EXTRACTION, BIOACTIVITIES AND IDENTIFICATION OF WATER-SOLUBLE PHYTOCHEMICALS FROM LOTUS (*Nelumbo nucifera*) RHIZOME OF MALAYSIA AND CHINA CULTIVARS

TAN SZE JACK

UNIVERSITI SAINS MALAYSIA

2015

EXTRACTION, BIOACTIVITIES AND IDENTIFICATION OF WATER-SOLUBLE PHYTOCHEMICALS FROM LOTUS (*Nelumbo nucifera*) RHIZOME OF MALAYSIA AND CHINA CULTIVARS

by

TAN SZE JACK

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science (Bioprocess Technology)

DECEMBER 2015

ACKNOWLEDGEMENTS

The very first of all, I would like to express my deepest gratitude to my supervisor, Dr. Gan Chee Yuen, and co-supervisor, Dr. Lee Chee Keong, who never cease to guide and encourage me throughout the research project. Their generosity in sharing knowledge and benevolence was indeed a great help and encouragement that keeps me motivated. To say them is an encouragement to me would be an understatement. I would also like to show my appreciation for the funding of the project: ERGS (203/CAATS/6730103).

Thanks to the members and staff in Centre for Advanced Analytical Toxicology Services (CAATS), especially Puan Noor Leenda Md Nasir and Puan Eliani, Ahmad Ghazali for their support and provision not only in materials and facilities, but also the moral support during challenging period. My sincere thanks and appreciation is extended to my best labmates, Siow Hwee Leng, Ngoh Ying Yuan, Nurul Hidayatul Afifah binti Saufi Sofian, and Chiang Pui Nyuk, who played an utmost important role in supporting and encouraging me. Thanks for always being there and the companionship offered that keeps me to go on.

Not to forget my family for their unconditional love and support, both financially and mentally, to keep me pursuing my study. Also, the housemates who never fail to provide ideas and advice. I am indeed a blessed individual. Without the love, support, patience, guidance, and encouragement given to me by all of you, the research will be not carried out successfully. Appreciate them from the very bottom of my heart.

Tan Sze Jack

TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3GT	3- glucosyl transferase
4CL	4-coumaroyl CoA-ligase
5GT	5- glucosyl transferase
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
ACE	angiotensin converting enzyme
AGE	advanced glycation endproducts
AMA	Antimycin A
AREs	antioxidant-response elements
AT	acyltransferases
ATP	Adenosine triphosphate
BBD	Box-Behnken design
BHA	butylated hydroxynisole
BHT	butylated hydroxytoluene
C4H	cinnamate-4-hydroxylase
CHI	chalcone isomerase
CHS	chalcone synthase
DFR	dihydroflavonol reductase
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-pieryl-hydrazyl
ESI	electrospray ionization
F3'5'H	flavonoid 3'5'-hydroxylase
F3H	flavanone 3-hydroxylase
FRAP	ferric reducing antioxidant power
FS	flavonol synthase
GAE	gallic acid equivalent
GSH	Glutathione
GSSG	gluthathione disulphide
GT	glycosyltransferases

HCT	hydroxycinnamoyl transferase
HD	Hydro distillation
HIV	Human immunodeficiency virus
HO-1	hemoxygenase-1
LC Q-TOF MS/MS	liquid chromatography quadropole time-of-flight tandem mass spectrometry
m/z	mass-to-charge ratio
MCP-1	monocyte chemoattractant protein-1
MFE	Molecular feature extraction
NQO1	quinoneoxidoredctase
Nrf2	nuclear factor erythroid 2-related factor 2
OMT	*O*-methyl transferase
PAL	phenylalanine ammonia lyase
PCE	pyrocathecol equivalent
PEP	phosphoenolpyruvate
RLAR	rat lens aldose reductase
ROS	Reactive oxygen species
RT	rhamnosyl transferase
SD	steam distillation
SE	Soxhlet extract
SOD	superoxide dismutase
TAL	tyrosine ammonia lyase
TBHQ	tertiary butyl hydroquinone
TFC	total flavonoid content
TNF	tumor necrosis factor
TPC	total phenolic content
UV	ultraviolet

LIST OF SYMBOLS

%	percentage
min	minute
h	hour
w/v	weight over volume
&	and
™	trademark
~	approximately
=	equal
<	less than
>	greater than
±	plus minus
℃	degree celcium
α	alpha
β	beta
μ	micro
®	registered trademark
γ	gamma

PENGEKSTRAKAN, BIOAKTIVITI DAN IDENTIFIKASI FITOKIMIA LARUT AIR DARIPADA AKAR TERATAI (*Nelumbo nucifera*) KULTIVAR MALAYSIA DAN CHINA

ABSTRAK

Akar teratai mempunyai potensi bagi mencegah penyakit kronik seperti penyakit darah tinggi, diabetis mellitus kelas dua atau kanser. Namun demikian, fitokimia yang terdapat dalam akar teratai belum lagi dicirikan dan dikenalpasti, khususnya akar teratai kultivar Malaysia. Pengekstrakan metabolit sekunder semulajadi dari akar teratai, *Nelumbo nucifera* yang diperoleh daripada dua jenis kultivar (China, SP 1 dan Malaysia, SP 2) menggunakan pelbagai jenis pelarut (metanol, etanol, heksana, aseton, etil asetat dan air) dan dengan kaedah pengekstrakan bantuan enzim (sellulase dan hemisellulase, 1% berat/isipadu) telah dijalankan dalam kajian ini. Kesan bagi pH, tempoh pengestrakan, suhu dan nisbah pelarut kepada sampel dengan pengestrakan fitokimia telah dikaji dan dioptimumkan menggunakan rekabentuk Box-Behnken. Fitokimia yang telah diekstrak kemudiannya dicirikan mengikut kandungan dan aktiviti antioksidan, antidiabetik, antihipertensi dan antibakteria. Ekstrak ini seterusnya dikenalpasti dan digabungkan ke dalam minyak ikan untuk mengkaji kestabilan oksidatif semasa penyimpanan. Pengestrakan air dilaporkan memberi aktiviti yang tinggi terhadap 2,2-difenil-pieril-hidrazil (DPPH) dan kuasa antioksidan penurunan ferik (FRAP) (35.2 % $DPPH_{sc}$ dan 2.39 mM $FeSO_4$). Keadaan optimum untuk jumlah kandungan flavonoid (TFC), %$DPPH_{sc}$, dan FRAP bagi SP 1 adalah pada pH 2.5, tempoh pengestrakan 0.5 jam, dan nisbah pelarut kepada sampel sebanyak 40 ml/g, manakala keadaan optimum untuk SP 2 adalah pada pH 2.4, tempoh pengekstrakan 0.5 jam, dan nisbah pelarut kepada sampel sebanyak 40 ml/g. Hasil pengestrakan optimum bagi SP1 dan

SP2 mencapai hasil sebanyak 28.5% dan 25.5%; 0.535 dan 0.755 mg setara asid galik (GAE)/ g jumlah kandungan fenolik; 0.67 dan 0.62 mg setara pirokatecol (PCE)/ g TFC; 42.6 dan 30.4% $DPPH_{sc}$; 84.66 dan 84.96% 2,2'-azino-bis(3-etilbenzotiazolina-6-sulforik asid) $(ABTS)_{sc}$; 1.9 dan 1.9 mM $FeSO_4$, masing-masing. Ekstrak SP 1 dan SP 2 juga mencapai perencatan aktiviti α-amilase sebanyak 99.3 dan 54.55 %; 52.16 dan 14.84 % untuk aktiviti α-glukosidase; 119.42 dan 112.65 % untuk aktiviti perencatan angiotensin pertukaran enzim (ACE) untuk aktiviti antidiabetik dan antihipertensi. SP 2 menunjukkan zon perencatan yang lebih tinggi terhadap pertumbuhan gram bakteria positif (*S. aureus*: 14 mm; *B. subtilis*: 13.2 mm; *B.cereus* 12.7 mm; *B. spizizenii*: 11.8 mm) berbanding dengan SP 1 (*S. aureus*: 9.0 mm; *B. subtilis*: 10.5 mm; *B.cereus* 9.2 mm; *B. spizizenii*: 9.8 mm). Semua bioaktiviti yang diperoleh disebabkan oleh kehadiran fitokimia dalam SP 1 and SP 2, iaitu 6 sebatian (DL-fenilalanina, DL-triptofan, floribundina, apohisina, asid pantotenik dan prosianidin B2) telah dikesan daripada kedua-dua SP1 and SP2. Hanya 5 sebatian (katesin, galokatesin, kolina, C16 sphinganina, dan dihidrokomarin) dikesan dalam SP 1 manakala 3 sebatian baru (anemonin, nizatidin dan juglanin) dijumpai dalam SP 2. Penggabungan campuran minyak ikan dengan ekstrak SP2 menunjukkan hayat penyimpanan yang paling lama dan penggabungan kedua-dua ekstrak dapat melambatkan proses oksidasi. Oleh yang demikian, akar teratai yang mempunyai sifat-sifat antioxidant, antidiabetic, antihipertensi dan antibakteria ini perlu diperbadankan kepada makanan untuk menghasilkan sumber makan alternatif semulajadi yang mempunyai unsur perubatan.

.

EXTRACTION, BIOACTIVITIES AND IDENTIFICATION OF WATER-SOLUBLE PHYTOCHEMICALS FROM LOTUS (*Nelumbo nucifera*) RHIZOME OF MALAYSIA AND CHINA CULTIVARS

ABSTRACT

Lotus root was found to have the potential in preventing chronic diseases, such as hypertension, type II diabetes mellitus, or even cancer. However, the phytochemicals in the roots were have not been characterized and identified, especially the root from the Malaysia cultivar. Thereofore, extraction of secondary metabolites from *Nelumbo nucifera* rhizome of two different cultivars (China, SP 1 and Malaysia, SP 2) using different solvents (i.e methanol, ethanol, hexane, acetone, ethyl acetate, and water) and enzyme-assisted method (cellulase and hemicellulase, 1% w/v) were conducted. Effects of pH, extraction time, temperature, and solvent-to-sample ratio to the extraction of phytochemicals were studied and optimized using Box-Behnken design. Extracted phytochemicals were characterized for their antioxidant content and activities, antidiabetic, antihypertensive, and antibacterial properties. They were then identified and incorporated into fish oil to study its oxidative stability during storage. Water extraction was reported to attribute high 2,2-diphenyl-pieryl-hydrazyl (DPPH) radical scavenging activity and ferric reducing antioxidant power (FRAP) (i.e 35.2%$DPPH_{sc}$ and 2.39 mM $FeSO_4$, respectively). The optimal conditions for total flavonoid content (TFC), %$DPPH_{sc}$, and FRAP of SP 1 were pH 2.5, extraction time of 0.5 h, and solvent-to-sample ratio of 40 ml/g, while the optimal conditions for SP 2 were pH 2.5, extraction time of 0.5 h, and solvent-to-sample ratio of 40 ml/g. Both optimized SP 1 and SP 2 extracts achieved 28.5% and 25.5% extraction yield; 0.535 and 0.755 mg gallic acid equivalent (GAE)/ g total phenolic content; 0.67 and 0.62 mg pyrocathecol equivalent (PCE)/ g TFC;

42.6 and 30.4%$DPPH_{sc}$; 84.66 and 84.96% 2,2'-azino-bis(3-ehtylbenzothiazoline-6-sulphonic acid) $(ABTS)_{sc}$; 1.9 and 1.9 mM $FeSO_4$, respectively. Extracts of SP 1 and SP 2 also achieved 99.3 and 54.55 % α-amylase inhibition; 52.16 and 14.84 % α-glucosidase inhibition; 119.42 and 112.65 % angiotensin converting enzyme (ACE) inhibition, respectively for their anti-diabetic and anti-hypertensive activities. SP 2 showed higher inhibition zone against growth of gram positive bacteria (*S. aureus*: 14 mm; *B. subtilis*: 13.2 mm; *B.cereus* 12.7 mm; *B. spizizenii*: 11.8 mm) compared to SP 1 (*S. aureus*: 9.0 mm; *B. subtilis*: 10.5 mm; *B.cereus* 9.2 mm; *B. spizizenii*: 9.8 mm). These bioactivities were attributed by the presence of phytochemicals in SP 1 and SP 2, where 6 common compounds (DL-phenylalanine, DL-tryptophan, floribundine, apohyoscine, pantothenic acid and procyanidin B2) were identified from both SP 1 and SP 2. 5 compounds (i.e. catechin, gallocatechin, choline, C16 sphinganine, and dihydrocoumarin) only found present in SP 1 and 3 novel compounds (i.e. anemonin, nizatidine and juglanin) were from SP 2. In term of application, fish oil incorporated with SP 2 extracts showed the longest shelf life and incorporation of both rhizome extracts delayed the oxidation process. Therefore, lotus rhizome extracts with the antioxidative, antidiabetic, antihypertensive and antibacterial properties should be incorporated into food product to produce an alternative source of natural medicinal food.

CHAPTER ONE

INTRODUCTION

A report in 2012 showed that 25% of the world population were suffered from diseases due to metabolic syndrome (Prasad et al., 2012). The metabolic syndrome represents a state of chronic inflammation, oxidative stress and insulin resistance. This could be further characterized by the condition of at least three cardiovascular risk factors: obesity, excessive visceral fat storage, dyslipidemia, hypertension, hyperglycemia or type 2 diabetes (Prasad et al., 2012). The most common approach in the metabolic syndrome treatment consists of the inhibition of α-amylase and α-glucosidase, which both of the enzymes are directly involved carbohydrate metabolism. Hence, there would be a reduction of postprandial hyperglycemia. Another therapeutic approach is the inhibition of oxidative stress generated during cell metabolism, where antioxidants would play the role.

Synthetic antioxidants like butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and butylated hydroxynisole (BHA) are usually added into food products to prevent lipid oxidation, enhance shelf-life and prevent growth of bacteria and fungus. However, report from Sun and Fukuhara (1997) discovered that synthetic antioxidants like BHA and BHT are potential carsinogens. This had led to a decrease in the usage of synthetic antioxidants in food industry and attention has been shifted to natural antioxidants, which do not require extensive safety examination before consumption.

However, natural products are usually pricy and less effective than synthetic antioxidants. To overcome this problem, extraction of natural antioxidants from agricultural sources is prioritized. Lotus is an ornamental plant and acts as a dietary

staple in many countries (Korea, Japan, China, and Southeast Asia countries like Malaysia). Lotus leaves are well known of their astringent and cooling actions and they are traditionally used as the cure for mild fever, diarrhea and hemorrboids (Ku-Lee et al., 2005). Ripe lotus seeds are used as a spleen tonic and the seeds are utilized as a cure for inflammation (Follett and Douglas, 2003; Bi et al., 2006).

From the finding of Jiang et al. (2014), lotus rhizome exhibits the highest antioxidant activities among various vegetables that include broccoli, garlic, potato, and parsley. A number of researches had been done on lotus rhizome and it was found that lotus rhizome also attributes antiproliferative activities (Park et al., 2009), anti-obesity (Ahn et al., 2013), and immunomodulatory potential (Mukherjee et al., 2010).

Up to date, there are very limited researches done on lotus rhizomes, from the extraction of phytochemicals to the identification of the phytochemical compounds, especially the cultivars from Malaysia. Difference from the appearance and morphological aspects between lotus rhizome from Malaysia cultivars and lotus rhizomes from other countries is easily noticed. Therefore it is believed that phytochemical components and activities in different cultivars of lotus rhizome would be varied.

The objectives of the research are:

1. To study and to optimise the effect of solvent, pH, extraction time, extraction temperature and solvent-to-sample ratio in the extraction of phytochemicals from lotus rhizomes followed by characterisation of the extracts based on the antioxidative, antidiabetic, antihypertensive and antibacterial activities.

2. To identify phytochemical compounds in lotus rhizome extracts using liquid chromatography quadropole time-of-flight tandem mass spectrometry (LC Q-TOF MS/MS) system.
3. To evaluate the oxidative stability of fish oil with lotus rhizome extract.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Nelumbo nucifera*

Lotus (*Nelumbo nucifera*) is a perennial ornamental plant, which produces rhizomes that are edible and has been widely favoured in eastern Asia, especially China. Lotus belongs to the genus *Nelumbo* and comes from the *Nymphaeaceae* family. Only two species are found in this genus, which are *Nelumbo nucifera* Gaertn that is widely distributed in Asia region and *Nelumbo lutea* that is spread in North and South America (Qichao and Xingyan, 2005).

Nelumbo nucifera Gaertn rhizomes are tuberous and thick in terms of diameter. Rhizomes grow at the bottom of ponds and their leaves are very large round or oval shapes. Lotus stems are long and having thorns on the surface. Lotus requires a little care, therefore, it could be grown under situations that are not favoured by other crops. In Southeast Asia, the environment requirements of growing lotus are similar as those to breed fish in the ponds.

Lotus is named differently in other countries (Table 2.1). In India where Buddhism is the main religion, lotus is also known as Sacred Lotus, or even named directly as Indian Lotus (Qichao and Xingyan, 2005). A European botanist, J. Gaertner who first discovered and named lotus, suggested that lotus might be originated from India. The name *Nelumbo* is a place located in Sri Lanka, therefore, an idea that this plant was initially existed in India. However, Japanese scientists believed that lotus was first cultivated in China and distributed to Iran, India, Japan, Korea and Southeast Asia countries like Malaysia and Vietnam (Xueming, 1987). The origin of lotus still remained debatable up to date, but it is understood that the

wild distribution was through the Caspian Sea from the west to China, Japan, and Korea in the east, then further to the southeast (Xueming, 1987).

Table 2.1 Different names of *Nelumbo nucifera* in other countries (Xueming, 1987; Qichao and Xingyan, 2005).

Country	Name of lotus
India	Sacred Lotus, Indian Lotus, East Indian Lotus
China	Asian Lotus, Lian, Lin ngau
Egypt	Egyptian Lotus, Blue Lotus of Nile
Japan	Hasu

2.1.1 Traditional uses of *Nelumbo nucifera*

Other than roots, leaves, seeds, fruits and other parts of lotus have been commonly utilised as traditional medicinal remedy and food sources (Ono et al., 2006). Lotus rhizomes are long sausage-like shape with hollow portions. They are usually boiled in soup, pickled or candied as a dessert. The rhizome is very starchy, after cooked, it becomes sweet in taste. The leaves of lotus are utilized to wrap food, for instance dish like lotus rice which is famous in Chinese community. Besides, the leaves could be eaten just like any vegetable with leaves. Lotus seeds, on the other hand, could be eaten raw, ripe or cooked. They are served as an ingredient of local desserts, as a thickener to soups or a snack like roasted chestnuts. Lotus seeds are dried and ground into powder, act as flour for pastry making. And the flower itself is fragrant. Lotus flowers are utilized for the production of fragrancy or perfume. Honey from bees that visit lotus flowers are developed into tonic that are able to cure eye disorders (Hanelt, 2001).

2.1.2 Research conducted on *Nelumbo nucifera*

Nelumbo nucifera is a good source of phenolic compounds and based on the finding of Jiang et al. (2014), lotus rhizome exhibits the highest antioxidant activities among various vegetables that include broccoli, garlic, potato, and parsley. The phenolic content of *Nelumbo nucifera* was reported to be high, and ranged from 31.9 to 77 g/100 g dry extract (Hu and Skibsted, 2002).

Total flavonoid content of *Nelumbo nucifera* was ranged from 7.96 to 33.0 rutin equivalent g/100 g according to the finding of Huang et al. (2011). Flavonoids are best known for the coloration pigment (blue, red, purple, and brown) they provide to flowers, fruits, seeds, and leaves. Flavonoids also exhibit the ability to absorb UV lights, giving UV protection and defense against radiation (Shirley, 1996). Different cultivars of *Nelumbo nucifera* were studied on their composition and content of anthocyanins, flavones and flavonols (Deng et al., 2013). According to their studies, among the four groups of flavones and flavonols identified, myricetin derivatives were the least abundant while the kaempferol derivatives were the most abundant, which are up to 52.17% of total flavones and flavonols content.

As for anthocyanins, five major aglycones were quantified, which are delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, petunidin 3-*O*-glucoside, peonidin 3-*O*-glucoside, and malvidin 3-*O*-glucoside (Deng et al., 2013). Amongst them, malvidin 3-*O*-glucoside was the most abundant anthocyanin (202.10 mg/kg), followed by delphinidin 3-*O*-glucoside and petunidin 3-*O*-glucoside, 109 and 55.57 mg/kg, respectively.

Table 2.2 represents the research conducted on various parts of *Nelumbo nucifera*. Methanolic extracts from the leaves of *Nelumbo nucifera* were discovered

potential to inhibit melanogenesis in theophylline-stimulated murine B16 melanoma 4A5 cells through the actions of alkaloids present in *Nelumbo nucifera* (*N*-methylasimilobine *N*-oxide and benzylisoquinoline alkaloids) (Nakamura et al., 2013). According to Liu et al. (2015), the leaves of *Nelumbo nucifera* had recorded in the earliest and well known Chinese traditional medicinal written document "Ben Cao Gang Mu" as a medicinal herb for treating blood clotting, dizziness and dysentery. In their findings, one of the leaf extracts from *Nelumbo nucifera*, nuciferine possessed pharmacological properties like anti-viral and anti-cancer. Treatment with *Nelumbo nucifera* leaves extract that mainly consisted of gallic acid, quercetin and rutin was confirmed to elicit tumor regression, thus showing the significant potential of *Nelumbo nucifera* as a chemotherapeutic alternative (Yang et al., 2011).

Table 1.2 Phytochemical extracts and compounds attributed to the bioactivities by lotus leaves, seeds and rhizomes

Parts of lotus	Properties	Compounds attributed to the activities	Reference
Leaves	Inhibit melanogenesis	*N*-methylasimilobine *N*-oxide (-)-lirinidine 2-hydroxy-1-methoxy-6a,7-dehydroaporphine	Nakamura et al., 2003
	Antiviral, anticancer	nuciferine	Liu et al., 2015
	Inhibit proliferation of breast cancer	gallic acid rutin quercetin	Yang et al., 2011
	Antidiabetic	quercetin-3-O-β-D-glucopyranoside Qc-3-O- β-D-glucoronopyranoside	Jung et al., 2008
	Anti-HIV	(+)-1(R)-Coclaurine (-)-1(S)-norcoclaurine quercetin 3- O-β-D-glucoronide	Kashiwada et al., 2005
	Hepatoprotective	catechin glycoside miricitrin-3-O-glucoside isoquercitrin quercetin-3-O-rhamnoside astragalin	Huang et al., 2010
	Inhibit pancreatic lipase and adipocyte differentiation	Trans-N-coumaroyltyramine Cis-N-feruloyltyramine -(6R, 6aR)-roemerine-Nβ-oxide liriodenine	Ahn et al., 2013

Table 2.2 (continued) Phytochemical extracts and compounds attributed to the bioactivities by lotus leaves, seeds and rhizomes

Seeds	Radiation-sickness prevention, reducing radiation-induced mortality	Procyanidin	Duan et al., 2010
	Protection from antimycin A-induced mitochondrial dysfunction	Lotus seed extract	Im et al., 2013
	Anti-cancer in Hep 3B cells	Neferine	Yoon et al., 2013
	Inhibition of proliferation of human osteosarcoma cells		Zhang et al., 2012
	Anti-amnesic		Jung et al., 2010
Rhizomes	Protection from lipid oxidation	Tannins	Huang et al., 2011
	Anti-adipogenic, antiobesity Memory enhancement	Lotus rhizome extract	Yang et al., 2008
	Stimulation of defense sytem by immunomodulation		Mukherjee et al., 2010
	Antidiabetic		Mukherjee et al., 1997

The leaves extract of *Nelumbo nucifera* also exhibited the antioxidant properties through prevention against oxidative stress (Jung et al., 2008). Jung et al. (2008) also found out that leaves extract of *Nelumbo nucifera* had an apparent inhibitory response towards rat lens aldose reductase (RLAR) and advanced glycation endproducts (AGE) formation, thus prevent diseases due to oxidative stress and diabetic problems.

Research conducted Kashiwada et al. (2005) reported alkaloids ((+)-1(R)-Coclaurine, (−)-1(S)-norcoclaurine, quercetin 3-O-β-D-glucuronide) and flavonoids compounds extracted from leaves of *Nelumbo nucifera* showed potent anti-HIV activities with EC_{50} values as low as <0.8 μg/ml. Besides aforementioned bioactivities, leaves extracts from *Nelumbo nucifera* also displayed inhibitory effects on pancreatic lipase and adipocyte differentiation attributed to the presence of megastigmanes alkaloids and flavonoids (Ahn et al., 2013).

Ethanolic extracts of *Nelumbo nucifera* leaves was also accessed for hepatoprotective activity against CCl_4 induced liver toxicity *in vitro* and *in vivo* (Huang et al., 2010). The finding showed significant potential of flavonoids and phenolic components of leaves extracts towards *in vivo* hepatoprotective activity at doses of 100 mg/kg, and this was comparable to the standard well-known hepatoprotective drug treatment, silymarin, at doses of 100 mg/kg as well.

Procyanidin isolated from seedpod of *Nelumbo nucifera* were accessed for its *in vivo* radioprotective activity against full body gamma irradiation (Duan et al., 2010). Procyanidins showed effective stimulation of endogenous spleen colony forming units, maintenance of spleen index to normal level, and promotion of red

blood cells, white blood cells and platelets level in peripheral blood at the dosage of 200 mg/kg, thus reducing the damage of radiation-induced diseases.

Antimycin A (AMA) tends to damage mitochondria in cell *via* inhibition of mitochondrial electron transport, thus, inducing apoptosis. Study from Im et al. (2013) proven the protective properties by *Nelumbo nucifera* seed extracts towards inhibition of p53, Bax, and caspase 3 activities, hence, granted protection to mitochondrial membrane and mitochondrial ATP production.

Another novel alkaloid obtained from the seed embryo of *Nelumbo nucifera*, neferine showed exquisite bioactivities like anti-cancer, inhibition of proliferation of human osteosarcoma cells and anti-amnesic (Jung et al., 2010; Zhang et al., 2012; Yoon et al., 2013). Neferine is a major bisbenzylisoquinoline and exhibited cytotoxicity against Hepatocellular carcinoma Hep 3B cells, which is a very aggressive cause of malignant diseases but resistant to chemotherapy. It is found that neferine attributed to inhibition on growth of human osteosarcoma cells, but no inhibition on non-neoplastic osteoblast cells. The direct effect of anti-tumour that neferine exhibited could be observed at the increased phosphorylation of p21 at Ser130. Neferine, moreoever, showed improvement in cognitive impairment in amnesia via antioxidant and anti-inflammatory mechanisms capacities.

Study of Huang et al. (2011) on porcine and bovine ground meat samples discovered that rhizome extracts of *Nelumbo nucifera* were significantly protective against lipid oxidation, therefore, prolong the shelf life of meat. Ethanolic extract of *Nelumbo nucifera* rhizome improved glucose tolerance, hence, reduced blood sugar to normal level (Mukherjee et al., 1997).

Another novel and interesting effects of *Nelumbo nucifera* rhizome extract were to improve memory and learning function, thus becoming a cure to cognitive dysfunction (Yang et al., 2008). Improvement on memory function is attributed to the enhancement in nerugonesis, thus more cell proliferation and differentiation in the dentate gyrus of the hippocampus.

2.2 Factors affecting the content of phytochemicals in plant extracts

Different types of extraction solvents could affect the extraction efficiency. Various studies show that high total phenolic content, total anthocyanin content, total flavonoid content and antioxidant activities were achieved using high polarity extraction solvents such as water and methanols. Solvents with low strength, such as hexane or chloroform do not produce good extraction efficiency (Liu et al., 2000). When methanol was used as the extraction solvent, phenolic components such as hydroxycinnamic derivatives, flavonols, flavan 3-ol monomers, and flavones showed a greater recovery (Tsao and Deng, 2004). Usage of water in combination with organic solvents like methanol and acetone able to contribute a moderately polar medium to ensure the extraction of polyphenolic compounds, however, the use of water solely interfere the yield of extraction due to the high content of impurities, which will further affect the identification and quantification analysis (Lapomik et al., 2005).

The addition of acid in the extraction solvent could be acted in a few ways. Acid increases the stability of the phenolic compounds such as anthocynanins (Escribano-Bailon et al., 2003), or facilitates the dissolution of phenolic compounds, which are initially imparted or bound to the cell wall constituents *via* a hydrolysis process. Acidic conditions also improve the desintegration of cell walls, thus

facilitating the solubilization and diffusion of phenolic compounds from plant cells (Naczk and Shahidi, 2004). In some particular cases of anthocyanins, acidic medium (~pH 1.8) was found to be the most suitable for the extraction process and anthocyanins are most stable at acidic condition (Markakis, 1982).

Combinations of organic solvents (i.e. methanol, ethanol and acetone) with water have been widely used for phenolic extraction. These combinations of solvents are believed to improve the extraction of phenolic components, for instance, phenolic glycosides, which are more water-soluble. However, research conducted by Chirinos et al. (2007) shows that water-to-solvent ratio equal or more than 50% do not ensure the recovery of higher antioxidant contents. Moreover, there is a minimum of 70% methanol has been reported in order to inactivate polyphenol oxidase and to allow maximum recovery of flavonoids in plants (Robards et al., 1999).

Research conducted by Chirinos et al. (2007) also showed that after 60 min, further increment in extraction time did not significantly improve the recoveries of phytochemical contents. Longer extraction times increase the risk of phenolic oxidation, thus losing the content, unless reducing agents are added to the extraction process (Naczk and Shahidi, 2004).

2.3 Extraction of phytochemicals

The very first and crucial step in the study of phytochemical analysis is the sample preparation. It is always desired to extract chemical constituents from plant matrices that will lead to further characterization and identification. Major extraction techniques of phythochemicals were reviewed and listed as conventional method, enzyme-assisted method, microwave-assisted method, ultrasonic extraction, supercritical fluid extraction, and solid phase extraction.

2.3.1 Conventional extraction method

Selection of extraction solvents and methods are essential for the analysis of phytochemicals of interest. For food sources, water as an extraction solvent are proven to be effective for the isolation of high-polar bioactive components, proteins and polysaccharides (Zhao et al., 2013). In some studies, water was added with additives (i.e salts and acids) to improve the separation of phytochemicals of acidic or basic components, such as phenolic acids, vitamin C and alkaloids (Chen et al., 2007). Hydro distillation (HD) and steam distillation (SD) were also applied to isolate volatile compounds such as terpenes and other essential oils using water as extraction solvent by increasing extraction temperature.

For extraction of low polarity phytochemicals (i.e flavonoids, flavonols, saponins, etc.), pure organic solvents like methanol, ethanol, hexane or acetone or a combination of organic solvents with different fractions were used. Reflux extraction and Soxhlet extract (SE) were the famous conventional extraction techniques for their isolation and improvement of analytes allow low volatility, high recovery and high thermal stability (Zhao et al., 2013).

However, long extraction hour and efficiency, large expenditure of extraction solvents, water and energy are the shortcomings of conventional extraction methods. Besides, water-soluble components are unable to be isolated using organic solvents. High extraction temperature will cause thermal damage and decomposition to some heat labile phytochemicals.

2.3.2 Enzyme-assisted method

Enzyme-assisted extraction methods are being more considered in past decades. Enzymes such as cellulose, hemicellulose and pectinase are usually

employed to break down the cell wall structure, facilitating and exposing intracellular phytochemicals for extraction process (Ranveer et al., 2013). Research conducted showed a significant increment in phytochemical content (range from 6-fold to 10-fold increase) from the extraction after the treatment of cellulose and pectinase, compared to non-enzyme treated extraction. However, the ability to extract phytochemicals was reduced when a combination of enzymes were used simultaneously. This suggests that there is a competitive adsorption to the cell wall polysaccharides and further leads to steric hindrance of binding positions of enzyme to substrate, which negatively influences the breakdown of cell-wall components (Hyunh et al., 2014).

From a mechanistic stand point, it has been discovered that xylan and xylo-oligomers have an affinity to cellulose and their adsorption on cellulose surface may inhibit cellulase digest cellulose (Zhang et al., 2012). The presence of lignin on the cell walls could be a reason of lower activity of the mixture of enzymes, where the accessibility of cellulase and hemicellulase to their substrate is greatly limited (Miron et al., 2013).

2.3.3 Other extraction methods

The principle of microwave-assisted extraction method is based on the elevation of temperature (up to 190 °C) then facilitate the partition of phytochemicals from plant matrices to extraction solvent through absorption of microwave energy by organic solvent molecules (Zhao et al., 2013). In the extraction of alkaloids from *Nelumbo nucifera* using microwave-assisted method, extraction efficiency was increased from 20% up to 50% when compared with regular extraction conditions.

Ultrasonic extraction utilized ultrasonic vibration, which is an energy source promotes the release of phytochemicals from plant cell matrices at the frequency of 16 kHz to 1 GHz. Generation and collapse of empty decayed area, along with the friction between the interfacial surfaces created a phenomena named 'acoustic pressure' (Romanik et al., 2007). This acoustic pressure increases the diffusion rate of phytochemicals to the extraction solvent. Ultrasonic extraction has a shorter extraction period, which normally range from 10 min to 1 h, and it is much shorter compared to other extraction methods. It also makes extraction for multiple samples at the same time possible while the recovery obtained is comparable to the recoveries from other extraction methods.

Supercritical fluid extraction which uses supercritical fluids that are high diffusive and low in viscosity to penetrate and dissolve phytochemicals. Carbon dioxide is the most common supercritical fluid extraction agent as it is low cost, having favourable critical parameters (T_c = 31.1 °C, P_c = 74.8 atm) and low toxicity. However, carbon dioxide is a non-polar extracting agent, extraction of polar phytochemicals required a mixture of carbon dioxide and other polar organic solvents to increase the solubility of analytes and prevent phytochemicals from adsorption on plant cell matrix (Zhao et al., 2013).

Reversed-phase C_{18} catridge are often used in solid phase extraction (SPE). SPE is a sample preparation method used to remove undesirable compounds that could interfere the purity of phytochemicals. However, SPE is not suitable to extract unknown compounds as phytochemicals of interest might be washed off during washing step. SPE is more recommended for purifying and enrichment of targeted phytochemicals.

2.4 Role of phytochemicals in biological activities

Non-enzymatic antioxidants react through scavenging radicals, chelating metals, and decomposing peroxides during lipid oxidation. Therefore, bioavailability, pharmacokinetics and metabolism mechanism of antioxidant activity must be considered before deduce from in vitro assays to in vivo situation.

Moure et al. (2001) divided classes of antioxidants into three, depending on their reaction: (1) antioxygen radical, (2) reducing agents, and (3) metal chelators. Antioxidant activity is therefore evaluated by different assays for different mechanisms. Antioxidant mechanisms are commonly measured through total oxidative DNA damage, the content of antioxidants, oxidative damage to lipids and proteins.

2.4.1 Role of phytochemicals as antioxidative agents

Reactive oxygen species (ROS) are generated by mitochondria in the body during energy production. ROS are chemically reactive molecules. The unpaired electrons that ROS contained are believed to be one of the reasons of aging. There is a solid correlation between levels of ROS, oxidative damage to body tissues and chronological age.

Excess amount of ROS activates the oxidation of proteins and fatty acids in human body, causing oxidative damage to DNA which will lead to infirmity, deterioration of cell functionality and illness (Linnane et al., 1989). Oxidative stress occurred due to the imbalance of ROS generation and neutralization and this leads to age-related diseases such as cancer, cardiovascular diseases and diabetes.

Phytochemicals have the potential to scavenge ROS due to the presence of phenolic hydroxyl groups. The ability to scavenge ROS depends on the position and number of hydroxyl group and their substituents, including glycosylation of phytochemicals (Nenadis et al., 2004). Phytochemicals with more hydroxyl groups like kaempherol derivatives (up to 4 hydroxyl substituents) at the fifth position have stronger antioxidant activities. In detailed study conducted by Cao et al. (1997), the 3',4'-di-OH substitution plays a significant role in antioxidant activity, where the flavonoids derivatives with the 3',4'-di-OH substitution achieved up to 3.57 times of trolox equivalent antioxidant capacity compared to the compounds without the substitution. Besides, substitution patterns at the 2,3-double bond in A-ring and B-ring, as well as the 4-oxo group in C-ring also influence the antioxidant activity of flavonoids.

Apart from direct scavenging of ROS, phytochemicals are reported to enhance the production of enzymatic antioxidants in human body (Suh et al., 2004). Glutathione (GSH) plays an essential role in ROS defense systems for its electron donating capacity. GSH exists as the reduced GSH and oxidized gluthathione disulphide (GSSG) form of water-soluble antioxidant. Accumulation of ROS that will lead to age-related diseases is due to the depletion of *de novo* GSH synthesis, recycling and ratio of GSH/GSSG. This is proven in the study of Suh et al. (2004) that the total GSH levels were reduced by half in the livers of aged mice.

Epicatechin, a group of flavonoid, showed extension of mouse lifespan through increasing the production and activity of enzymatic antioxidant superoxide dismutase (SOD) (Si et al., 2011). SOD converts superoxide to hydrogen peroxide during detoxification of cellular damage, which then hydrogen peroxide will be catalysed and neutralized to form water and oxygen by enzymatic actions.

Phytochemicals also prolong survival through regulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathways. Nrf2 is a transcriptional factor that regulates various Phase II antioxidant enzymes (i.e hemoxygenase-1 (HO-1), GSH S-transferase, and quinoneoxidoredctase (NQO1) and initiates detoxification actions (Si and Liu, 2014). Nrf2 inducers such as ROS dissociate the Nrf2/Kelch-like ECH-associated scaffold proteins (Keap-1) complex by modifying the thiol group in Nrf2 through kinase activity. Dissociated Nrf2 complex is then translocated to nucleus and binding of Nrf2 to the antioxidant-response elements (AREs) activates the expression of detoxifying enzymes such as SOD, GSH, and HO-1.

2.4.2 Role of phytochemicals as anti-inflammatory and antidiabetic agents

Inflammation happens when adipose tissue layer is invaded by foreign macrophages, thus pro-inflammatory mediators like interleukin (IL)-1β,IL-6, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF) will be released. As a result, ROS are produced and this inducing T-cell defensive responses. The interaction between immune cells with adjacent adipose tissues exaggerates the inflammation.

Curcumin is a phytochemical which is well known for its anti-inflammatory properties. Its activity is attributed to the inactivation of HIF-1 by down-regulating HIF-1α and HIF-1β (Choi et al., 2006), and subsequently, inhibition of angiogenesis. Research of Ohara et al. (2009) also showed that curcumin able to inhibit TNF-α-induced expression of interleukins, enhance the release of adiponectin, and inhibit glucose transport.

Catechins, which consist of catechin, epicatechin, gallocatechin, epigallocatechin, and their gallates are a group of flavanols. They are discovered to

lower blood pressure and diabetes mellitus in the past decades (Kean, 1944). It modulates inflammation activity by reducing the expression of IL-6 and MCP-1, thus enhancing the production of the anti-inflammatory adiponectin. Chung et al. (2001) suggested epigallocatechin gallate could interfere with mitogen-activated protein kinase activity, resulting inhibition of nuclear factor-kappa B.

Another group of flavonols, quercetin is also known to activate phosphorylation of eukaryotic initiation factor 2 by different kinases, therefore stopping cellular translation (Du et al., 2010) and restricting the expression of HIF-1α. Quercetin also activates the accumulation of HIF-1α through metal chelation, thereby inhibiting and degrading the activity of HIF-1 proline hydroxylase.

2.4.3 Role of phytochemicals as antihypertensive agents

Flavonoids inhibit lipid peroxidation as superoxide anions and hydroxyl radicals scavengers due to their structural activity. The presence of a hydroxyl group in the 3rd position at the C ring of catechin inhibits lipid peroxidation through 3-OH substitution. Hydrogenation of the double bond between carbons 2 and carbon 3 (C-2 and C-3) at the C ring decreases the antiperoxidative effects (Cholbi et al., 1991).

Hydroxyl groups of flavonoids at the position C-5 and C-7 of the A ring, C-3' and C-4' of the B ring, as well as the position at the C-3 of the C ring contribute to the inhibition of lipid peroxidation. On the other hand, flavonoids aglycones like quercetin and myricetin exhibit stronger inhibition of melondialdehyde production due to their presence of sugar moiety. Sugar moieties show steric hindrance and thus reduce the antiperoxidation efficiency (Cholbi et al., 1991). According to Cholbi et al. (1991), flavonoids may inhibit lipid peroxidation by protecting or delaying α-tocophenol in low density lipoproteins from oxidation.

Flavonoids appear to participate in cardioprotective action by increasing the platelet cyclic AMP and inhibiting platelet aggregation by antagonizing thromboxane receptor function and its formation (Tzeng et al., 1991). Flavonoids scavenge the free radicals and bind to the platelet membranes, thereby restore the biosynthesis of endothelial prostacyclin which are inhibited by free radicals.

CHAPTER THREE

METHODOLOGY

3.1 Materials

Fresh lotus rhizome cultivars (SP 1 was imported from China and SP 2 was cultivated in Malaysia) (Fig. 3.1) were purchased from the local market located in Jelutong, Penang, Malaysia. The lotus rhizomes were free from damage and pesticides. Samples were rinsed with deionised distilled water and cut into small cubes and lyophilized to remove moisture. Lyophilised samples were then ground into powder prior to further experiments. All chemicals used in this study were of analytical grade and listed in Table 3.1.

Figure 3.1: Lotus (*Nelumbo nucifera*) rhizomes a) SP 1 (China Cultivar); b) SP 2 (Malaysia cultivar)

Table 2.1 Chemicals used in this study

Chemical	Brand	Country	CAS No
Methanol	QReC	New Zealand	67-56-1
Ethanol	QReC	New Zealand	E7045-1-2500
Acetone	QReC	New Zealand	67-64-1
Ethyl Acetate	Fisher Scientific	UK	141-78-6
Hexane	QReC	New Zealand	N3057-1-2501
Hydrochloric acid	Sigma-Aldrich	Malaysia	7647-01-0
Sodium nitrite	Sigma-Aldrich	Malaysia	7632-00-0
Sodium chloride	Merck	Germany	7647-14-5
Sodium hydroxide	Sigma-Aldrich	Malaysia	1310-73-2
2,2-diphenyl-1-pieryl-hydrazyl	Sigma-Aldrich	Malaysia	217-591-8
Gallic acid	Sigma-Aldrich	Malaysia	149-91-7
Tripyridyltriazine	Sigma-Aldrich	Malaysia	3682-35-7
Iron (II) chloride	Sigma-Aldrich	Malaysia	7758-94-3
Acetic acid	Fisher Scientific	UK	64-19-7
Folin-Ciocalteau	Sigma-Aldrich	Malaysia	10377-48-7
Sodium bicarbonate	Sigma-Aldrich	Malaysia	144-55-8
Aluminium chloride	Sigma-Aldrich	Malaysia	7446-70-0
Pyrocathecol	Sigma-Aldrich	Malaysia	120-80-9
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid	Sigma-Aldrich	Malaysia	30931-67-0
Potassium persulphate	Fluka	US	7727-21-1
Sodium phosphate	Sigma-Aldrich	Malaysia	342483
3,5-dinitrosalicylic acid	Sigma-Aldrich	Malaysia	609-99-4
α-amylase	Sigma-Aldrich	Malaysia	9014-71-5
α-glucosidase	Sigma-Aldrich	Malaysia	9001-42-7
p-nitrophenyl-α-D-glucopyranoside	Sigma-Aldrich	Malaysia	3767-28-0
Angiotensin converting enzyme	Sigma-Aldrich	Malaysia	35115-60-7
Hippuryl-histidyl-leucine	Sigma-Aldrich	Malaysia	31373-65-6
Ampicillin	Sigma-Aldrich	Malaysia	69-53-4
Lecithin	Sigma-Aldrich	Malaysia	8002-43-5
N-hexane	Fisher Scientific	UK	110-54-3
p-anisidine	Sigma-Aldrich	Malaysia	104-94-9
Omega-3 fish oil	Biolife	Malaysia	8016-13-5

3.2 Phase one: Screening of extraction solvent based on antioxidant activity

3.2.1 Extraction of phytochemicals

Extraction parameters were based on the methods of Boulila et al., (2015). Sample (1 g) was added into extraction solvents at the ratio of 20 ml/g. Different solvents including methanol, ethanol, acetone, ethyl acetate, hexane, water or enzyme solution (cellulase or hemicellulase, 1% w/v) were investigated in order to obtain the most efficient extraction solvent for the study. The mixture was homogenised and incubated at 40 °C for 2 h and constantly agitated at 250 rpm. After extraction, the mixture was then centrifuged at 4500 *g* for 15 min at 4 °C. Supernatant was collected and reconstituted to 50 ml with extraction solvent. The crude extract was stored in -20 °C for future analysis. All samples were prepared in three replicates.

3.2.2 2.2-diphenyl-1-pieryl-hydrazyl (DPPH) radical scavenging activity assay

Extracts were analysed with DPPH radical scavenging assay. The scavenging activity on DPPH radical of lotus rhizome extracts was determined using the modified methods of Liu *et al.* (2009). Each extract (5 µl) was added to 150 µl of DPPH solution (0.1 mM). The mixtures were incubated for 30 min in the dark at 30 ^{0}C and discolourations were determined at 517 nm. A standard curve using gallic acid was plotted and deionised water was used as control. All samples were analyzed in three replicates. %DPPH scavenged (%DPPH$_{sc}$) was calculated using the equation below:

$$\%DPPH_{sc} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100\% \qquad Eq.\ (1)$$