"PHYTOCHEMICAL PROFILING, ANTIOXIDANT PROPERTY, ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF MEDICINAL FERN *Christella dentata*"** was prepared by LAM WENG YEE and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biotechnology at University Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **LAM WENG YEE** (ID No: **11ADB02046**) has completed this final year project entitled **"PHYTOCHEMICAL PROFILING, ANTIOXIDANT PROPERTY, ALPHA-AMYLASE AND ALPHA-GLUCOSIDASE INHIBITORY ACTIVITIES OF MEDICINAL FERN *Christella dentata*"** under the supervision of DR NOR ISMALIZA BINTI MOHD ISMAIL (Supervisor), from the Department of Biological Science, Faculty of Science, and DR CHAI TSUN THAI (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,

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(LAM WENG YEE)

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
ANOVA	Analysis of variance
BE	1, 2-benzopyrone equivalents
BF	*n*-Butanol fraction
CAE	Caffeic acid equivalents
DNA	Deoxyribonucleic acid
DM	Diabetes mellitus
DPPH	1,1-diphenyl-2-picrylhydrazyl
EAF	Ethyl acetate fraction
EC_{50}	Effective concentration
GAE	Gallic acid equivalents
HCAs	Hydroxycinnamic acids
HIV	Human immunodeficiency virus
LE	Leucocyanidin equivalents
LSD	Fisher's least significant difference
OAE	Oleanolic acid equivalents
PPA	Porcine pancreatic α-amylase
*p*NPG	*p*-Nitrophenyl-α-D-glucopyranoside
QE	Quercetin
QEE	Quercetin equivalents
SE	Standard errors

TCou	Total coumarin
TF	Total flavonoid
THC	Total hydroxycinnamic acid
TP	Total phenolic
TPro	Total proanthocyanidin
TSap	Total saponin
WE	Water extract
WF	Water fraction

CHAPTER 1

INTRODUCTION

A potential medicinal fern, namely *Christella dentata*, was chosen as the sample in this study. Investigation on fern was initiated because it has been used as folkloric medicines since the olden days (Tanzin, et al., 2013). Ferns contain different bioactive phytochemicals which contribute to various promising bioactivities (Li, et al., 2008; Chang, Sushim and Tsay, 2010; Sospeter, et al., 2013).

Phytochemical profiling of *C. dentata* was done through the determination of total phenolic (TP), total flavonoid (TF), total proanthocyanidin (TPro), total coumarin (TCou), total hydroxycinnamic acids (THC) and total saponin (TSap) contents based on phytochemical assays. The main concerns of this study were to determine the potential bioactivities of *C. dentata* such as antioxidant property, α-amylase and α-glucosidase inhibitory activities toward the management of diabetes mellitus (DM). Correlation analyses were performed to evaluate the association between the phytochemical contents and the antioxidant property, α-amylase and α-glucosidase inhibitory activity of *C. dentata.*

All types of DM are characterized by hyperglycemia (American Diabetes Association, 2010). The prolonged exposure to hyperglycemia will promote the formation of free radicals, which will subsequently incur oxidative stress toward cells (Colman, Wang and Lafferty, 1989). Clinical evidences reported that the onset of DM is linked with oxidative stress due to the generation of reactive oxygen species (Rosen, et al., 2001; Johansen, et al., 2005). Hence, antioxidant evaluation was one the concerns in this study.

α-Glucosidase is responsible in the breakdown of carbohydrates in conjunction with α-amylase (Hizukuri, Abe and Hanashir, 1996; National Library of Medicine, 2011). It has been reported earlier that the carbohydrate digestion in the mammalian system is in a progressive manner begins with α-amylase and then α-glucosidase in yielding glucose (Dhital, et al., 2013). The inhibition of these two enzymes can be one of the alternative therapeutic approaches against DM (Krentz and Baile, 2005). Therefore, another concern of this study was at the point of targeting these two enzymes through inhibition leading to lower rate of carbohydrates breakdown. Consecutively, the mode of enzymatic inhibition was further evaluated by constructing a Lineweaver-Burk plot.

The objectives of this study were:

1) To perform phytochemical profiling of *C. dentata* water extract and solvent fractions.
2) To evaluate the antioxidant, α-amylase and α-glucosidase inhibitory activity of *C. dentata* water extract and solvent fractions.
3) To determine the mode of enzymatic inhibition based on α-glucosidase inhibitory assay and Lineweaver-Burk plot.
4) To perform correlation analyses between the phytochemical contents of *C. dentata* and antioxidant property, α-amylase and α-glucosidase inhibitory activity.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetes Mellitus

DM refers to a group of chronic disease characterized by impairment in insulin secretion, insulin action or both leading to increased blood glucose levels, which is known as hyperglycemia (Geethalakshimi and Sarada, 2010). People who are suffered from DM will have high blood glucose levels as glucose is not being utilized by cells but resided in the bloodstream of the body (National Diabetes Information Clearinghouse, 2013).

According to World Health Organization (2013), there are approximately 170 million people around the world who were chronically suffered from DM in the year 2000 and the value is estimated to reach 366 million by the year 2030 due to population growth, unhealthy lifestyle, obesity and aging. Currently, the effective DM drugs in the market such as sulfonylureas, biguanides, thiazolidinediones, meglitinides and so on carry side effects of upset stomach, skin rash, nausea, liver problems, and bloating (Albany Medical Centre, 2014).

Hence, this has strengthened the keen on finding an alternative therapeutic agent against DM.

DM can be categorized into three types - Type 1 DM, Type 2 DM and gestational DM (American Diabetes Association, 2010). The insufficiency of insulin in the body due to beta cells destruction leads to the development of type 1 DM. Also, it is partially due to genetic susceptibility leading to autoimmune responses, which causes the destruction as antibodies failed in the recognition of own cells (Yoon and Jun, 2005). Type 2 DM, which is more common, develops due to beta cells dysfunction and insulin resistance. This will lower the efficiency of body system to utilize insulin. As a result, beta cells in the pancreas can no longer supply sufficient amount of insulin to counter the impairment (LeRoith, 2002). Another type of diabetes is gestational DM. It will only develop during pregnancy due to hormonal changes, metabolic demands as well as genetic and environmental factors (National Diabetes Information Clearinghouse, 2013).

2.2 Ferns

Ferns (Division: Pteridophyta) have evolutionary succeed in surviving more than 350 million years and they have a remarkable history of living through various harsh periods such as climatic and topographic changes (Sharma and Vyas, 1985). Therefore, they can adapt and grow in wider habitats as compared

to other plants. Notably, one of the important features of pteridophytes is that they are resistance to microbial and pathogens attacks (Gracelin, Britto and Kumar, 2012). Ferns have been used mainly in the olden days as medicine, foods, fibers, building materials, abrasives, decorations and so on (Kamini, 2007).

Only 1 % of the known plant species, including ferns, in the world have been studied for their medicinal values and properties (Gurib-Fakim, 2006). There are approximately 13,000 species of ferns which are widely distributed all over the world and it is estimated that 1136 species of ferns are found in Malaysia (Bidin and Jaman, 1999). There are very few scientific investigations have been done on ferns. Therefore, the vast array of medicinal values and the useful aspects of most ferns are still scarce (Tanzin, et al., 2013). Most of the medicinal values of ferns are based on traditional postulation and establishment (Vasishta, 2012). Modern investigations have reported that ferns possess bioactivities and medicinal values such as antimicrobial, antiviral, antioxidant, anti-inflammatory, antitussive, antitumor, anti-HIV, enzymes modulation and stimulation, hormonal action, interference on DNA replication and physical action (Chang, Sushim and Tsay, 2010; Sospeter, et al., 2013). Many researches are gradually being carried out from time to time to investigate and identify phytochemicals which are associated with health benefits toward human for the development of potential drugs which are capable of treating different types of diseases (Krishnaraju, et al., 2005).

Promising results have been reported on earlier studies which utilized ferns as the sources of bioactivities targeting on antioxidant property, α-amylase and α-glucosidase inhibitory activities. In brief, the leaf and rhizome extracts of *Phymatopteris triloba* and *Gleichenia truncata* were found to have outstanding antioxidant property and alpha-glucosidase inhibitory activity (Chai, et al., 2013). Solvent extract from several common ferns in Malaysia, namely *Cyathea latebrosa, Cibotium barometez, Drynaria quercifolia, Blechnum orientale and Dicranopteris linearis,* which exhibited high TP contents, has been regarded as potential antioxidant (Lai and Lim, 2011). Besides that, two ferns species which are native to Korea, namely *Pyrrosia lingua* and *Osmunda cinnamomea*, have shown inhibition toward α-glucosidase (Kim, Chi and Lee, 2013). Also, an indigenous fern which is native to China, *Pteris vitata*, has been reported of possessing α-amylase inhibitory activity (Paul and Banerjee, 2013).

2.3 Traditional Uses and Medicinal Values of Ferns

Ferns have contributed to several health benefits toward mankind since the ancient time, especially toward indigenous peoples who are resided in the rural areas (Fernandez, Revilla and Kumar. 2010). Different regions of the world manipulate ferns differently against different ailments. Based on ethnobotanical studies, ferns have been traditionally used in treating wounds, fever, mental disorders, tumors, inflammation, throat disorders, stomach

disorders, menstrual, gonorrhea, contraception disorders and many more (Kamini, 2007).

For the treatment of wound, bleeding and poison, the fresh leaves of *Nephrolepis cordifolia* were used in India to promote blood coagulation due to cuts (Shanker and Khare, 1994) and *Adiantum lunulatum* were used as antidote to counter the poison due to snakebite (Singh, et al., 1989). In China, the rhizomes of various ferns namely *Drynaria fortune*, *Pseudodrynaria coronans*, *Davallia divaricata*, and *Humata griffithiana* were used in combination to cure inflammation, blood stasis and bone injuries (Chang, et al., 2007). Also, the rhizomes of *Dryopteris crassirhizoma* were used against hemoptysis, epistaxis and also bloody stools (Williams, 2012). In Philippines, a typical fern *Lycopodium clavatum* was used as emetic to induce vomit and counter food poisoning (Holdsworth and Giheno, 1975). Besides that, *Dryopteris crassirhizoma* was used in New Guinea to treat ulcer, boils and wounds (Kamini, 2007).

For the treatment of pain, fever, throat disorders, skin diseases and mental disorder, the blades of *Cheilanthes farinose* was used against mental disorder (Shanker and Khare, 1994) and *Ophioglossum reticulatum* was used to get rid of headache in India (Singh, et al., 1989). In Malaysia, crushed *Dicranopteris linearis* leaves were used to control fever and a common edible ferns, *Diplazium esculentum*, was used to treat fever, dermatitis, measles, coughs and

as supplement by woman after childbirth (Ong, 2011). The pastes from the fronds of *Adiantum capillus-veneris* were used in Nepal to relieve headaches and chest pains (Kamini, 2007).

For the expulsion of parasites, the rhizome of *Dryopteris crassirhizoma* was used in China against intestinal parasites (Williams, 2012). Similarly, the same fern was used in some other countries such as Indochina to expel intestinal worms (Kamini, 2007).

2.4 *Christella dentata* (Forssk.)

Common names: Binung, Downy maiden fern (Snyder and Bruce, 1986)

Synonym: *Polypodium dentatum*, *Dryopteris dentata*, *Thelypteris dentata*, *Cyclosorus dentatus*, *Polypodium nymphale* (De Lange, et al., 2010).

C. dentata is a medium-size perennial fern, with plant height in the range of 0.25 - 0.8 meters (Ferns of Western Australia, 2013). It is native to Asia, Africa, Australia and New Zealand (McCarthy, 1998). The habitats of *C. dentata* are wetlands, sheltered lowlands, foothills and shady stream banks (Piggott, 1988). It has also been found growing in sandstone rocks and limestone substrates (Correll, 1976). Based on earlier studies, *C. dentata* has been proven to have antibacterial properties against *Bacillus subtilis*,

Escherichia coli, Salmonella typhi and *Staphylococcus aureus* (Kumar and Kaushik, 2011). Also, it possesses antihyperglycemic and antinociceptive potential (Tanzin, et al., 2013). The basic structural parts and botanical descriptions of *C. dentata* have been summarized in Table 2.1.

Table 2.1: Botanical description of *Christella dentata* (Palmer, 2003; Ferns of Western Australia, 2013)

Parts	Description
Rhizome	Around 0.7 cm in diameter, short and extends along the surface of ground.
Frond	50 – 100 cm long with diameter around 0.7 m.
Blade	40 – 100 cm long, evergreen, egg-shaped, lanceolate.
Pinnae	6 – 14 cm long, 15 – 30 lobes, linear-lanceolate, tips acuminate, 0.2 – 0.4 mm short white hairs on adaxial surface, shorter on abaxial surface, deflected and reduced lower pinnae
Stipe	20 – 50 cm long, purplish brown and occasionally pale yellow, presence with hairs.
Rachis	Purplish brown, occasionally pale yellow, attaches with 20 – 33 pinna pairs.
Spore	Tawny, circular, extends in the middle, cover with hairy indusium

2.4.1 Taxonomy

Figure 2.1 illustrates the complete *C. dentata* plant and its pinna portion.

(A) (B)

Figure 2.1: A-B**:** *Christella dentata*: A, complete plant; B, pinna portion (Brownsey and Chinnock, 1973)

The scientific classification of *C. dentata* are summarized as following,

Domain	:	Eukaryota
Kingdom	:	Plantae
Phylum	:	Pteridophyta
Class	:	Polypodiopsida
Order	:	Polypodiales
Family	:	Thelypteridaceae
Genus	:	*Christella*
Specific epithet	:	*dentata*
Botanical name	:	*Christella dentata*

2.5 Phytochemical Compounds in Study

Phytochemicals are large group of plant-derivative compounds, which are synthesized by plants through wide network of metabolic pathways known as primary metabolism and secondary metabolism (Cseke, et al., 2006). Primary metabolism is an essential process which forms the fundamental elements for growth and survival of plant whereas secondary metabolism are required for plant adaptation, defense, in responses to biotic and abiotic stress (Dewick, 2009).

Based on the assumption of traditional medical systems such as Ayurvedic medicine, traditional Chinese medicine and European phyotherapy, the synergy of all phytochemicals in plants will exert the maximum therapeutic efficacy as the plant resources have more than one active compound that give rise to their medicinal values (Ulrich-Merzenich, et al., 2010). In modern phytochemical researches, the synergies of phytochemicals which responsible for different bioactivities are still remain largely unexplored. This is because all phytochemicals vary in molecular size, polarity and solubility, which will eventually affect their distribution in the cells, organs, tissues and subcellular organelles (Liu, 2003). Also, different compounds of similar effects may lead to greater, equivalent or weakened effects. Thus, the interactions may term differently as synergy, antagonistic or additive effects (Wagner and Ulrich-Merzenich, 2009).

Recently, ferns have been reported to contain of bioactive phytochemicals such as phenols, flavonoids, alkaloids, terpenoids, steroids, amino acids and fatty acids (Li, et al., 2008). Besides that, phenolic compounds, flavonoids, proanthocyanidins, coumarin, hydroxycinnamic acid and saponin have been identified to possess potent bioactivities (Chai and Wong, 2012; Kostova, et al., 2011; Pietta, 2000; Wu, 2007).

Phenolic compounds can be defined chemically as a compound which contains an aromatic ring (C6) with at least one or more hydroxyl substituents (Cseke, et al., 2006). Phenolic compounds with more than one hydroxyl substituents are term as polyphenols (Michalak, 2006). Phenolic compounds are primarily involved in protection against ultraviolet damage and cell death due to dimerization and breakage of DNA (Dey and Harborne, 1997). Also, they are vital in plant growth, pigmentation, reproduction and defense (Vincenzo, et al., 2006). Phenolic compounds can be further subdivided into phenolic acids, flavonoids, stibenes, coumarins and tannins (Cseke, et al., 2006). Phenolic compounds are usually soluble in polar organic solvents due to the presence of hydroxyl group and their relative solubility increase with increasing number of hydroxyl groups which present in the compound (Harborne, 1989). The molecular size and length of hydrocarbon will also influence the solubility of phenolic compounds in solvent (Daniel, et al., 2008).

Next, flavonoids are generally water-soluble phenolic compounds, which contain two benzene rings separated by a three-carbon propane unit and they are usually presence in the form of glycosides (bound to sugar) in plants (Cseke, et al., 2006). Flavones, flavonols, flavanones, anthocyanins, isoflavones, chalcones are some examples of which belong to the flavonoid class (Salunkhe and Chavan, 1989). The distinguishable features among flavonoids are based on the hydroxyl groups and additional oxygen-containing heterocyclic rings (Harborne, 1989). Flavonoids act as attractants to pollinators, protection against ultraviolet irradiation, alleolochemical, antimicrobial and anti-herbivory factors (Richard and Giulio, 2010)

Tannins are high molecular weight phenolic compounds which possess the ability of binding and precipitate proteins, alkaloids and heavy metals leading to the formation of insoluble copolymers (Praveen and Kumud, 2012). Tannins serve as protective function in the bark of roots, steams and outer layers of plants (Clinton, 2009). Tannins are chemically divided into two major classes, namely condensed tannins and hydrolysable tannins (Cseke, et al., 2006). Condensed tannins are also known as proanthocyanidins (Harborne, 1989). Proanthocyanidins are formed from the polymerization of the basic building block known as flavan-3-ols (Salunkhe and Chavan, 1989). The bondings between all the flavan units are either 4-6 or 6-8 carbon-carbon bonds, which are highly resistant toward cleavages and hydrolysis (Jess, 1995). Proanthocyanidins can be broken down into anthocyanidins through hot acid treatment (Harborne, 1989). For hydrolysable tannins, they consist of hydroxyl

groups which are partially esterified with phenolic groups. Hence, hydrolysable tannins are highly susceptible toward enzymatic or non-enzymatic cleavage by weak acids or bases (Praveen and Kumud, 2012).

Hydroxycinnamic acids (HCAs) are phenolic compounds which belong to the phenylpropanoids class (Teixeira, et al., 2013) and are hydroxyl derivatives of cinnamic acid consisting of a phenol compound which is attached to a three-carbon side chain (Cseke, et al., 2006). Naturally, HCAs of plants are associated with developmental processes (e.g. flowering, fruiting, seeding) and responses toward viral and fungal infections (Slocum and Flores, 1991). HCAs are usually found in esterified form bounding to cell wall or proteins because the esterification of HCAs will result in the modification of the cell wall polysaccharides. This will build up resistance toward the action of lytic enzymes produced by fungal and pathogens (Hanley, Russell and Chesson, 1993).

Coumarins are phenolic compounds which belong to the benzopyrone family (Kontogiorgis, Detsi and Hadjipavlou-Litina, 2012). Structurally, they consist of at least one benzene ring that is attached to one or several pyrone ring (Joshi, et al., 2012). Pyrone ring is an unsaturated six membered rings which possess an oxygen atom and a ketone functional group (Harborne, 1989). Coumarins are derivative of o-hydroxycinnamic acids that form through cyclization and ring closure between the carboxyl groups and o-hydroxyl group via oxidation-

reduction and isomerization (Macheix, Fleuriet and Billot, 1990; De Amorim, et al., 2012). Naturally, coumarins play a vital role in alleolochemical interactions by influencing the physiologies of other species through the inhibition or stimulation of biochemical processes (Zobel and Brown, 1995). The syntheses of coumarins in plants are greatly enhanced when plants are exposed to biotic and abiotic stresses such as wounding, microbial attacks and environmental changes (Zobel and Brown, 1995).

Saponins refer to high molecular weight tripterpene glycosides (600-2000 daltons), which consist of a glycone (sugar group) linked to either an aglycone (sterol) or a genin (triterpene) via glycosidic ether or ester link (Cseke, et al., 2006; Arora, 2010). They are amphiphilic molecules characterized by foamy or detergent-like properties due to the asymmetric distribution of hydrophobic aglycone group and hydrophilic sugar group (Rahman, 2000). Saponin can be classified into three groups based on the aglycone moiety, which can be a steroid, triterpene or steroidal glycoalkaloid (Arora, 2010). Based on studies, saponins are bioactive compounds with anti-cancerous, anti-inflammatory, antioxidant activities and hypo-cholesterolemic effects (Rao and Gurfinkel, 2000). They are also served as anti-feedants, anti-microbial, antibacterial, insecticidal, piscicidal, antihelminthic and molluscicidal in response to biotic stresses (Ustundag and Mazza, 2007).

2.6 Roles of Plant Antioxidant on Diabetes Mellitus

An antioxidant is molecule that is stable enough in donating electrons and neutralizing free radicals or terminating chain reactions. This will lower the capacity in causing cellular damages and protecting damages caused by free radical-induced oxidative stress (Fang, et al., 2002). Free radical is unstable and highly reactive due to the presence of unpaired electron (Ionita, 2003). Phytochemicals associate with antioxidant are capable of suppressing the formation of free radicals and scavenge radicals by preventing the initiation and propagation of oxidizing chain reactions (Lobo, et al., 2010). Some examples of natural antioxidants are ascorbic acid, vitamin A, α-tocopherol, β-carotene, lutein, lignin, lycopene, selenium and phenolic compounds (Hamid, et al., 2010).

Previous studies have reported that DM is associated with the increased formation of free radicals or reactive oxygen species (ROS) and decreased antioxidant capacity, leading to oxidative damage on vital cellular components. (Bashan, et al., 2009). The oxidative stress induced by hyperglycemia plays a vital role in the development of various diabetic complication, insulin resistance and pancreatic beta-cells dysfunction (Uchiyama, et al., 2002). The excessive production of free radicals in the body are also linked with other diseases such as cardiovascular diseases, cancer, skin inflammation, Alzheimer's disease, liver disease, kidney disease, arthritis, anemia and aging (Halliwell, 1996). In addition, the oxidative stress caused by hyperglycemia

has been reported to induce apoptosis of endothelial cells both *in vitro* and *in vivo* (Kangralkar, Patil and Bandivadekar, 2012). Hence, the inhibition of intracellular free radicals or ROS formation by antioxidant would provide a therapeutic approach in preventing oxidative stress, which prevent the occurrence of DM, DM complications and other diseases as mentioned earlier (Bajaj and Khan, 2012).

2.7 Inhibition of Alpha-amylase and Alpha-glucosidase

α-Amylase and α-glucosidase are two enzymes that are involved in the breakdown of carbohydrates into simple monosaccharides (National Library of Medicine, 2011). α-amylase breakdowns carbohydrates through endohydrolysis by acting on the inner α-1,4-glucosidic bonds (Hizukuri, Abe and Hanashiro, 1996) whereas α-glucosidase involves in the exohydrolysis of complex carbohydrates by acting on the terminal non-reducing 1,4-α-linkages (National Library of Medicine, 2011). Both endohydrolysis and exohydrolysis of carbohydrates will yield smaller monosaccharides consisting of α-glucose units (Hizukuri, Abe and Hanashiro, 1996). The carbohydrate digestion in the mammalian system is in a progressive manner begins with α-amylase and then α-glucosidase in yielding glucose (Dhital, et al., 2013). Sequentially, the mammalian α-amylase catalyzes main step digestion of carbohydrates into simpler monosaccharides (Gupta, et al., 2003) whereas α-glucosidase catalyzes the end step digestion and absorption of carbohydrates by anchoring in the mucosal brush border of the small intestine (Anam, Widharna and Kusrini,

2009). The inhibition of both α-amylase and α-glucosidase will reduce the rate of carbohydrate breakdown, which in turn lower the amount of glucose being digested and absorbed in the intestine (Sindhu, Vaibhavi and Anshu, 2013). This will lead to the reduction in postprandial glycemic levels and the range of postprandial glucose levels (Chiasson, et al., 1994; Lebovitz, 1998). Most importantly, the inhibition of these two enzymes is one of the alternative therapeutic approaches against DM (Krentz and Baile, 2005).

Several inhibitors of α-amylase and α-glucosidase which exist today, such as acarbose and sulfonylureas, have adverse side effects of liver toxicity, gastrointestinal symptoms such as diarrhea, nausea, stomach pain and so on which may rise the risk of heart diseases (Tewari, et al., 2003). Based on studies, the adverse effects might due to excessive inhibition of α-amylase which results in the unusual fermentation of carbohydrates by bacteria (Bischoff, 1994). Plants, animals and microorganisms are presence with various α-amylase and α-glucosidase inhibitors in regulating the activity of both enzymes in their system (Kumar, et al., 2011). Therefore, the discovery of novel α-amylase and α-glucosidase inhibitors with the minimal side effects on human system to treat DM is the main concern of various studies despite the sources of isolation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Instruments

All the chemicals and instruments used in this study were provided by Department of Biological Science, University Tunku Abdul Rahman (Perak campus). The details of the chemicals and instruments are listed in Table 3.1 and Table 3.2, respectively.

Table 3.1: Chemicals used in this research

Chemicals	Brands
Acarbose	LKT Laboratories
Acetic acid	Systerm
Aluminium chloride hexahydrate	Ajax Finechem
Ammonium iron (III) sulphate	Systerm
Ascorbic acid	Sigma
Caffeic acid	Sigma
Coumarin	Acros Organics
Di-potassium hydrogen phosphate anhydrous	QRëC™
Ethanol	Copens Scientific
Ethyl acetate	R & M Chemicals
Folin-Ciocalteu reagent	R & M Chemicals
Gallic acid	R & M Chemicals
Hydrochloric acid	Prolabo
Lead (II) acetate	Scharlau
Methanol	RCI Labscan

Table 3.1 Continued

Chemicals	Brands
n-Butanol	R & M Chemicals
Oleanolic acid	MP biomedicals
Phenol	Calbiochem
p-Nitrophenol	Bio Basic
p-Nitrophenyl-α-D-glucopyranoside (*p*NPG)	Acros Organics
Quercetin	Acros Organics
Saponin	Nacalai tesque
Sodium carbonate	QRëC™
Sodium dihydrogen phosphate	QRëC™
Sodium hydroxide	MERCK
Sodium iodide	R & M Chemicals
Sodium molybdate	R & M Chemicals
Sodium nitrite	QRëC™
Sodium sulphate	MERCK
Starch azure	Sigma
Sulphuric acid	Prolabo
Vanillin	R & M Chemicals
1,1-Diphenyl-2-picrylhydrazyl (DPPH)	Sigma
α-Glucosidase	Sigma
α-Amylase	Sigma

Table 3.2: Instruments used in this research

Instruments	Brands
Bench top freeze dryer	LaboGene
Centrifuge machine	Hettich
Digital water bath	Memmert
Electronic balance	Copens Scientific
Ice machine	Hoshizaki
Magnetic stirrer	Laboratory Medical Supplies
Microcentrifuge machine	Thermo Scientific
Oven	Memmert
pH meter	Eutech Instruments
Refrigerator	Pensonic
Rotary evaporator	BUCHI R-200
UV-VIS spectrophotometer	Thermo Scientific

3.2 Plant Materials

Christella dentata (Family: Thelypteridaceae) specimens were provided by Dr. Chai Tsun-Thai (Department of Chemical Science, University Tunku Abdul Rahman). The species of fern was authenticated by Professor Ong Hean Chooi (Institute of Biological Sciences, University Malaya). The leaves of fern sample were oven-dried to constant weight at 45 °C and then ground into powder using a blender.

3.3 Preparation of *Christella dentata* Water Extract

To prepare the water extract (WE) of *C.dentata*, the fern powder was suspended in deionized water at the ratio of 1:20 (dry weight: volume). Next, the mixture was incubated in a 90 °C water bath for 1 hour with agitation every 15 minutes. Then, the mixture was vacuum-filtered and the filtrate was centrifuged at 7830 rpm for 5 minutes. The supernatant was then freeze-dried. The freeze-dried WE was then redissolved in water, aliquoted, and kept at 4 °C until further use.

3.4 Solvent-Partitioning of Water Extract

Before proceed to the solvent-partitioning process, the freeze-dried WE (2.5 g) was firstly redissolved 50 ml of deionized water. Then, the solution was partitioned sequentially in a separatory funnel with equal volume of ethyl acetate and *n*-butanol, hence yielding ethyl acetate fraction (EAF), *n*-butanol fraction (BF) and water fraction (WF). Both EAF and BF were concentrated in a vacuum evaporator at 37 °C and then oven-dried to constant weight at 37 °C. WF was freeze-dried. All dried samples were dissolved in distilled water, aliquoted and stored at 4 °C for further analysis. Figure 3.1 illustrates the schematic flow of the extraction and solvent-partition processes of *Christella dentata*.

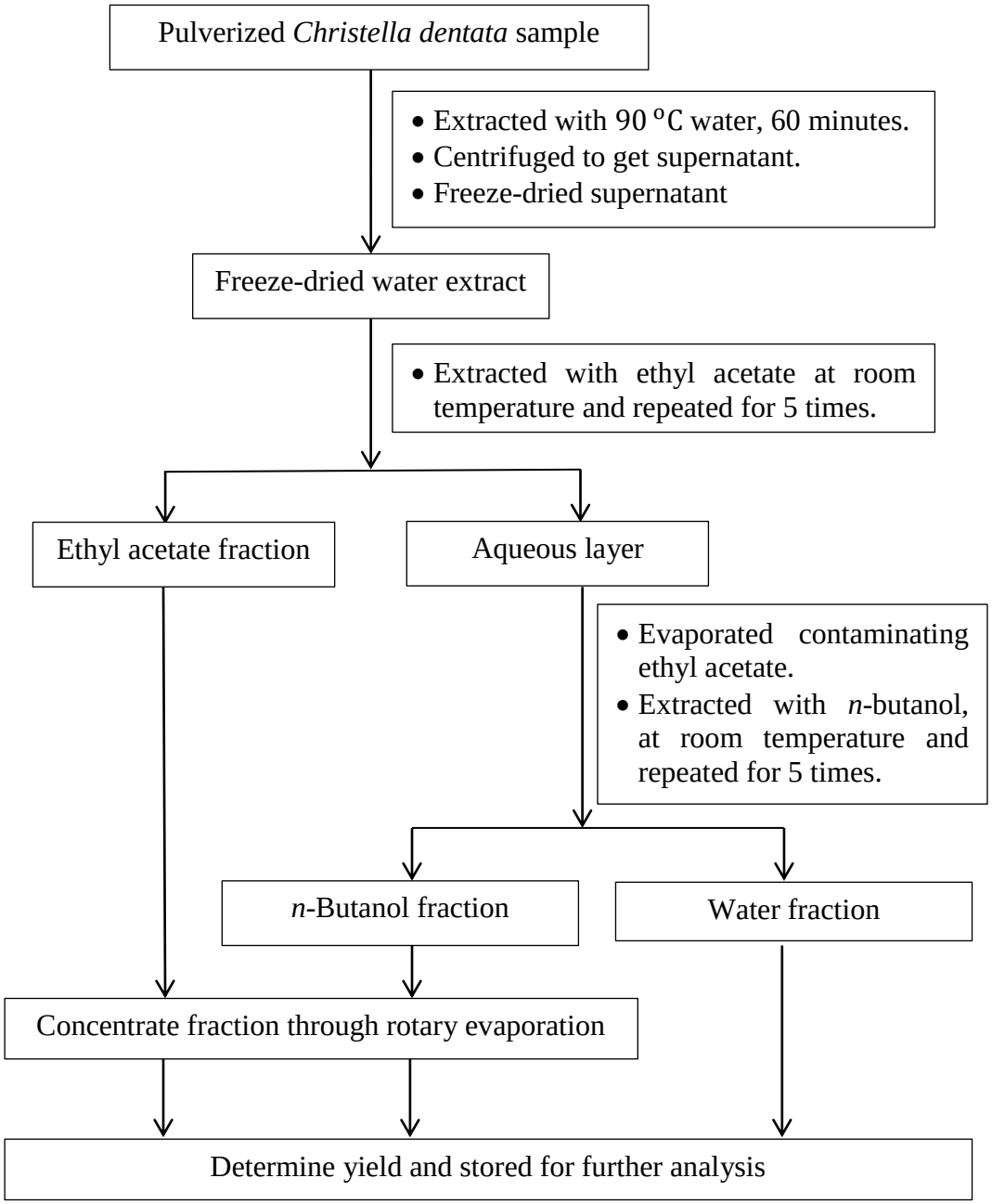

Figure 3.1: Schematic diagram on the extraction and solvent-partition of *Christella dentata.*

3.5 Yield Determination

The respective yields of WE, EAF, BF and WF were calculated using Formula 3.5.1 and Formula 3.5.2. Yields were expressed in percentage (%).

Formula 3.5.1:

$$\text{Yield of WE (\%)} = \frac{\text{Dry weight of WE obtained (g)}}{\text{Dry weight of } C.dentata \text{ powder extracted used(g)}} \text{ X } 100 \text{ \%}$$

Formula 3.5.2:

$$\text{Yield of solvent fractions (\%)} = \frac{\text{Dry weight of solvent fraction obtained (g)}}{\text{Dry weight of WE used for fractionation(g)}} \text{ X } 100 \text{ \%}$$

3.6 Phytochemical Analysis

3.6.1 Total Phenolic Content

Total phenolic (TP) contents of WE, EAF, BF and WF were determined by using the Folin-Ciocalteu assay (Waterhouse, 2001). Firstly, a mixture of 200 µl of sample, 800 µl of deionized water and 100 µl of Folin's reagent was prepared. The mixture was vortexed and incubated for 3 minutes. Next, 300 µl of 5 % sodium carbonate was added and the mixture was incubated in darkness at room temperature for 2 hours. Absorbance of the mixtures was then determined at 765 nm. TP content of samples was expressed in mg gallic acid equivalents (GAE) per gram of sample, based on a standard curve prepared with 0 - 100 µg of gallic acid/ ml.

3.6.2 Total Flavonoid Content

Total flavonoid (TF) contents of WE, EAF, BF and WF were determined by using an aluminium chloride colorimetric assay (Zou, et al., 2004). Firstly, a mixture of 200 µl of sample and 150 µl of 5 % sodium nitrite was prepared. The mixture was vortexed and incubated for 6 minutes at room temperature. Next, 150 µl of 10 % aluminium chloride hexahydrate was added, followed by another incubation of 6 minutes. Then, 800 µl of 10 % sodium hydroxide was added to terminate the reaction and the mixture was allowed to stand for 15 minutes at room temperature. Absorbance of the reaction mixtures was determined at 510 nm. TF content of samples was expressed in mg quercetin equivalents (QEE) per gram of sample based on a standard curve prepared with 0 - 500 µg of quercetin/ ml.

3.6.3 Total Proanthocyanidin Content

Total proanthocyanidin (TPro) contents of WE, EAF, BF and WF were determined by using an acid-butanol assay (Porter, et al., 1986). Firstly, 200 µl of sample was added to 1200 µl of acid-butanol (*n*-butanol and concentrated hydrochloric acid at a ratio of 19:1 v/v) followed by 40 µl of iron reagent (2 % ferric ammonium sulphate in 2M hydrochloric acid). The mixture was vortexed and then incubated in a boiling water bath for 50 minutes. Upon 50 minutes, the mixture was cooled on ice to room temperature and its absorbance was determined at 550 nm. TPro content of samples was calculated with the

assumption that effective $E^{1\%\ 1\,cm,\ 500\,nm}$ of leucocyanidin is 460 and expressed in mg leucocyanidin equivalents (LE) / g sample.

3.6.4 Total Coumarin Content

Total coumarin (TCou) contents of WE, EAF, BF and WF were determined by using the method described by De Amorim, et al. (2012). Briefly, a mixture of 100 µl sample, 200 µl of deionized water, 100 µl of 5 % lead (II) acetate and 1000 µl of 0.1M hydrochloric acid was prepared. The mixture was vortexed and left standing at room temperature for 30 minutes in darkness before its absorbance was determined at 320 nm. TCou content of the samples was expressed as mg 1, 2-benzopyrone equivalents (BE) per gram of sample, based on a standard curve prepared with 0 - 1000 µg of 1, 2-benzopyrone/ ml.

3.6.5 Total Hydroxycinnamic Acid Content

Total hydroxycinnamic acid (THC) contents of WE, EAF, BF and WF were determined by using the method described by Matkowski, et al. (2008). Firstly, 200 µl of 0.5 M hydrochloric acid, 200 µl of Arnow's reagent (10 % sodium molybdate and 10 % sodium nitrite), 200 µl of 8.5 % sodium hydroxide and 200 µl of water were added to 200 µl of sample. Absorbance of the mixture was 490 nm. THC content was expressed in mg caffeic acid equivalents (CAE)

per gram of sample, based on a standard curve prepared with 0 - 200 µg of caffeic acid/ ml.

3.6.6 Total Saponin Content

Total saponin (TSap) contents of WE, EAF, BF and WF were determined by using a vanillin-sulphuric acid assay (Hiai, et al., 1976). Firstly, a mixture of 100 µl of sample, 300 µl of 8 % vanillin and 3000 µl of 72 % sulphuric acid was prepared. The mixture was vortexed and incubated in a $60^{o}C$ water bath for 10 minutes. The mixture was then cooled on ice. Absorbance of the mixture was determined at 538 nm. TSap was expressed in mg oleanolic acid equivalents (OAE) per gram of sample, based on a standard curve prepared with 0 - 1000 µg oleanolic acid/ ml.

3.7 DPPH Radical Scavenging Activity

DPPH radical scavenging activity of the samples was assessed according to Chai and Wong (2012). A mixture of 0.5 ml of sample and 0.5 ml of DPPH (0.1 mM in methanol) was prepared and kept in darkness for 30 minutes at room temperature. Absorbance of the mixture was determined at 517 nm. DPPH radical scavenging activity was calculated using Formula 3.7, where A_{sample} is the absorbance in the presence of fern extract/ fractions, whereas $A_{control}$ is the absorbance of control reaction, where fern extract/ fractions

were substituted with deionized water. Ascorbic acid (AA) was used as positive control for this assay. EC_{50}, the concentration of sample required to achieve 50 % of radical scavenging activity, was determined through linear regression analysis.

Formula 3.7:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \text{X } 100\ \%$$

3.8 Alpha-Glucosidase Inhibitory Activity and Mode of Inhibition

3.8.1 Alpha-glucosidase Inhibition Assay

α-Glucosidase inhibitory activity of sample was assessed as previously described in Chai, et al. (2013). A reaction mixture of 250 µl 100 mM potassium phosphate buffer (pH 7.0), 150 µl of 0.5 mM *p*NPG (0.5 mM in 100 mM phosphate buffer, pH 7.0), 50 µl of sample and 150 µl of α-glucosidase (0.1 unit/ml in 10 mM potassium phosphate buffer, pH 7.0) was prepared and incubated in a 37 °C water bath for 30 minutes. The reaction was terminated by adding 600 µl of 200 mM sodium carbonate. Absorbance of the reaction mixture was determined at 400nm. α-Glucosidase inhibitory activity was calculated using Formula 3.8.1, where A_{sample} is the absorbance in the presence of fern extract/ fractions, whereas $A_{control}$ is the absorbance in the absence of fern extract/ fractions. A reaction blank was prepared for each measurement by replacing α-glucosidase with 10 mM potassium phosphate

buffer. Quercetin (QE) was used as positive control for this assay.EC_{50}, the concentration of sample required to achieve 50 % of the inhibitory activity, was determined through linear regression analysis.

Formula 3.8.1:

$$\alpha - \text{Glucosidase inhibitory activity (\%)} = \left(1 - \frac{A_{sample}}{A_{control}}\right) \text{X } 100\ \%$$

3.8.2 Determination of Mode of Inhibition of Alpha-glucosidase by WF

To determine the mode of inhibition of α-glucosidase, the α-glucosidase inhibition assay described above was carried out by varying the *p*NPG concentration between 0 - 2 mM, using 10.83 µg/ml of WF as the source of inhibitor. For control (inhibitor omitted), WF was replaced with deionized water. The amount of product formed through the enzymatic reaction was determined from a standard curve prepared with *p*-nitrophenol, with concentration range between 0 - 0.5 mM. The reaction velocity (mM/min) of the enzymatic reaction was calculated using Formula 3.8.2. A Lineweaver-burk plot of $\frac{1}{V}$ against $\frac{1}{[S]}$ was constructed to identify the mode of inhibition, where $\frac{1}{V}$ was the reciprocal of reaction velocity, whereas $\frac{1}{[S]}$ was the reciprocal of *p*NPG (substrate) concentration.

Formula 3.8.2:

$$\text{Reaction velocity (mM/min)} = \frac{\text{Amount of product formed (mM)}}{\text{Reaction time (min)}}$$

3.9 Porcine Pancreatic Alpha-amylase Inhibitory Activity

Porcine Pancreatic α-amylase (PPA) inhibitory activity of sample was assessed as described in Hansawasdi, et al. (2000) with slight modifications. Firstly, a reaction mixture of 200 µl of 20 mg/ml starch azure (In 20 mM sodium phosphate buffer, pH 7.0), 200 µl of sample and 300 µl of PPA (2.1 unit/ml in 20 mM phosphate buffer, pH 7.0) was prepared and incubated at 37^{o}C water bath for 20 minutes. The reaction was terminated by adding 500 µl of 50 % acetic acid. The mixture was centrifuged at 10,000 rpm for 5 minutes. The absorbance of the supernatant was determined at 595 nm. A blank was prepared for each sample by replacing PPA with 20 mM sodium phosphate buffer. PPA inhibitory activity was calculated using Formula 3.9.1, where A_{sample} is the absorbance in the presence of plant extract/ fractions, whereas $A_{control}$ is the absorbance in the absence of extract/ fractions. A reaction blank was prepared for each measurement by replacing PPA with 20 mM sodium phosphate buffer. Acarbose was used as positive control for this assay. EC_{50}, the concentration of sample required to achieve 50 % of inhibitory activity, was determined through linear regression analysis.

Formula 3.9.1:

$$\alpha - \text{Amylase inhibitory activity } (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \text{ X } 100\ \%$$

3.10 Statistical Analysis of Experimental Result

Experiments were carried out in triplicates and data are reported as mean $\pm$ standard errors (SE). Data for WE, EAF, BF and WF were analyzed by using Analysis of Variance (ANOVA) test. Subsequently, Fisher's Least Significant Difference (LSD) test was used for post-hoc analysis. A two-tailed Student's T test was used to compare the means of extract and solvent fractions with that of positive control. Differences were considered significant at $P < 0.05$. ANOVA test, LSD test and two-tailed Student's T test were performed using SAS Version 9.2 (SAS Institute Inc.). Linear regression analyses were performed using Microsoft Excel 2010.

CHAPTER 4

RESULTS

4.1 Yield of extract and fractions of *Christella dentata*

The respective yields of WF, EAF, BF and WF were calculated by applying Formula 3.5.1 and Formula 3.5.2 as listed in Section 3.5 (Materials and Methods) and summarized in Table 4.1.

Table 4.1: Yield of extract and fractions

Samples	Yield (%)
WE	15.2
EAF	10.4
BF	9.6
WF	50.0

4.2 Phytochemical Analysis

4.2.1 Total Phenolic Content

Figure 4.1 illustrates the TP contents of WE, EAF, BF and WF of *C. dentata.* Relative abundances of phenolics in descending order were as follows: EAF > BF > WE > WF. TP content of EAF was nearly 2-fold higher than those of WE and BF as well as being 4-fold higher than that of WF.

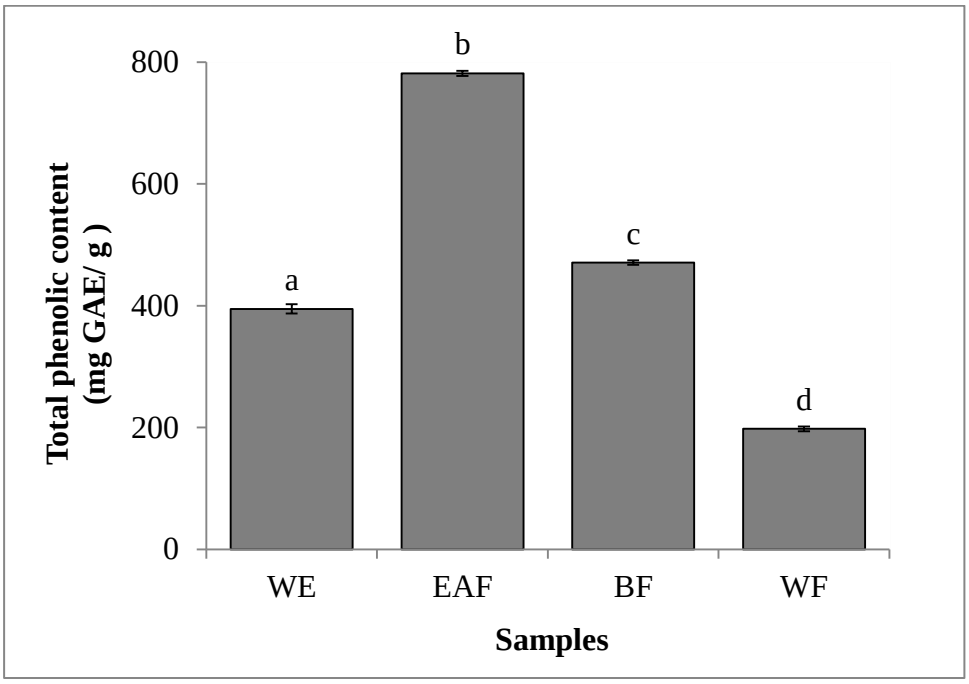

Figure 4.1: TP contents of WE, EAF, BF and WF of *C. dentata.* Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

4.2.2 Total Flavonoid Content

Figure 4.2 illustrates the TF contents of WE, EAF, BF and WF of *C. dentata.* Relative abundances of flavonoids in descending order were as follows: BF, EAF > WE > WF. TF contents of EAF and BF were almost 2-fold greater compared with WE and 2.5-fold higher than WF.

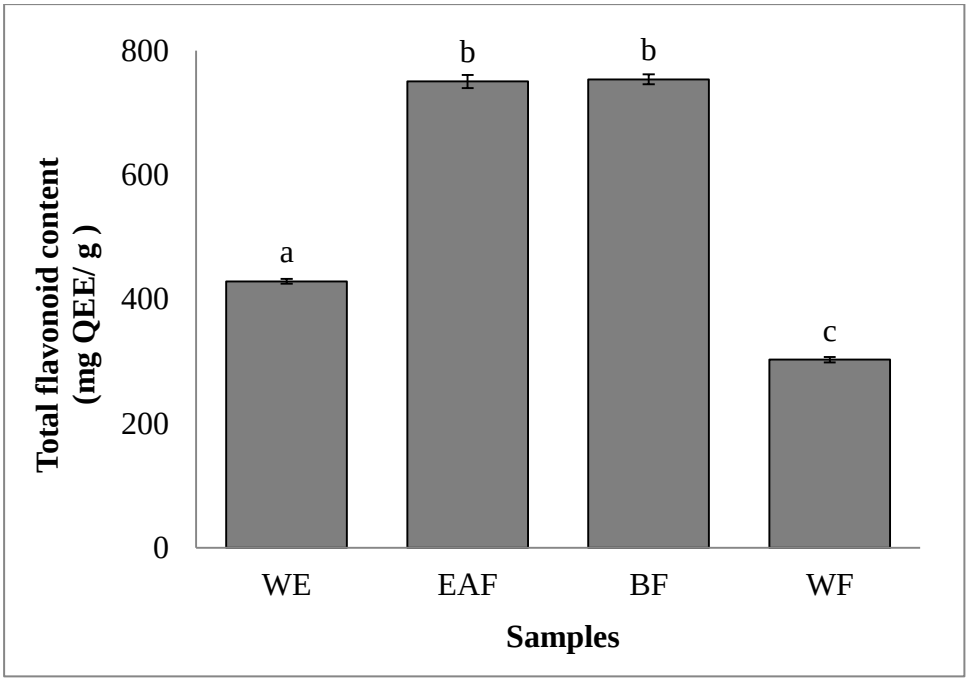

Figure 4.2: TF contents of WE, EAF, BF and WF of *C. dentata.* Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

4.2.3 Total Proanthocyanidin Content

Figure 4.3 illustrates the TPro contents of WE, EAF, BF and WF of *C. dentata.* Relative abundances of proanthocyanidins in descending order were as follows: EAF > BF > WE > WF. There was a 1.3-fold difference in TPro contents between EAF and WF.

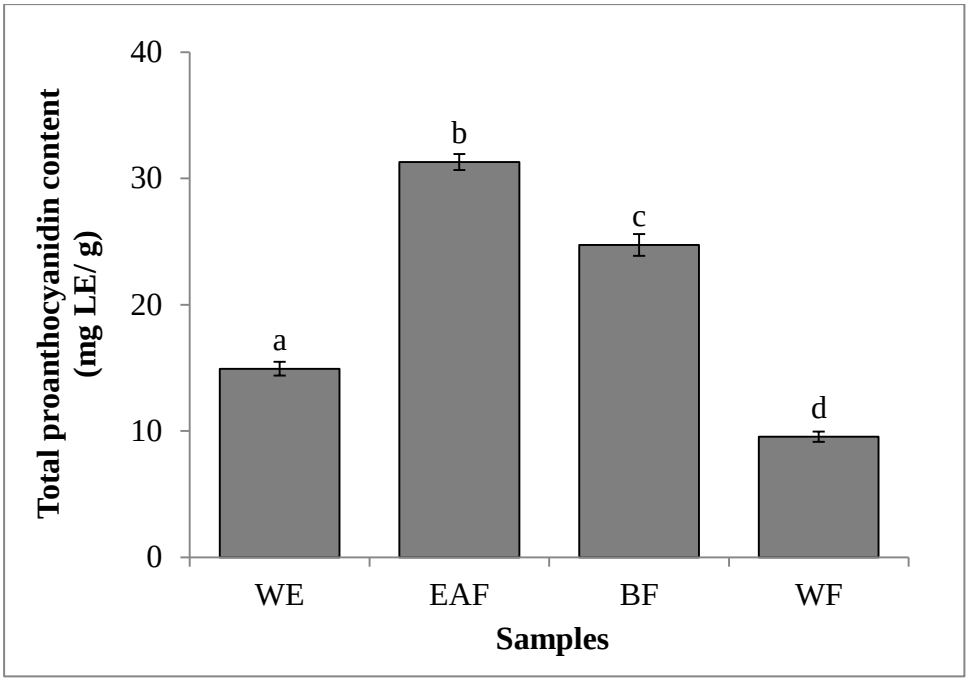

Figure 4.3: TPro contents of WE, EAF, BF and WF of *C. dentata.* Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

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4.2.4 Total Coumarin Content

Figure 4.4 illustrates the TCou contents of WE, EAF, BF and WF of *C. dentata*. Relative abundances of coumarins in descending order were as follows: EAF > BF > WE > WF. TCou content of EAF was 2-fold higher than BF as well as 2.7-fold and 5-fold higher than WE and WF, respectively.

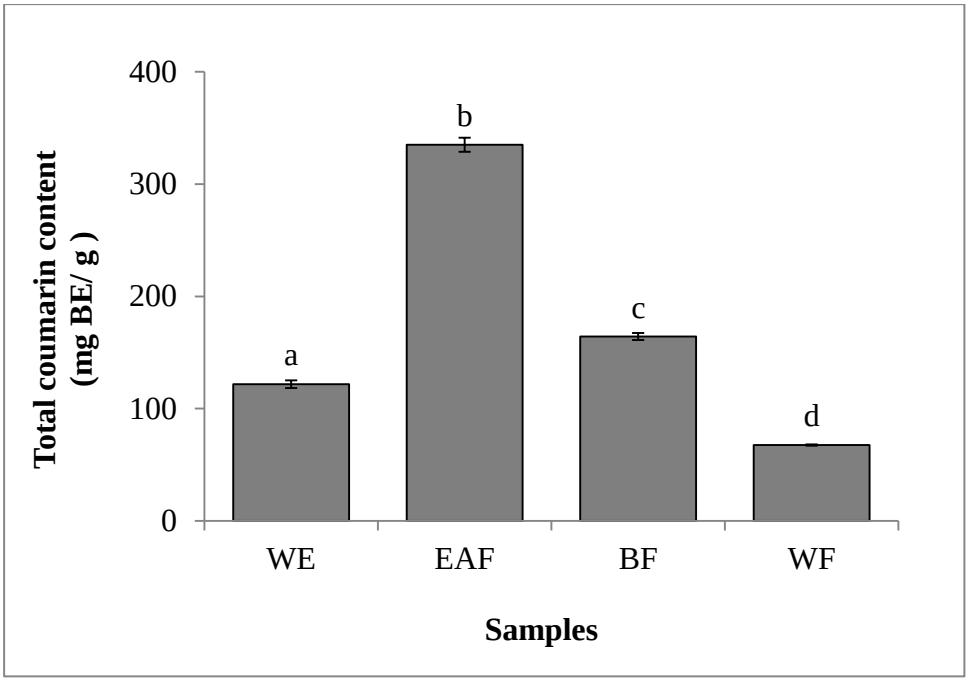

Figure 4.4: TCou contents of WE, EAF, BF and WF of *C. dentata*. Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

4.2.5 Total Hydroxycinnamic Acid Content

Figure 4.5 illustrates the THC contents of WE, EAF, BF and WF of *C. dentata.* Relative abundances of hydroxycinnamic acids in descending order were as follows: EAF > BF > WE > WF. THC content of EAF was about 1.5-fold higher than BF as well as 3-fold and 6.4-fold higher than WE and WF, respectively.

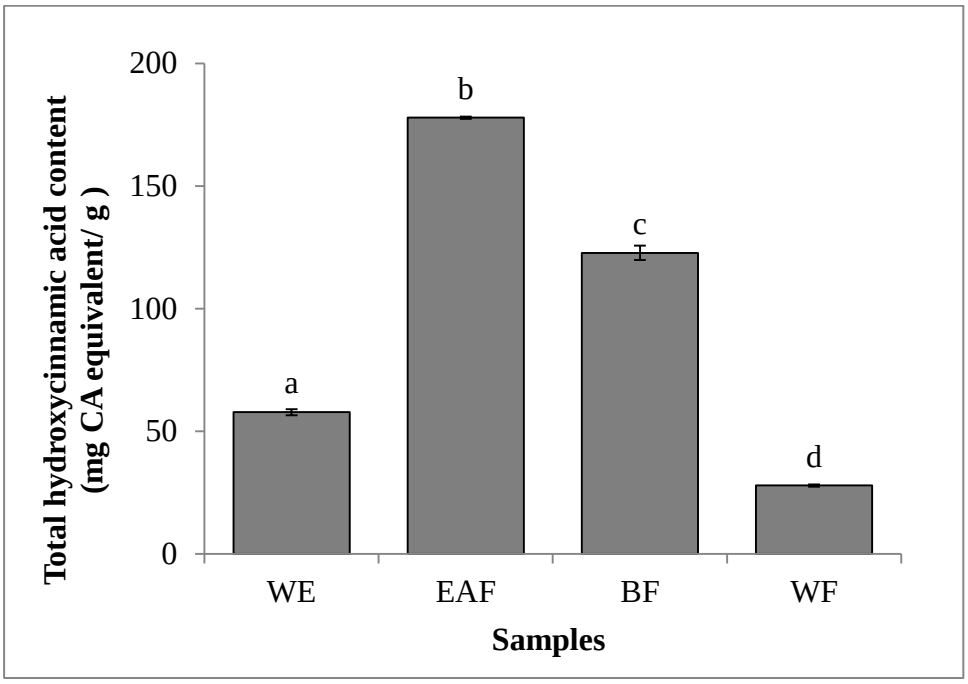

Figure 4.5: THC contents of WE, EAF, BF and WF of *C. dentata.* Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

4.2.6 Total Saponin Content

Figure 4.6 illustrates the TSap contents of WE, EAF, BF and WF of *C. dentata.* Relative abundances of saponins in descending order were as follows: BF > EAF > WE > WF. TSap content of BF was nearly 1.5-fold higher than EAF as well as 1.7-fold and 2.2-fold higher than WE and WF, respectively.

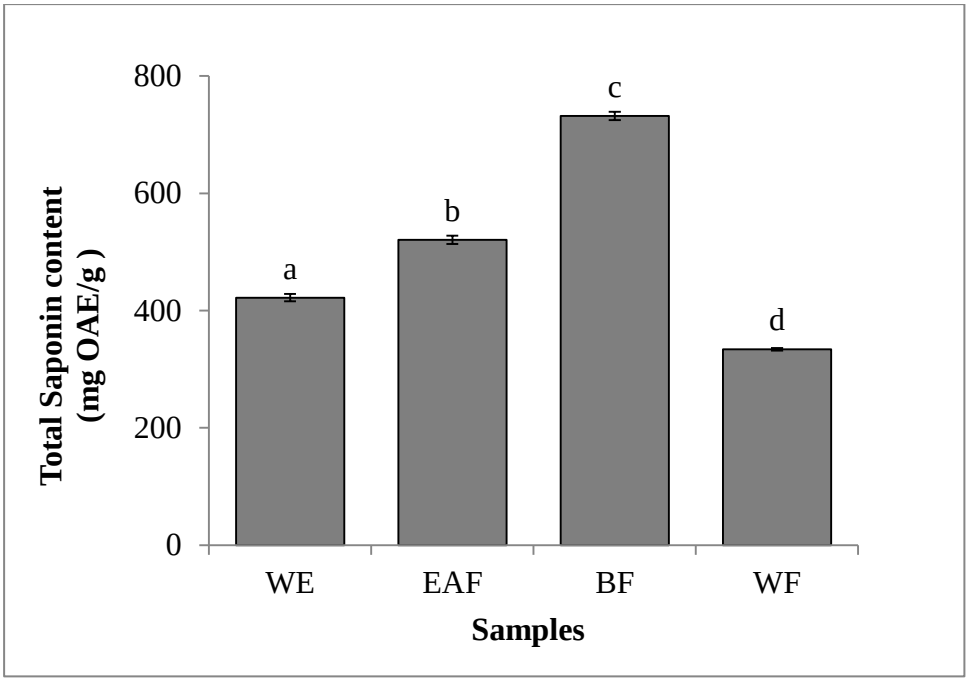

Figure 4.6: TSap contents of WE, EAF, BF and WF of *C. dentata.* Data are presented as mean ± standard error (n=3). Data points indicated by different letters are significantly different at $P < 0.05$, as determined by using Fisher's LSD test.

4.3 DPPH Radical Scavenging Activity

Figure 4.7 illustrates the relative DPPH radical scavenging activity of WE, EAF, BF, WF and ascorbic acid (AA) while Table 4.2 illustrates the EC_{50} values of the extract and fractions of *C. dentata* in DPPH radical scavenging assay. Overall, the DPPH radical scavenging activity in descending order based on EC_{50} values was as follows: EAF > BF > WE > WF. The EC_{50} values ranged between 10.75 - 26.35 µg/ml. In comparison, the EC_{50} values of EAF, BF, WE and WF were 3-fold, 4.3-fold, 4.7-fold and 7.5-fold lower than AA, respectively.

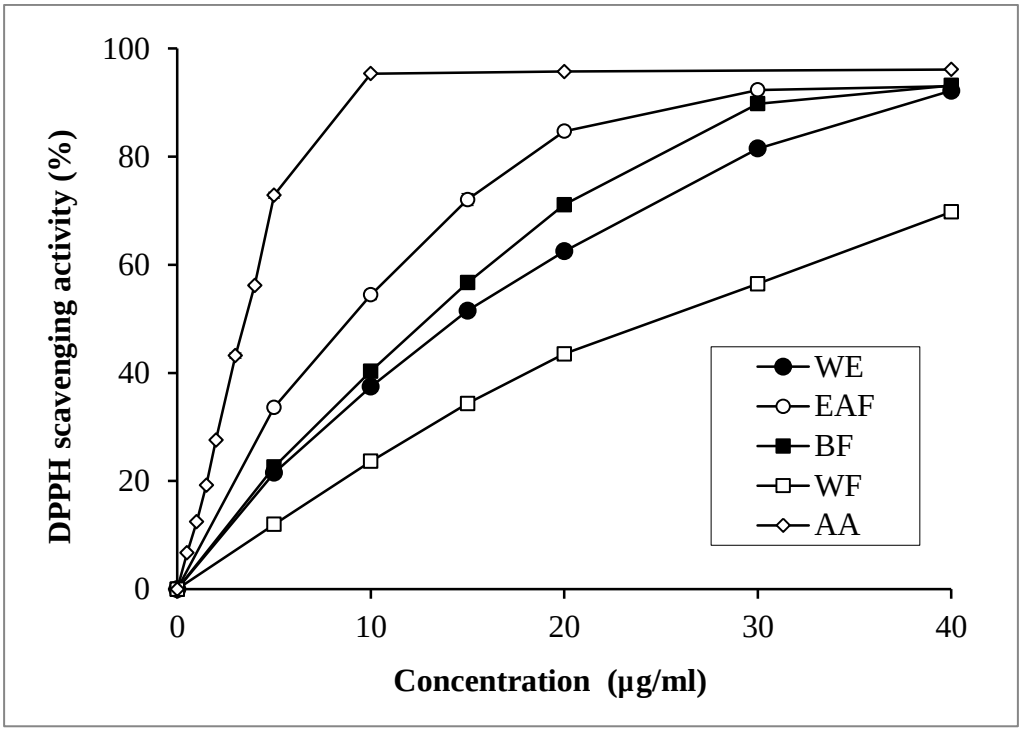

Figure 4.7: DPPH radical scavenging activity of WE, EAF, BF, WF and AA. Data are presented as mean ± standard error (n=3).

Table 4.2: EC_{50} Values of Samples in DPPH Radical Scavenging Assay

Samples	EC_{50} (µg/ml)
WE	$16.67 \pm 0.02^{a,*}$
EAF	$10.75 \pm 0.10^{b,*}$
BF	$15.05 \pm 0.12^{c,*}$
WF	$26.35 \pm 0.14^{d,*}$
AA	3.51 ± 0.02

Data was expressed as mean ± SE values (n=3). Values for extract and fractions followed by different superscript letters are significantly different as determined by Fisher's LSD test ($P< 0.05$). * indicates mean values of extract and fractions which are significantly different ($P < 0.05$) from that of the mean value of AA, as determined by using Student's T test.

4.4 Porcine Pancreatic α-Amylase Inhibitory Activity

Figure 4.8a illustrates the respective PPA inhibitory activity of WE, EAF, BF, and WF whereas Figure 4.8b illustrates the PPA inhibitory activity of acarbose. Table 4.3 illustrates the EC_{50} values of WE, EAF, BF, WF and acarbose in PPA inhibitory assay. Overall, the PPA inhibitory activity in descending order based on EC_{50} values was as follows: WF, WE, BF > EAF. The EC_{50}values ranged between 12.83 - 66.13 mg/ml. The EC_{50} values of WE, BF and EAF had 13.8 %, 80.0 % and 415.4 % higher than WF, respectively. The difference in EC_{50} values between WF and EAF was about 5-fold. WE, EAF, BF and WF had EC_{50} values of 66-fold, 300-fold, 105-fold and 58-fold lower than acarbose, respectively.

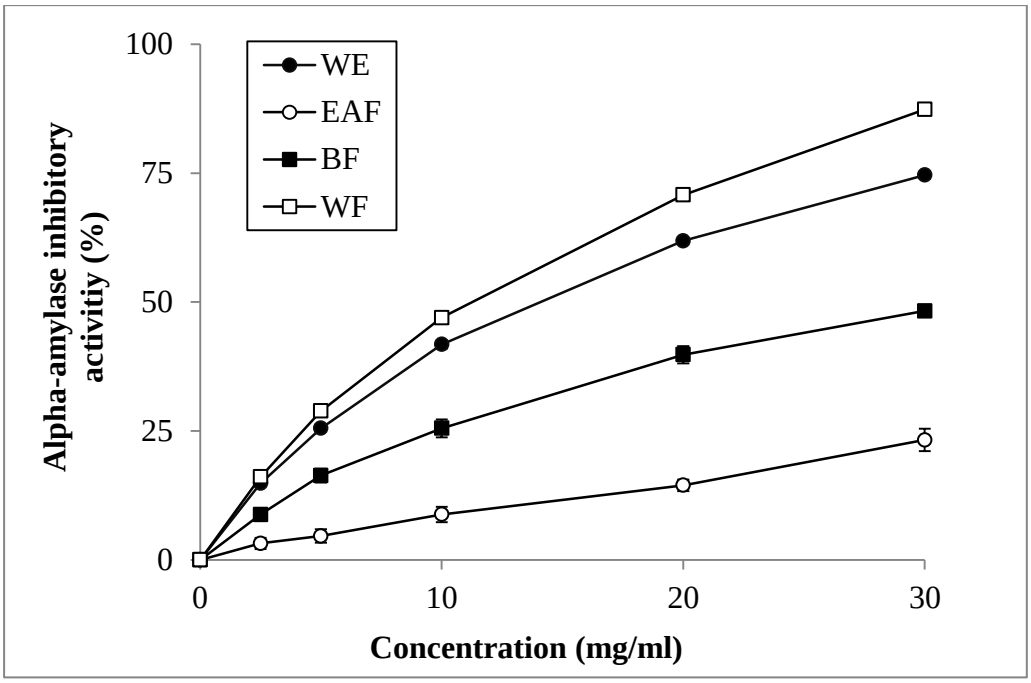

Figure 4.8a: PPA inhibition activities of WE, EAF, BF and WF. Data are presented as mean ± standard error (n=3).

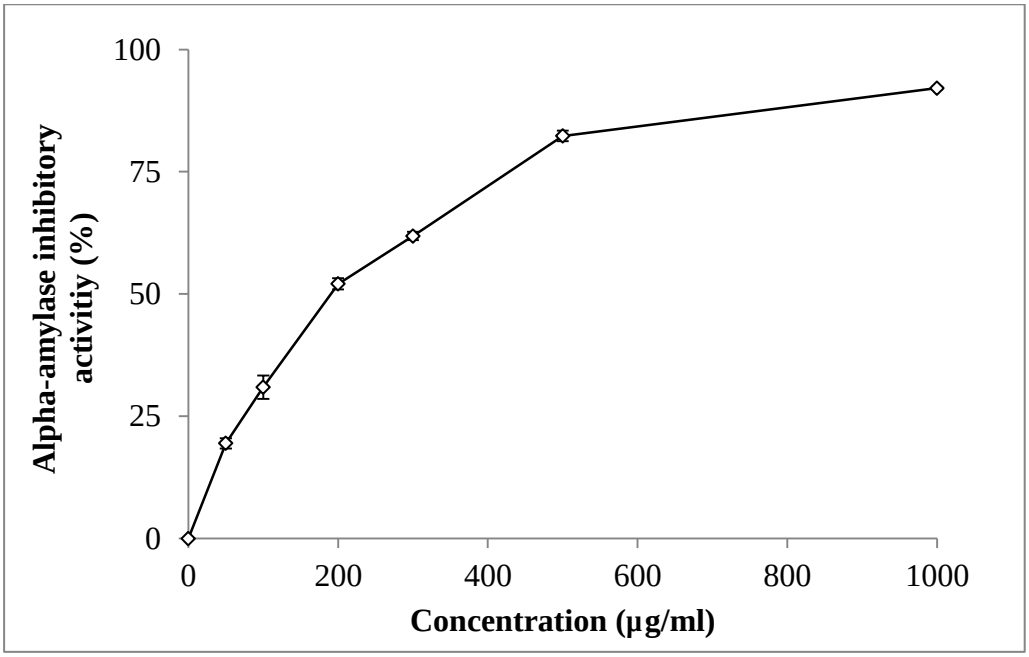

Figure 4.8b PPA inhibitory activity of acarbose. Data are presented as mean ± standard error (n=3).

Table 4.3: EC_{50} Values of Samples in PPA Inhibitory Assay

Samples	EC_{50} (mg/ml)
WE	$14.60 \pm 0.14^{a,*}$
EAF	$66.13 \pm 7.28^{b,*}$
BF	$23.10 \pm 1.03^{a,*}$
WF	$12.83 \pm 0.10^{a,*}$
Acarbose	0.22 ± 0.00

Data was expressed as mean ± SE values (n=3). Values in the same column followed by different superscript letters are significantly different as determined by Fisher's LSD test ($P< 0.05$). * indicates mean values of extract and fractions which are significantly different ($P < 0.05$) from that of the mean value of positive control, as determined by using Student's T test.

4.5 α-Glucosidase Inhibitory Activity

Figure 4.9a illustrates the respective alpha-glucosidase inhibitory activities of WE, EAF, BF, and WF whereas Figure 4.9b illustrates the α-glucosidase inhibitory activity of QE and Table 4.4 illustrates the EC_{50} values of WE, EAF, BF, WF and QE in alpha-glucosidase inhibitory assay. Overall, the α-glucosidase inhibitory activity in descending order based on EC_{50} values was as follows: WF, WE > BF> EAF. The EC_{50} values ranged between 10.83 - 405.30 µg/ml. In comparison, the EC_{50} values of WE, BF and EAF were approximately 2-fold, 3-fold and 40-fold of WF respectively. WE, BF and WF had EC_{50} values of 4.4-fold, 1.3-fold and 7.9-fold lower than QE, respectively.

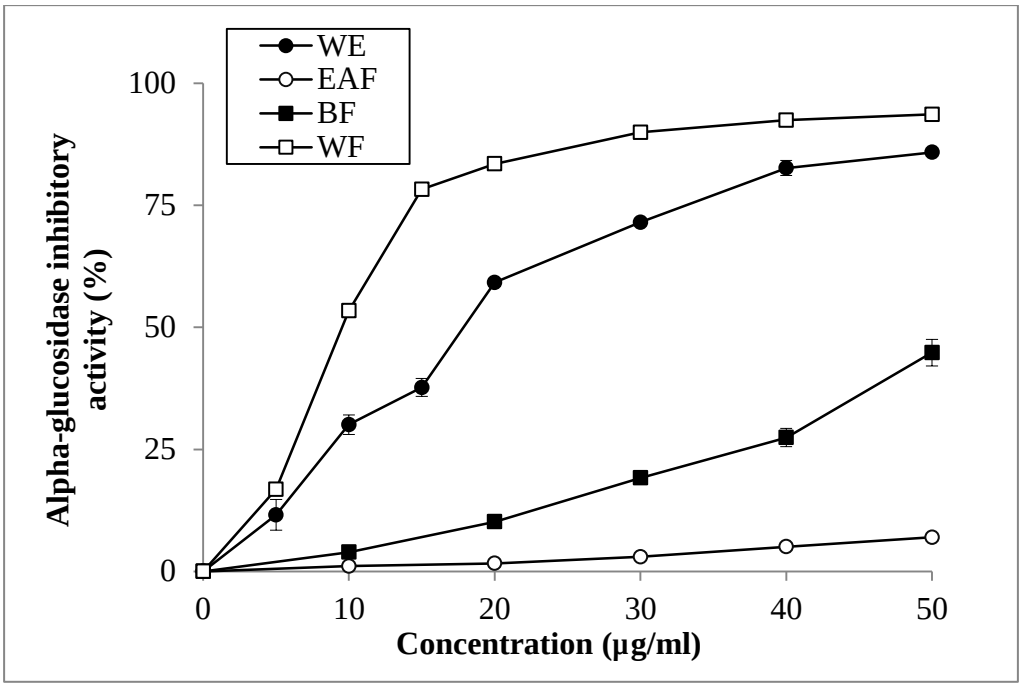

Figure 4.9a: α-Glucosidase inhibitory activities of WE, EAF, BF and WF. Data are presented as mean ± standard error (n=3).

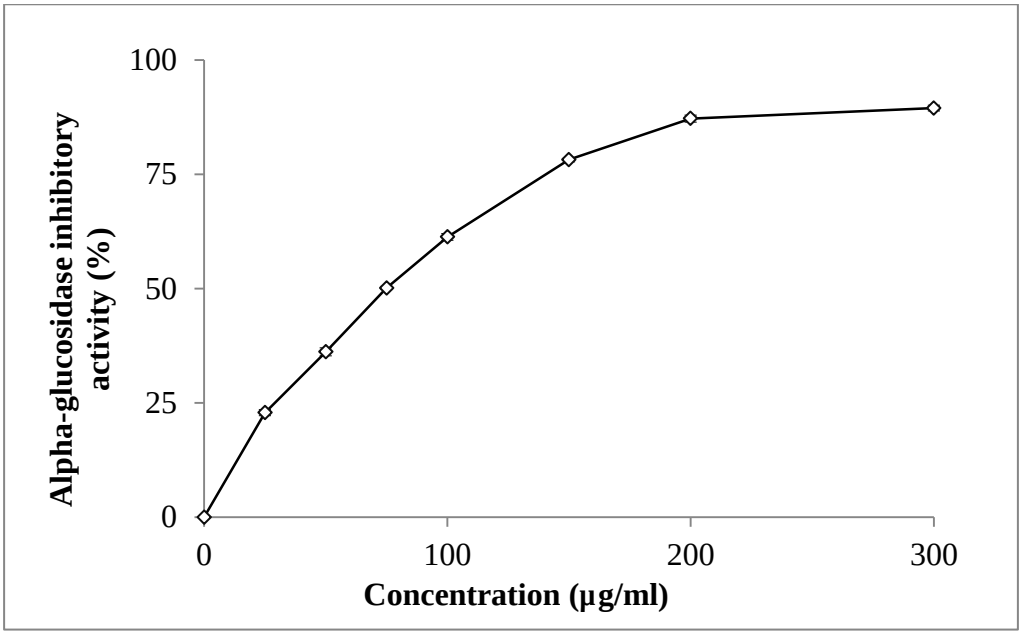

Figure 4.9b: α-Glucosidase inhibitory activity of QE positive control. Data are presented as mean ± standard error (n=3).

Table 4.4: EC_{50} Values of Samples In α-glucosidase Inhibitory Assay

Samples	EC_{50} (µg/ml)
WE	$19.40 \pm 0.08^{a,*}$
EAF	$405.30 \pm 26.58^{b,*}$
BF	66.41 ± 2.65^{c}
WF	$10.83 \pm 0.03^{a,*}$
QE	85.95 ± 0.24

Data was expressed as mean ± SE values (n=3). Values in the same column followed by different superscript letters are significantly different as determined by Fisher's LSD test ($P< 0.05$). * indicates mean values of extract and fractions which are significantly different ($P < 0.05$) from that of the mean value of positive control, as determined by using Student's T test.

4.5.1 Mode of Inhibition of Alpha-glucosidase by WF

Figure 5.0 demonstrates a Lineweaver-burk plot of $\frac{1}{V}$ against $\frac{1}{[S]}$ which illustrates the mode of inhibition of WF at EC_{50} concentration of 10.83 µg/ml as compared to a control of no inhibitor and Table 4.5 illustrates the K_m and V_{max} values in the presence and absence of inhibitor. Based on the Lineweaver-burk plot, the trend exhibited a slight increase in $\frac{1}{V}$ value in the presence of inhibitor whereas for $\frac{1}{[S]}$ value, the value remained almost the same as compared to the control.

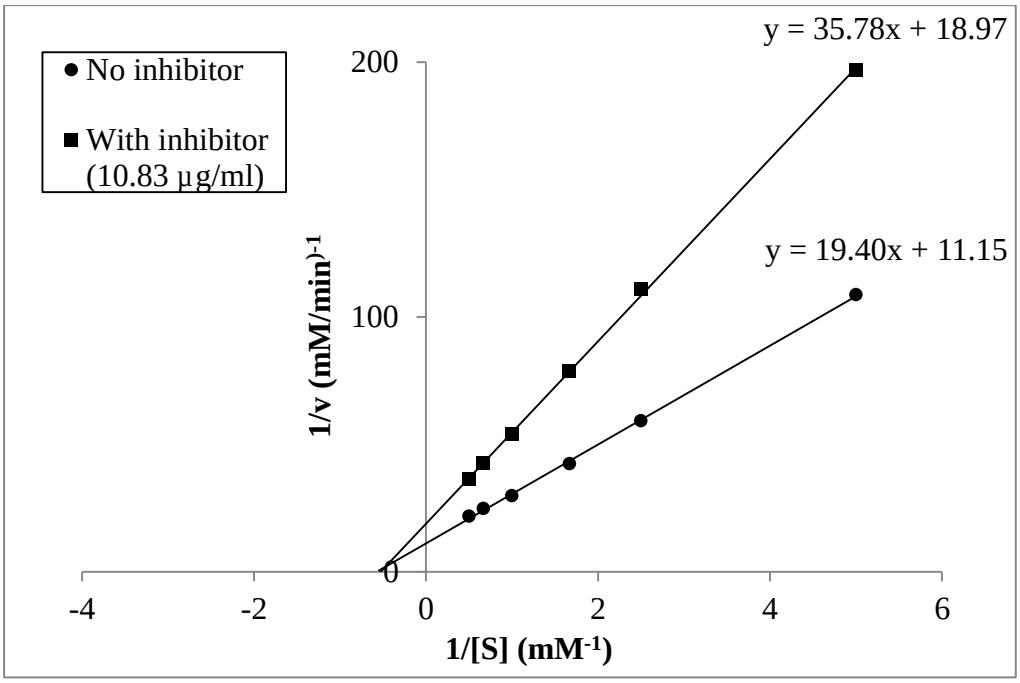

Figure 4.10: Mode of inhibition of WF at EC_{50} concentration and positive control

Table 4.5: K_m and V_{max} values of WF and control

Samples	K_m (mM)	V_{max} (µM/min)
WF (Inhibitor)	1.97 ± 0.25^a	56.40 ± 10.38^a
Control (No inhibitor)	1.75 ± 0.09^a	90.55 ± 6.24^b

Data was expressed as mean ± SE values (n=3). Values in the same column followed by different superscript letters are significantly different as determined by Fisher's LSD test ($P < 0.05$).

4.6 Correlation Analyses

Table 4.6 shows the outcomes of the correlation analyses. TP, TPro, TCou and THC contents were correlated well with the EC_{50} values for DPPH scavenging activity. However, no correlations were found between the phytochemical contents and the EC_{50} values of α-glucosidase and α-amylase inhibitory activity,

Table 4.6: Coefficient of determinations of phytochemical contents and bioactivity

Phytochemical contents	Coefficient of determination (R^2)		
	EC_{50} values for DPPH scavenging activity	**EC_{50} values for α-glucosidase inhibitory activity**	**EC_{50} values for α-amylase inhibitory activity**
TP	0.99	ns	ns
TF	ns	ns	ns
TPro	0.86	ns	ns
TCou	0.91	ns	ns
THC	0.87	ns	ns
TSap	ns	ns	ns

Values presented are statistically significant ($P < 0.05$). ns, not statistically significant.

CHAPTER 5

DISCUSSION

5.1 Yield of Extract and Fractions of *Christella dentata*

Based on the percentage yields calculated for WE and solvent fractions in Section 4.1 (Materials and Methods), WF had the highest percentage yield of 50 % as compared to WE, EAF and BF with only 15.2 %, 10.4 % and 9.6 %, respectively. Higher extraction yield of WF than non-polar fractions was complied with other study as reported by Erol (2009). Also, WF had the highest yield as compared to other solvent fractions which partitioned with petroleum ether, chloroform, acetone and ethanol in other study (Jarald, Joshi, and Jain, 2009). The yields of our WE, EAF, BF and WF were similar as the yields of WE, EAF, BF and WF of *Actinidia arguta* as reported by Lee, et al. (2014). Based on the trends from previous studies, it shows that the percentage yield of extracts and fractions increase along with the increasing of solvent polarity (Erol, 2009; Jarald, Joshi, and Jain, 2009; Lee, et al., 2014). Therefore, WF, which had the highest yield and lower yields of non-polar solvent fractions in our study complied with other studies.

The key factors which correspond to the yields of extract and fraction are the chemical properties of different phytochemicals and the types of solvents being used in the solvent-partitioning process (Tiwari, et al., 2011). This is because the solvent will act on the plant materials and solubilize different compounds of the same polarity (Ncube, Afolayan and Okoh, 2008). The partitioning processes using solvents are based on the chemistry principle "like dissolving like" which states that solutes will tend to dissolve in a solvent which has almost the same polarity. It is due to the differences in polarity of the solvents and compounds leading to the partitioning of polar and non-polar compounds into different solvents (Hussin, et al., 2011). The yield of extract and fractions will also vary depend on the particle size of the compounds, the extraction temperature and the ratio of solvent with the sample extraction (Herodez, et al., 2003).

In this study, three solvents were involved in the extraction and solvent-partitioning processes, namely water, ethyl acetate and *n*-butanol. The polarity of these solvents in descending order is as follows: water > *n*-butanol > ethyl acetate (Reichardt, 2003). This specifies that the less polar phytocompounds would be present in the EAF and BF whereas the more polar compounds would be present in the WF. Partitioning of the crude extract with ethyl acetate was reported to target at the separation of non-polar phytochemicals (Misbah, Aziz, and Aminudin, 2013).

5.2 Phytochemical Analysis

Based on the results, EAF contained the highest TP, TCou, THC and TPro contents followed by BF, WE and lastly WF. TCou, THC and TPro contents exhibited the same trends as in TP contents. This complied with the literature because coumarin, hydroxycinnamic acid and proanthocyanidin are phenolic compounds (Harborne, 1989). The main concern of determining different types of phenolics in our study was to evaluate the association of phenolics with antioxidant, α-amylase and α-glucosidase inhibitory activities. Besides that, same amount of flavonoids was observed in EAF and BF. This is because flavonoids have well known of being able to dissolve well in ethyl acetate and *n*-butanol (Yang, 2007). On the other hand, BF had the highest TSap content as compared to WE, EAF and WF. This is because saponins are polar compounds and can easily be extracted with lower aliphatic alcohols such as ethanol and *n*-butanol (Goel, 2008). However, certain saponins are lipophilic, thus they are more preferable to be extracted with less polar solvent such as ethyl acetate (Xu, Ye and Zhao, 2012).

Higher concentration of phytochemicals was observed in fractions which were partitioned with less polar solvent such as ethyl acetate and *n*-butanol. This is due to the abilities of the less polar solvents in partitioning the mid-polar compounds which are present in the crude *C. dentata* WE such as flavanols and theaflavins than polar solvent (Larger, et al., 1998). For example, *n*-butanol will separate the mid-polar polyphenols and flavonol glycosides whereas water

will separate the highly polar phenolic compounds such as polymerized polyphenols and tannins (Sujatha, 2012). Previous finding has indicated the presence of phenolics, saponins, tannins, carbohydrates and carboxylic acids in the chloroform extract of *C. dentata* (Mithraja, et al., 2012). Our study complied with the previous findings as high concentration of phenolics such as coumarins, hydroxycinnamic acids and proanthocyanidins were detected in the ethyl acetate (relative polarity proximate to chloroform) fraction of *C. dentata*. Also, the TP content of our study exhibited the same trend as reported in other fern study (Lai, Lim and Kim, 2010). Their study has reported the presence of flavonoids, terpenoids and tannins in EAF, BF and WF. Therefore, all these has led to a hypothesis that *C. dentata* contains a higher concentration of mid-polar or less polar phytochemicals such as flavonoids, and saponins (triterpene group) as compared to polar phytochemicals such as tannins. Our result complied with the hypothesis as only low amount of proanthocyanidins (31 mg LE/g), which belong to the tannin group, was presence in EAF as compared to high amount of flavonoids (750 mg QE/g) in EAF.

In this study, different colorimetric assays were performed to determine the phytochemical contents of *C. dentata.* The TP contents of *C. dentata* were evaluated using Folin-Ciocalteu assay (Waterhouse, 2001). Folin-Ciocalteu reagent is a mixture of phosphotungstic acid and phosphomolybdic acid, will react with phenolic compounds and form a blue color solution under alkaline condition. This is due to the reduction of phosphotungstic acid and phosphomolybdic acid complexes to blue oxides of tungsten and molybdene

(Peterson, 1979). The formation of blue color solution allows the quantification of phenolic content which presence in the samples by measuring absorbance at 765 nm. However, the main drawback of Folin-Ciocalteu reagent is its non-specificity of detecting all phenolic compounds and extractable proteins in extracts. Also, the presence of reducing substances can interfere with the determinations of this assay (Otles, 2011).

The TF contents of *C. dentata* were evaluated using aluminium chloride colorimetric assay (Zou, Lu and Wei, 2004). In this assay, aluminium chloride will react with flavonoids and form an acid stable complexes with the keto group (C=O) or hydroxyl group (O-H) of flavonoids leading to the formation of orange or brown solution due to spectrum shifting from the UV region to the visible light region upon the formation of complexes (Rupasinghe, 2012).

The TPro contents of *C. dentata* were determined through acid butanol assay. Acid-butanol and iron reagent are required to add into the reaction mixture to induce the hydrolysis of proanthocyanidins to form anthocyanidins (Porter, et al., 1986). The addition of ferric ammonium sulphate is to supply Fe^{2+} ions and Fe^{3+} ions, which are responsible in enhancing the reproducibility and yield of the conversion process (Otles, 2011). The formation of anthocyanidins will lead to the development of red color in the sample. The intensity of the red color indicates the amount of anthocyanidins being produced.

The TCou contents of *C. dentata* were evaluated based on the methods described by De Amorim, et al. (2012). This assay involves the ionization of the phenolic hydroxyls of coumarins leading to the bathochromic shift of the absorption of coumarin at 280 nm to 320 nm. Spectrophotometric quantification of coumarins can be done at 320 nm.

The THC contents of *C. dentata* were evaluated based on the assay as described by Matkowski, et al. (2008). The formation of complex due to the reaction of Arnow's reagent with the ortho-diphenols of hydroxycinnamic acids allows the spectrophotometric quantification of hydroxycinnamic acids at 490 nm (Vladimir-Knezevic, et al., 2012).

The TSap contents of *C. dentata* were determined through vanillin-sulphuric acid assay (Hiai, et al., 1976). Vanillin-sulphuric acid reagent is required in the derivatization of saponins at the C-3 hydroxyl (O-H) group to yield chromogens which can be quantified spectrophotometrically at 538 nm. (Makkar, Siddhuraju and Becker, 2007). In the presence of saponins, greenish-brown solution will be formed. The intensity of greenish-brown solution will correspond to the amount of saponins which presence whereby higher intensity will indicate higher amount of saponins.

5.3 DPPH Radical Scavenging Activity

Scavenging free radicals is the basis and assessment of an antioxidant assay. The free radical used in this study is DPPH. DPPH is a stable free radical as a result of the delocalization of electrons all over the molecule (Molyneux, 2004). The delocalization of electrons in DPPH results in a deep violet color and upon the reduction of DPPH by any hydrogen donating compounds, the violet color of DPPH will fade and lead to the formation of pale yellow hydrazine. This is due to the shifting of wavelength in the visible spectra from 517 nm to 330 nm (Ionita, 2003). Hence, the level of antioxidants which correspond to the reduction of DPPH in the samples can be quantified by measuring absorbance at 517 nm. Figure 5.1 shows the reduction process of DPPH by hydrogen donating compound.

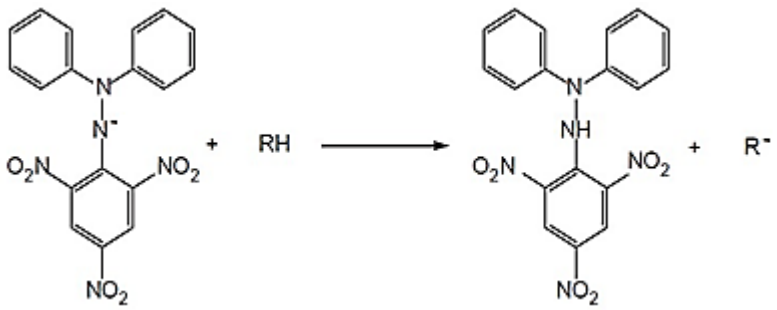

Figure 5.1: Reduction process of DPPH free radical (Dureja and Dhiman, 2012)

The antioxidant activity was expressed as EC_{50}. Lower EC_{50} values will signify lower concentration of *C. dentata* extract or solvent fractions which are required to scavenge 50 % of the free radicals. In this study, high DPPH scavenging activities were shown in EAF (EC_{50} of 10.75 ± 0.10 µg/ml) and BF

(15.05 ± 0.12 µg/ml). Based on the results, EAF has the highest TP, Cou, THC, and TPro contents followed by BF. Correlation analyses suggested that the antioxidant activity may be attributed to TP, TPro, TCou, THC contents of *C. dentata.*

The antioxidant properties of *C. dentata* have not been determined in other studies. However, the antioxidant properties of phytochemicals such as phenolic, coumarin, hydroxycinnamic acid and proanthocyanidin have been mentioned in other studies (Kostova, et al., 2011; Pietta, 2000; Wu, 2007). This may imply that high TP, TCou, THC and TPro contents may serve as an indicator of high antioxidant activity in plants. A previous study on *Polypodium leucotomos* fern has revealed a prominent antioxidant property due to the presence of high TP content, whereby the antioxidant property increases in a concentration-dependent manner (Gombau, et al., 2006). Besides that, the WE, EAF and BF of *Blechnum orientale*, a tropical fern which diversifies mainly in the southern hemisphere, showed strong antioxidant property (Lai, Lim and Kim, 2010). Our study on the antioxidant property of the WE, EAF and BF of *C. dentata* (EC_{50} of 10.75-16.67 µg/ml) demonstrated a comparable range of result with the antioxidant property of the WE, EAF and BF of *B. orientale* (EC_{50} of 8.7-13.0 µg/ml). This complied with our previous hypothesis regarding the attribution of TP content to the antioxidant property of fern.

5.4 Porcine Pancreatic Alpha-amylase Inhibitory Activity

The PPA is closely resembles the human α-amylase (Sudha, Zinjarde, Bhargava and Kumar, 2011). Hence, PPA was used to evaluate the inhibitory activity of *C. dentata* extracts and solvent fractions. In this assay, starch azure (covalently linked with Remazol Brilliant Blue R) was used as the substrate for PPA. Under specific conditions (pH 6.9, 37 °C and dark condition), PPA will break down the insoluble dye-coupled starch azure into soluble color product (Zemek, Kuniak and Matusova., 1985). This allows the determination of PPA activity spectrophotometrically at 595 nm.

PPA inhibitory activity was expressed as EC_{50}. Lower EC_{50} values will signify lower concentration of *C. dentata* extract or solvent fractions which are required to achieve 50 % of the PPA inhibitory activity. Based on the results, WE, BF and WF were not significantly different in the inhibitory activities but they had greater inhibitory activities than EAF. Correlation analyses suggested that PPA inhibitory activity was not attributed to TP, TF, TPro, TCou, THA and TSap contents of *C. dentata*. WF (EC_{50} of 12.83 ± 0.10 mg/ml) which had greater inhibitory activity out of WE, EAF and BF accounted for 60-fold lower activity as compared to acarbose (EC_{50} of 0.22 mg/ml). The inhibition of α-amylase by WF was considered as ineffective due to the vast different in EC_{50} as compared to acarbose. Previous study on other fern, namely *Pteris vitata,* has reported that the PPA inhibitory activity is due to phenolic compounds (Paul and Banerjee, 2013). Similarly, phytochemical analysis of other studies

on other medicinal plants revealed that alkaloids, tannins, cardiac glycosides, flavonoids, saponins and steroids are responsible for the PPA inhibitory activity (Sudha, Zinjarde, Bhargava and Kumar, 2011). Hence, this suggested that only low amount of PPA inhibitors are present in *C. dentata* despite the high TP content in EAF, which accounted for the lowest PPA inhibitory activity among the extract and fractions in our study.

5.5 Alpha-Glucosidase Inhibitory Activity and Mode of Inhibition

5.5.1 Alpha-glucosidase Inhibition Assay

In α-glucosidase inhibitory assay, *p*NPG was supplied to the reaction mixture as the substrate for α-glucosidase (Gurudeeban, Satyavani and Ramanathan, 2012). Under specific conditions (pH 7.0 and 37 °C), α-glucosidase will catalyze the conversion of *p*NPG to D-glucose and *p*-nitrophenol. As a result, yellow solution will form due to the presence of *p*-nitrophenol (Wilson and Walker, 2000). This allows the determination of α-glucosidase activity spectrophotometrically at 400 nm. The higher the intensity of the yellow product will correspond to greater α-glucosidase activities. Figure 5.2 shows the conversion process of *p*NPG to D-glucose and *p*-nitrophenol by α-glucosidase.

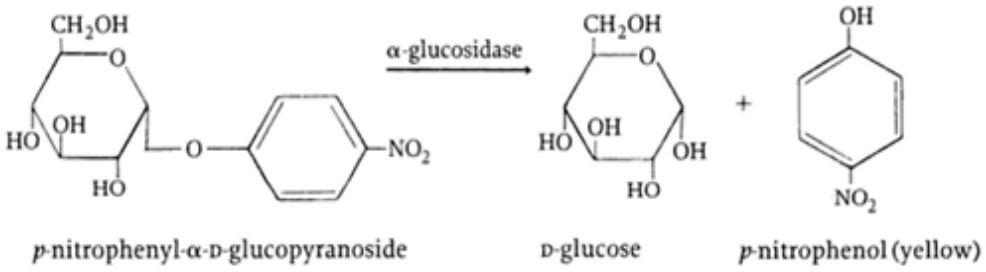

Figure 5.2: The conversion process of *p*NPG to D-glucose and *p*-nitrophenol by α-glucosidase (Wilson and Walker, 2000)

Based on the results, α-glucosidase inhibitory activity was expressed as EC_{50}. Lower EC_{50} values will signify lower concentration of *C. dentata* extract or solvent fractions which are required to achieve 50 % of α-glucosidase inhibitory activity. Both WE and WF of *C. dentata* exhibited a good inhibitory activity against α-glucosidase with low EC_{50} as compared to EAF and BF. The α-glucosidase inhibitory activities of WE and WF were approximately 4.5-fold and 8-fold higher than quercetin, respectively, which has been reported as a powerful α-glucosidase inhibitor previously (Li, et al., 2009). Statistical analysis showed that the inhibitory effects of WE and WF were not significantly different. In addition, correlation analyses suggested that the α-glucosidase inhibitory activity cannot be attributed to TP, TF, TPro, TCou, THC and TSap contents of *C. dentata.*

As no studies on the α-glucosidase inhibitory activity from the genus *Christella* have been carried out, our study was compared with other studies on other ferns. A study on ferns has reported that few species of ferns, namely *Dicranopteris curranii*, *Gleichenia truncata* and *Phymatopteris triloba*

exhibited great α-glucosidase inhibitory activity based on their crude water extracts (Chai, et al., 2013). Similarly, WE of *Actinidia arguta* has been reported of having good inhibitory activity against α-glucosidase (Lee, et al., 2014). Correlation analyses on other study also revealed that the α-glucosidase inhibitory activity is not directly attributed to TP and TF contents (Farsi, et al., 2011). Hypothetically, the polar phytocompounds from *C. dentata* may possess anti-diabetic properties due to promising α-glucosidase inhibitory activity.

5.5.2 Determination of Mode of Inhibition of Alpha-Glucosidase by WF

The study on the enzymatic mode of inhibition was initiated on WF which had the highest α-glucosidase inhibitory activity. The mode of inhibition of α-glucosidase by WF was evaluated through Lineweaver-Burk plot. Lineweaver-Burk plot is a commonly used method for linearizing enzyme kinetic data and determining vital terms such as K_m and V_{max} in enzyme kinetics. K_m represents the concentration of substrate at which the reaction rate is at half maximum or affinity of enzyme towards its substrates whereas V_{max} represents the maximum rate of the enzymatic reaction. By comparing the Lineweaver-Burk equation of $\frac{1}{V} = \frac{K_m}{V_{max}}\left(\frac{1}{S}\right) + \frac{1}{V_{max}}$ with the general mathematical linear equation Y = mX + C, the m represent the gradient of the slope and C is the y-intercept. The graph is of $\frac{1}{V}$ against $\frac{1}{[S]}$, thus the y-intercept is actually representing $\frac{1}{V_{max}}$ and the x-intercept is representing $-\frac{1}{K_m}$. The gradient is the division of K_m with V_{max} (Price and Stevens, 1999).

Based on the Lineweaver-Burk plot, the reaction velocity of the enzymatic reaction was reduced in the presence of inhibitor (WF). The α-glucosidase inhibitory activity by WF demonstrated a non-competitive pattern of inhibition, whereby no significant differences of K_m but significant differences of V_{max} were suggested based on statistical analyses.

Non-competitive inhibition will interact with both enzyme and enzyme-substrate complex without competing with substrate for free enzyme. This is because non-competitive inhibitors will bind to the allosteric site of enzyme rather than the active site. This will result in the alteration of V_{max} value whereby K_m will remain unaffected (Krogsgaard-Larsen, Liljefors and Madsen, 2002). Increasing the concentration of substrate does not serve to overcome the effect of inhibition on the velocity (Price and Stevens, 1999). This suggested that the WF of *C. dentata* contains phytochemicals which correspond to anti-diabetic property based on the ability of inhibiting α-glucosidase through non-competitive binding. The unidentified α-glucosidase inhibitor may be substrates such as carbohydrates or peptides which can competitively inhibit α-glucosidase as previously reported (Azevdeo, et al., 2008; Yuan, Gu and Tang, 2008; Li, Gao, Peng and Feng, 2009; Kim, et al., 2010). However, such constituents possibly were not the normal substrates for α-glucosidase, hence not binding to the active site of the enzyme or else the enzymatic inhibition mode will exhibit a competitive pattern. Anyhow, the identity of the α-glucosidase inhibitor was not determined in this study.

5.6 Further Studies and Suggestions

As EAF showed promising antioxidant property and WF showed outstanding α-glucosidase inhibitory activity, it is vital to understand the compounds which present and responsible for the activities. Purification and identification of compounds from EAF and WF are highly encouraged for further evaluation. Also, the *in vitro* α-glucosidase inhibitory activities may not correspond to *in vivo* α-glucosidase inhibitory activities (Subramanian, Asmawi and Sadikun, 2008). Hence, further *in vivo* validations are required on animal models to identify more information about the role of α-glucosidase inhibitor from *C. dentata* towards the management of DM both *in vivo* and *in vitro*.

CHAPTER 6

CONCLUSION

Various biochemical assays were used to evaluate the phytochemical contents, antioxidant property, α-amylase and α-glucosidase inhibitory activities of *C. dentata*. Overall, EAF contained the highest TP, TCou, THC and TPro contents whereas BF contained the highest TSap content and the same amount of TF content as in EAF. Besides that, WF had showed low value for all the phytochemical contents. EAF exhibited the greatest antioxidant activity (EC_{50} of 10.75 ± 0.10 µg/ml) whereas WF exhibited the greatest α-amylase inhibitory activity (EC_{50} of 12.83 ± 0.10 mg/ml) and α-glucosidase inhibitory activity (EC_{50} of 10.83 ± 0.03 µg/ml). The inhibition of α-amylase by WF was considered as ineffective when compared with acarbose (EC_{50} of 0.22 mg/ml). The inhibitory activity of α-glucosidase by WF showed a non-competitive mode of inhibition with K_m of 1.97 ± 0.25 mM and V_{max} of 56.40 ± 10.38 µM/min. Further purification of EAF and WF of *C. dentata* can be carried out in future for further evaluation on the bioactivities. Testing on animal models can be done to evaluate the *in vivo* antioxidant property and the role of α-glucosidase inhibitor from *C. dentata* toward the management of DM *in vivo*.

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