Antioxidative properties of *Diplazium esculentum* extract by using pressurized hot water extractor: optimization study

by

Nur Azirah Binti Jamial

(0731110218)

A thesis submitted in fulfilment of the requirements for the degree of Master of Science (Bioprocess Engineering)

School of Bioprocess Engineering

UNIVERSITI MALAYSIA PERLIS

2010

UNIVERSITI MALAYSIA PERLIS

DECLARATION OF THESIS

Author's full name : ……………………………………………………………………………

Date of birth : ……………………………………

Title : ……………………………………………………………………………

……………………………………………………………………………

……………………………………………………………………………

Academic Session : ……………………………………

I hereby declare that the thesis becomes the property of Universiti Malaysia Perlis (UniMAP) and to be placed at the library of UniMAP. This thesis is classified as :

☐ **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*

☐ **RESTRICTED** (Contains restricted information as specified by the organization where research was done)*

☐ **OPEN ACCESS** I agree that my thesis is to be made immediately available as hard copy or on-line open access (full text)

I, the author, give permission to the UniMAP to reproduce this thesis in whole or in part for the purpose of research or academic exchange only (except during a period of _____ years, if so requested above).

Certified by:

____________________	____________________
SIGNATURE	**SIGNATURE OF SUPERVISOR**
____________________	____________________
(NEW IC NO. / PASSPORT NO.)	**NAME OF SUPERVISOR**
Date :________________	Date : ________________

NOTES : * If the thesis is CONFIDENTIAL or RESTRICTED, please attach with the letter from the organization with period and reasons for confidentially or restriction.

ACKNOWLEDGEMENT

Alhamdulillah, thanks to Allah S.W.T., with His willing gave me the opportunity to complete my research in fulfilment of Master of Science requirements. I would like to thanks to my supervisor, the Dean of School of Bioprocess, Assoc. Prof. Dr. Mohamed Zulkali Mohamed Daud for the guidance and ideas throughout the research. To my co-supervisor, Ms. Khairul Farihan Kasim; a sincere gratitude's for the quality time, guidance, advice and knowledge. Plus, the financial support for the opportunity to worked as research officer under the ScienceFund grant from Ministry of Science, Technology and Innovation.

I would like to acknowledge and thanks to the School of Bioprocess Engineering, Universiti Malaysia Perlis for the utilization of the facilities. Thanks to Pn. Hafiza Shukor and all staffs for your knowledge, help and advice during the research and class sessions. Special gratitude to my family for encourages and supports me through all the years. Last but not least, to all my friends; thanks for the great moments being with you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DPPH	2,2-diphenyl-1-picrylhydrazyl
TPC	Total phenolic contents
TFC	Total flavonoids contents
DOE	Design of experiments
PHWE	Pressurized hot water extraction
PHW	Pressurized hot water
BAW	Butanol-acetic acid-water
PC	Paper chromatography
HCL	Hydrochloric acid
$AlCl_3$	Aluminium trichloride
SEM	Standard error measurement

Ciri-Ciri Antioksidatif Daripada Ektrak *Diplazium esculentum* Menggunakan Pengkestrakan Air Panas Bertekanan: Kajian Pengoptimuman

ABSTRAK

Pengekstrakan air panas bertekanan (PHWE) telah dijalankan untuk mengurangkan penggunaan pelarut organik bertoksik untuk mengekstrak sebatian bioaktif dari *Diplazium esculentum*. Perbandingan antara kaedah-kaedah pengekstrakan telah dilakukan dan keputusan menunjukkan PHWE mempunyai keupayaan penyingkiran radikal bebas, 2,2-diphenyl-1-picrylhydrazyl (DPPH) (70%) dan jumlah kandungan fenolik (118.83µg catechin setara /mg ekstrak kering) yang tinggi berbanding dengan pengautoklafan, pendidihan, perendaman dan sonikasi dan pengekstrakan Soxhlet. Pengekstrakan perendaman mempunyai jumlah kandungan flavanoid keseluruhan tertinggi (28.03µg rutin setara /mg ekstrak kering). Untuk mendapatkan ekstrak PHWE dengan aktiviti antioksida yang terbaik, kaedah permukaan gerak balas dengan reka bentuk Box-Behnken telah dilakukan. Keputusan menunjukkan keadaan optimum yang diperlukan ialah pada suhu 175^{o}C, 21 minit masa pengekstrakan dengan 50mL air yang ditambahkan kepada 2g sampel *D. esculentum* yang telah dikeringkan. Keupayaan antiradikal DPPH untuk ekstrak *D. esculentum* menunjukkan nilai EC_{50} ialah 1241.14µg /mL dan masa untuk mencapai kestabilan (T_{EC50}) ialah 79.83 minit. Maka, ekstrak dikategorikan sebagai lambat dalam penyingkiran radikal bebas DPPH. Flavonoid yang terkandung di dalam ekstrak telah dikenalpasti melalui HPLC iaitu quercetin dan myricetin.

Antixoxidative Properties of *Diplazium esculentum* Extract By Using Pressurized Hot Water Extractor: Optimization Study

ABSTRACT

Pressurized hot water extraction (PHWE) was implemented in attempt to reduce the use of toxic organic solvent in extracting bioactive compounds from *Diplazium esculentum*. Comparison extraction methods were done and the results shows that the PHWE had higher 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity (70%) and total phenolic contents (118.83µg catechin equivalent/mg dry samples) compared to autoclaving, boiling, soaking, sonicating and Soxhlet extraction. Soaking extraction had the highest total flavonoids contents (28.03µg rutin equivalent/mg dry extract). By applying Box-Behnken design of response surface methodology, the optimized condition for the best antioxidant activity of PHWE was at 175°C, 21 minutes extraction time and 50mL water volume added to 2g of dried ground *D. esculentum*. The DPPH antiradical efficiency was analyzed where the *D. esculentum* extracts had EC_{50} value of 1241.14µg/mL and time to reach steady state (T_{EC50}) was 79.83 minutes. Thus, the extract was categorized as slow in scavenging the DPPH free radicals. Through HPLC, the identified flavonoids in the crude extracts were quercetin and myricetin.

CHAPTER 1

INTRODUCTION

1.2 Overview

Herbal medicines are defined as any plant materials or extracts which are used to treat illness or to improve man's health. All cultures through out the world posses local plants as alternatives medicines (White & Foster, 2000). There are more than 2000 plants in Malaysia highly rich in medicinal value such as kacip Fatimah (*Labisia pumilia*), pegaga (*Centella asiatica*), misai kucing (*Orthosiphon stamineus*) and mas cotek (*Ficus deltoidea*) (Bernama, 2009).

The reasons of herbals usage among locals are the availability and lower cost which affordable by the lower income group, plus the impression of natural source is safe compared to chemically synthesize modern medicines. Even though the active compounds extracted from plants have contributed more than 60-70% in the modern medicines developments; further research should be carried out to study the effectiveness, the toxicity and side effects as remedy (Abas-Husin, 2001).

Diplazium esculentum is among thousands of Pteridophytes species where their fronds are eaten and believes to have antimalarial effects, treat jaundice, constipation, earache, to treat fever, measles and dermatitis (Baas *et al.,* 1990; Kala, 2005; Roosita *et al.*, 2008). Local community in Malaysia collects the edible ferns to sell in the market or cook at home. They normally grow at partially shaded riverbanks, wet fields and can also be found on drier sites (Noweg *et al.*, 2003).

Extraction of plant materials for the preparation of alternative medicines previously were protracted, low selectivity and low extraction yield, and also require huge amounts of toxic organic solvents. Newer extraction process has overcome these drawbacks such as supercritical fluid extraction and subcritical water extraction (Herrero *et al.*, 2006). Alternative green solvent in replacing organic solvents such as carbon dioxide and water, commonly used in extracting natural compounds are highly favourable nowadays. Water with non-toxicity and non-flammable characteristics make it easy to handle the plant extracts in terms of processing, analyzing and production. Therefore, water has been chosen as solvent in the present research (Carlos & Crespo, 2005; Snoeyink & Jenkins, 1980).

1.2 Problem statements

1.2.1 Local communities in poor countries use *D. esculentum* to treat leprosy, a skin disease caused by *Mycobacterium leprae* (Sarker & Hossain, 2009). Previous researched shows toxicity symptom of feeding the plants on animals and no antimicrobial activity of the plant extracts (Somvanshi *et al*., 2006; Kankamol *et al*., 2006; Nanasombat & Teckchuen, 2009). Does the antioxidant activity of the extracts aid in treating the skin disease?

1.2.2 The organic solvents are toxic, expensive and not environment friendly in terms of extraction and waste disposal (Herrero *et.al*. 2006). So, what are the alternative solvents?

1.3 Scope of research

The research focuses on the extraction of *D. esculentum* active compounds which contribute in DPPH antiradical activity. Various water extraction methods and Soxhlet extraction were made for comparison. Pressurized hot water extractions were carried out in a range of parameters and optimization was made. All extracts were subjected to antioxidant analysis which comprise of DPPH scavenging activity, total phenolic contents and total flavonoids contents. The optimized extract was tested on DPPH antiradical efficiency and the bioactive compounds in the crude extract were purified and identified.

1.4 Research objectives

The objectives are itemized below:

i. To determine the best antioxidant activity of *D. esculentum* from various extraction methods.
ii. To screen the antioxidant activity of pressurized hot water extraction from *D. esculentum* at various condition.
iii. To optimized the condition of pressurized hot water extraction from *D. esculentum*.
iv. To study the antiradical efficiency of optimized *D. esculentum* extract.
v. To identify the flavonoids group in the optimized *D. esculentum* extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 *Diplazium esculentum* (Retz.) Swartz

2.1.1 Introduction

D. esculentum (Retz.) Swartz (Fig. 2.1) also known as *Athyrium esculentum* (Retz.) Copel is in the family of Athyriaceae. This vegetable ferns (known as *pucuk paku* in Malaysia) grows wildly at moist or wet areas such as partially shaded river banks or homestead ground. The diploid plant occurs throughout Asia and Oceania (Indonesia, Philiphines, Thailand, Papua New Guinea, Bangladesh, Japan and Fiji) (Noweg *et al.,* 2003; Sarker & Hossain, 2009; Takamiya *et al.*, 1999).

Figure 2.1: Photo of *D. esculentum*

D. esculentum usually grows in a large bunch with upright stem and can grow up to one meter high. The feather-like fronds are curled and can be divided to two or three times. The leaf are saw-like shape on the edge and sharp at the tip which is about eight centimeter long and one centimeter wide. As other pteridophytes species, this forest fern grown from spores at high humidity in about 21^{o} C (French, 1986). *D. esculentum* phylogeny tree is as follow (USDA, Plant Database):

Kingdom	Plantae-Plants
Subkingdom	Tracheobionta –Vascular plants
Division	Pteridophyta – Ferns
Class	Filicopsida
Order	Polypodiales
Family	Dryopteridaceae –Wood fern family
Genus	Diplazium Sw. – twinsorus fern
Species	*Diplazium esculentum* (Retz.) Sw. – vegetable fern

2.1.2 The usage of *D. esculentum*

Local communities collect the young shoots of this edible ferns through all the year for home consumption or to be sold in the market. The young fronds were fried with garlic and anchovies among people who live at Crocker Range, Sabah though cooked as *masak lemak* in Peninsular Malaysia (Noweg *et al.*, 2003). In Bangladesh, the young fronds are cooked with shrimps, fishes or as curry ingredients. It is also consumed as a laxative to treat constipation or abdominal diseases. Another usage is as remedy for skin diseases such as leprosy, a contagious bacteria disease (Sarker & Hossain, 2009).

However, dried fronds is more favoured to be used as animal bedding during winters by the villagers at Nanda Devi Biosphere Reserve zone in India (Misra *et al.*, 2008). The rhizomes parts are placed in the grain stores to avoid from insects and pests and the fronds as in most countries are eaten as vegetables by the people of Himalaya, India (Upreti *et al.,* 2009).

2.1.3 Researched study on *D. esculentum*

There is no research on severely toxic compound extracted from *D. esculentum* published to this date. The concentrations of ptaquiloside (carcinogenic glycoside) were investigated in India based on the high and low-altitude area. Most samples showed no ptaquiloside in *D. esculentum* with an exception for a sample from high-altitude area of Harsil-Ganotri which contained 19 mg/kg vegetables (Somvanshi *et al*., 2006).

Ironically, feeding *D. esculentum* causes toxicity symptoms to rabbits and died after 11 days of feeding. As mentioned by Seth *et al*., (2000) "The effects were reduced body and organ weights, alternations in haematological parameters with degenerative changes and haemorrhages in brain and visceral organs and disrupted myocardial fibres in heart". On the other hands, feeding frozen *D. esculentum* on rats caused mild pathologic effects but encouraged fatality with moderate type of clinicopathological effects in guinea pigs (Gangwar & Somvanshi, 2004).

The moisture contents of *D. esculentum* is high up to 90-93%. In 100g of edible portion of *D. esculentum* contained 88kJ energy, 3.6g of protein, 4.4mg of iron and 25mg of Vitamin C (French, 1986). According to Irawan *et al*., (2006), *D. esculentum* contained small amount of fat, fiber, folic acid (6.3ppm) and vitamin C (21.72 mg/100g) but high in β-carotene (74.04ppm), of fresh vegetables.

Supported by the research on wild vegetables in Vietnam, *D. esculentum* extracts contained only 27µg folate/100g of sample which is low since green leafy vegetables are believed to contain high folate contents. Vegetables are natural sources of nutrients to the villagers in poor country such as Vietnam and lacking of folate concentration in human dietary could increase the risk of cardiovascular and cancer diseases (Ogle *et al.*, 2001). Study in Southern Thailand has found out that *D. esculentum* contains heat-stable and heat-labile antithiamin. There is only trace amount of thiamine contained in fresh *D. esculentum* (Taungbodhitham, 1995).

The *D. esculentum* extracts have no antimicrobial activities where there are no growth inhibition of several bacterial strains tested (Kankamol *et al.*, 2006; Nanasombat & Teckchuen, 2009). Despite the negative findings on *D. esculentum*, it is still been use as remedy to treat diseases (Sarker & Hossain, 2009) by traditional medicine practitioners. Thus, it would be advantageous to find out on how does the plant treat the diseases.

2.1.4 Ferns as medicines

There are thousands of Pteridophytes or ferns species throughout the world. Some are used as traditional remedies to treat various diseases. *Lygodium japonicum* (Thun b.), one of valued traditional Chinese medicines in treating various illness has strong antioxidant activities against liposome peroxidation, DPPH free radicals and hydrogen peroxide scavenging (Li *et al.,* 2006). Four *Adiantum* ferns species showed potent antimicrobial activities against *Escherichia coli, Trichophyton rubrum and Aspergillus terreus* has support the use of these ferns in traditional treatments among locals in India (Singh *et al.,* 2008).

2.2 Evolution of extraction methods

Extraction is a process of removing components from one substance into another using solvent or gas (Mason, 1999). *D. esculentum* were freshly used, boiled with water or soaked with organic solvents (hexane, acetone and alcohols) prior to analyses (Irawan *et al.,* 2006; Taungbodhitham, 1995; Rahmat *et al.,* 2003; Somvanshi *et al.,* 2006; Kankamol *et al.,* 2006; Nanasombat & Teckchuen, 2009). There is no recent extraction method has been applied on this plant up to this date.

Herbal medicinal practitioners mostly used fast and immediate extract preparation. Infusion is where the plant materials are boiled with water. Heating caused the plant cell wall to expand allowing plants constituents to diffuse out of the membrane cell. Maceration is a method of extraction which softly breaks up the plant material by soaking in a liquid such as water for a long time (weeks) with agitation at room temperature (Mason, 1999; Merken & Beecher, 2000; Raaman, 2006).

Conventional Soxhlet extraction is a standard and well-established extraction technique that has been used since decades ago as a comparison to the later extraction methods. The basic principle is on distillation process where the heated solvent in the solvent's flask flow upward to the condenser (Fig. 2.2). The condensed solvent is filled in the Soxhlet extractor until it reaches maximum level and unloads back into the solvent flask carrying the extracts. The solvent will redistill while the solutes will remain in the solvent flask. It is cheap and simple extraction methods but require long extraction time plus high amount of solvent (Wang & Weller, 2006).

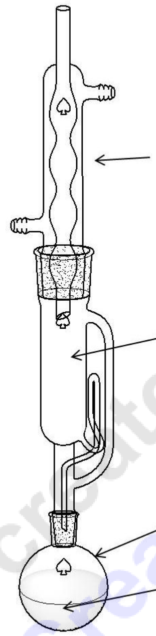

Figure 2.2: Soxhlet extraction apparatus

Ultrasonication-assisted extraction has been applied in solvent extraction for better yield of extracts. The principle lies is on the soundwave that induce cavitational forming bubbles in water bath. When the bubbles collapse near the cell walls, the solvent is driven into the walls dissolving the compounds and transport them outside (Mason, 1999).

Recent extraction method is supercritical fluid extraction (SFE) implementing solvent at high temperature and pressure up to critical points. Carbon dioxide is enormously utilized due to low toxicity, low critical temperature (31^{o}C) and pressure (72bar). Simpler principle than SFE is the pressurized liquid extraction (PLE), implementing organic solvents (e.g. ethanol) at the range of boiling and critical temperature. Similar concept to that of PLE, pressurized hot water extraction (PHWE) is a green alternative method due to feasibility used of water as solvent (Turner, 2006).

2.3 Pressurized hot water extraction (PHWE)

2.3.1 Introduction

Pressurized hot water (PHW) extraction also known as subcritical water, superheated water extraction or pressurized low polarity water refers to the water at the temperature between the boiling and critical temperature which is 100^{o}C and 374^{o}C. The implementation of water as solvent brings great interest of researchers in the use of PHW extraction in several fields of applications (Smith, 2002).

PHW extractions have been applied on soils and environmental solid waste treatment. For example the extraction of polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), benzophurans and pesticides residue for the treatment of industrial soils and municipal waste. Recently, there has been a vast of interest in application of PHWE on extraction of natural compounds from plant matrices. A few reviews had been done on application of PHWE (Herrero *et al.,* 2006; Pronyk & Mazza, 2009; Smith, 2002).

2.3.2 Water as green solvent

Water is odourless, almost tasteless, non-toxic and non-flammable makes it as environmental friendly extraction solvent (Snoeyink & Jenkins, 1980). It is cheap and easily available and therefore could reduce the costs for the production of commercial natural compounds and also the cost for the organic solvents' disposal and waste treatment (Carlos & Crespo, 2005). Due to these benefits, water is an alternative green