UNIVERSITI PUTRA MALAYSIA

ANTIOXIDATIVE PROPERTIES AND NUTRITIONAL CONTENTS OF SELECTED INDIGENOUS MICROALGAE

NATRAH FATIN MOHD IKHSAN

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Dedicated to

All of my supervisors
Who inspired me the most

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ANTIOXIDATIVE PROPERTIES AND NUTRITIONAL CONTENTS OF SELECTED INDIGENOUS MICROALGAE

By

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Microalgae are known to contain various beneficial pigments and are high in nutritional contents. The present studies were done to identify and explore the potential of indigenous microalgae as new natural sources for antioxidants with superior proximate and biochemical values.

Fourteen microalgae were isolated, purified and cultured from fresh and brackish waters. The ability of microalgae as natural resources of antioxidants were studied through screening test using three antioxidative chemical assays (ferric thiocyanate (FTC), thiobarbituric acid (TBA) and 1,1'–diphenyl-2-picrylhydrazyl (DPPH)). Preliminary screening results showed that methanol extracts from six microalgae (*Isochrysis galbana, Chaetoceros calcitrans, Scenedesmus quadricauda, Chlorella vulgaris, Nannochloropsis oculata* and *Tetraselmis tetrathele*) were active in inhibiting the lipid peroxidation of linoleic acid. Among all the microalgae, *I. galbana* and *C. calcitrans*

showed highest antioxidative activities (>90%) in FTC and TBA assays indicating possibility of active constituents for the protection from lipid peroxidation.

Selected microalgae with high antioxidant values (*I. galbana* and *C. calcitrans*) were partitioned into different solvent fractions (hexane, dichloromethane, ethyl acetate, butanol and aqueous extracts) to separate components of wide mixture into group of compounds based on polarity and were re-tested with the antioxidative chemical assays. Dichloromethane extracts of *I. galbana* and *C. calcitrans* showed highest level of antioxidative activities with 97.1±0.1% and 97.4±0.1% linoleic acid peroxidation (LAP) inhibition in FTC assays respectively. Similar trends of high level antioxidative activities in these two microalgae were obtained in TBA assays.

Effects of selected microalgal extracts on up-regulation of antioxidant enzymes gene expression in murine monocytic macrophage (RAW 264.7) cell lines were studied. Both dichloromethane extracts of *I. galbana* and *C. calcitrans* showed high up-regulation in the expression of all antioxidant enzymes genes tested in RAW 264.7. Liquid chromatography-mass spectrometry was employed to identify the constituents which were responsible for all of the antioxidative activities. Fucoxanthin and its isomers were identified as the major constituents in both microalgal species.

Finally, nutritional analysis (proximate and biochemical) were done on microalgae with high antioxidative activities (*I. galbana* and *C. calcitrans*) in order to investigate their nutritive values. Both microalgae were found to be rich in nutrients with protein:

carbohydrate: lipid percentage composition of 47.9±2.5: 26.8±0.2: 14.5±1.4 for *I. galbana* and 36.4±1.7: 27.4±3.0: 15.5±0.9 for *C. calcitrans* respectively. Both species contained high level of n-3 HUFA (28.0%±0.7: *I. galbana* and 28.5%±1.4: *C. calcitrans*) and n-6 HUFA (6.5%±1.8: *I. galbana* and 23.0%±2.5: *C. calcitrans*). Amino acid analyses also showed both microalgae contained high composition of essential amino acids. This study illustrated that some microalgae such as *I. galbana* and *C. calcitrans* have the potential for effective natural sources of antioxidants with high nutritional values.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

CIRI ANTIOKSIDASI DAN KANDUNGAN NUTRISI MIKROALGA TEMPATAN YANG TERPILIH

Oleh

NATRAH FATIN MOHD IKHSAN

Disember 2006

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Mikroalga dikenali sebagai organisma yang mengandungi pelbagai pigmen berguna dan mempunyai kandungan nutrisi yang tinggi. Penyelidikan ini dilakukan untuk mengenalpasti potensi mikroalga setempat sebagai sumber baru antioksida yang mempunyai nilai proksimat dan biokimia yang tinggi.

Empat belas mikroalga telah dipisahkan, dipencil dan dikultur dari air tawar dan air payau. Kemampuan mikroalga sebagai sumber asli antioksida telah dikaji melalui ujian saringan menggunakan tiga jenis asai kimia antioksidasi (ferric thiocyanate (FTC), thiobarbituric acid (TBA) dan 1,1'–diphenyl-2-picrylhydrazyl (DPPH)). Keputusan ujian awal mendapati bahawa ekstrak methanol dari enam jenis mikroalga, (*Isochrysis galbana, Chaetoceros calcitrans, Scenedesmus quadricauda, Chlorella vulgaris, Nannochloropsis oculata* dan *Tetraselmis tetrathele*) adalah aktif di dalam perencatan oksidasi lipid jenis asid linoleik. Antara keseluruhan mikroalga yang dikaji, *I. galbana*

dan *C. calcitrans* menunjukkan aktiviti antioksidasi yang tertinggi (>90%) di dalam asai FTC dan TBA yang menunjukkan terdapat kemungkinan kewujudan komponen aktif di dalam mikroalga untuk perlindungan dari oksidasi lipid.

Mikroalga yang mempunyai kandungan antioksida yang tertinggi (*I. galbana* dan *C. calcitrans*) telah dipisahkan kepada beberapa fraksi pelarut yang berbeza (hexane, dichloromethane, ethyl acetate, butanol dan ekstrak akueus) untuk memisahkan kumpulan komponen campuran yang luas kepada kumpulan komponen yang lebih kecil bergantung kepada keterlarutan komponen kepada pelarut berkutub dan telah diuji semula dengan asai kimia antioksidasi. Ekstrak dichloromethane untuk sampel *I. galbana* dan *C. calcitrans* menunjukkan tahap antioksidasi yang tertinggi di dalam asai FTC berbanding ekstrak pelarut yang lain dengan aktiviti perencatan oksidasi lipid sebanyak 97.1±0.1% dan 97.4±0.1% setiap satu. Keputusan yang sama juga telah diperolehi melalui asai TBA untuk kedua-dua mikroalga.

Kebolehan microalga untuk menambah ekspresi gen enzim antioksidasi di dalam sel makrofaj (RAW 264.7) telah dikaji. Kedua-dua ekstrak dichloromethane *I. galbana* dan *C. calcitrans* menunjukkan pertambahan ekspresi gen enzim antioksidasi di RAW 264.7. Kajian cecair kromatografi telah digunakan untuk mengenalpasti komponen yang berkemungkinan bertanggungjawab ke atas semua aktiviti antioksidasi. Fucoxanthin dan isomernya telah dikenalpasti sebagai komponen yang utama di dalam kedua-dua spesis.

Akhir sekali, analisis nutrisi (proksimat dan biokimia) telah dilakukan ke atas mikroalga yang menunjukkan kandungan aktiviti antioksidasi yang tinggi (*I. galbana* dan *C. calcitrans*) dengan tujuan untuk menyelidiki nilai nutrisinya. Kedua-dua mikroalga didapati kaya dengan nutrisi dengan peratusan komposisi protein: karbohidrat: lipid pada 47.9±2.5: 26.8±0.2: 14.5±1.4 untuk *I. galbana* dan 36.4±1.7: 27.4±3.0: 15.5±0.9 untuk *C. calcitrans* setiap satu. Kedua-dua spesis juga didapati mengandungi tahap n-3 HUFA (28.0±0.7%: *I. galbana* dan 28.5±1.4%: *C. calcitrans*) dan n-6 HUFA (6.5±1.8%: *I. galbana* dan 23.0±2.5%: *C. calcitrans*) yang tinggi. Analisis asid amino juga menunjukkan kedua-dua mikroalga mengandungi komposisi asid amino yang tinggi di dalam jenis asid amino perlu. Penyelidikan ini menggambarkan bahawa sebahagian mikroalga seperti *I. galbana* dan *C. calcitrans* mempunyai potensi untuk sumber efektif antioksida yang semulajadi.

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I certify that an Examination Committee has met on _____ to conduct the final examination of Natrah Fatin Mohd Ikhsan on her degree thesis entitled "Chemical Analysis of Antioxidative Fractions and Nutritional Contents of Selected Microalgae" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date: 10 May 2007

DECLARATION

I hereby declare that the thesis 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 UPM or other institutions.

NATRAH FATIN MOHD IKHSAN

Date:

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LIST OF ABBREVIATIONS

AABA	alpha amino butyric acid
$ABTS^{+}$	2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonate)
AH	antioxidant
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
ASP	amnesic shellfish poisoning
B-actin	beta actin
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BuOH	butanol
ca.	about
CAT	catalase
cDNA	complementary deoxyribonucleic acid
CH_4	methane
COX-2	cyclooxygenase
CuZnSOD	copper-zinc superoxide dismutase
DAD	diode array detector
DCM	dichloromethane
DHA	docosahexaenoic acid

DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides
DPPH	1,1'–diphenyl-2-picrylhydrazyl
DSP	diarrhetic shellfish poisoning
EPA	eicosapentaenoic acid
ESI	electrospray
EtoAc	ethyl acetate
FAME	fatty acid methyl esters
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
$FeCl_2$	ferrous chloride
FCS	fetal calf serum
FR	free radical
FRAP	ferric reducing/ antioxidant power
FTC	ferric thiocyanate assay
GAPDH	glyceroldehyde-3-phosphate
GPS	global positioning system
GPX	gluthathione peroxidase
GSH	glutathione
GSSG	oxidized glutathione
H^+	hydrogen
H_2O	water

H_2O_2	hydrogen peroxide
HPLC-DAD-MS	high performance liquid chromatography-diode array detector-mass spectrometry
IFN- γ	interferon-gamma
iNOS	nitric oxide synthase
$L^{\cdot}$	resultant alkyl free radical
LAP	linoleic acid peroxidation
LC-MS	liquid chromatography-mass spectrometry
LCPUFAs	long-chain polyunsaturated fatty acids
LH	fatty acid/lipid
$LOO^{\cdot}$	unstable peroxy free radical
LOOH	hydroperoxide
LOOL	non radical products
LP	lipid peroxidation
MA	moisture and ash
MeOH	methanol
$MgCl_2$	magnesium chloride
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MUFA	monounsaturated fatty acids
MW	molecular weight
m/z	mass-to-charge ratio
NaCl	natrium chloride

NaOH	natrium hydroxide
$NaSO_4$	natrium sulfate
NCBI	national center for biotechnology information
NH_3	ammonia
NH_4SCN	ammonium thiocyanate
NO	nitric oxide
NO_2	nitrogen dioxide
Non SeGPX	non-selenium dependent glutathione peroxidase
OD	absorbance
O_2	oxygen
$O_2^{\cdot-}$	superoxide anion
$OH^{\cdot}$	hydroxyl radical
$OONO^{\cdot}$	peroxynitrite
PCR	polymerase chain reaction
PDA	photo diode array detector
PG	propyl gallate
PGE_2	prostaglandin E_2
PHGPX	phospholipid hydroperoxide glutathione peroxidase
PITC	phenyl isothiocyanate
PLGPX	plasma glutathione peroxidase
PSP	paralytic shellfish poisoning
PTC-amino acids	phenylthiocarbamyl amino acids
RAW 264.7	murine monocytic macrophage

RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNS	reactive nitrogen species
ROH	alcohol
ROS	reactive oxygen species
RT	retention time
RT-PCR	polymerase chain reaction
SCP	single cell protein
SeGPX	selenium-dependent glutathione peroxidase
SF	sulfated polysaccharides
SFA	saturated fatty acids
SOD	superoxide dismutase
SPSS	statistical package for the social sciences
TBA	thiobarbituric acid
TBHQ	tert-butylhydroquinone
TEAC	trolox equivalent antioxidant capacity
TIC	total ion chromatogram
TNF-α, IL-1, IL-6	cytokines
TPTZ	(Fe^{2+}/2,4,6-tripridyl-s-traizine
UV	ultraviolet
XDH	xanthine dehydrogenase
XO	xanthine oxidase

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background of study

As a tropical country with ample sunlight and rain throughout the year, Malaysia is blessed with a myriad of natural resources that can be sustainably utilized. These resources which range from terrestrial to aquatic organisms (plant, bacteria, fungi and animals) provide an indispensable value for various industrial applications which include medicine, pharmaceutical, nutraceutical, cosmetics and aquaculture (Borowitzka and Borowitzka, 1988; Young, 1999). The value of natural products become higher due to the inability to find the cure of certain diseases such as cancer, AIDS, Alzheimer's disease and arthritis (to name a few). Since natural products offer a vast source of chemical diversity with unusual chemical structures, they could provide the solution alleviating these diseases. From 1960 to 1982, over 180,000 microbial-derived, some 16,000 marine organism-derived, and over 114,000 plant-derived extracts have been screened for anti-tumor activity which resulted in the discovery of a number of clinically effective chemotherapeutics agents (Cragg *et al*., 1999).

Over the millennia, research on natural products has mainly focused on plants which have been proven as an excellent source for the treatment of several diseases. Plants are more fully studied than animals in terms of their pharmacological and therapeutical