UNIVERSITI PUTRA MALAYSIA

METABOLOMIC PROFILING OF ANTIOXIDANT AND ANTI INFLAMMATORY PROPERTIES IN DIATOM Chaetoceros calcitrans EXTRACTS USING NMR AND UHPLC-MS COUPLED WITH CHEMOMETRIC ANALYSIS

AWANIS BINTI AZIZAN

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By

AWANIS BINTI AZIZAN

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Chairman : Associate Professor Faridah Abas, PhD
Institute : Bioscience

Chaetoceros calcitrans is a diatom microalga that is known to be rich of amino acids, lipids, fatty acids and natural pigments identified as potentially important natural antioxidant and anti-inflammation. Nevertheless, little is known about the metabolome and the antioxidative and anti-inflammatory ability of the indigenous microalga, *C. calcitrans*. The main objectives of this study were to evaluate the metabolites that contributed to the antioxidant activity (DPPH*), nitric oxide (NO) inhibitory activity and total phenolic content (TPC) of *C. calcitrans*, extracted with different solvent polarities, including 70% ethanol, methanol, hexane, acetone, and chloroform using multi-platform metabolomics approaches. Nuclear magnetic resonance (NMR) coupled to multivariate data analysis (MVDA) was applied for the metabolomics profiling and relative quantification of the extracts. Further confirmation of the metabolites identification and quantitation were performed using ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS). The results showed that acetone and chloroform ($CHCl_3$) extracts of *C. calcitrans* revealed higher levels of TPC with 30.79 and 25.41 mg GAE/g dw, respectively. Both extracts also displayed moderate activity of DPPH radical scavenging inhibition with 43.01 and 35.03% at concentration 333 µg/ml. Furthermore, the $CHCl_3$ extract inhibited the release of NO production from the LPS-activated RAW 264 cells with an IC_{50} value of 3.46 µg/ml. Twenty-nine metabolites were identified via NMR analyses from *C. calcitrans* extracts including 6 fatty acids, cholesterol, 11 amino acids, 2 sugars and 1 sugar-alcohol, 6 carotenoids and 2 chlorophylls. The structures of the compounds were also confirmed using tandem mass spectrometry. The main identified secondary metabolites were carotenoids including fucoxanthin, lutein, astaxanthin, canthaxanthin, zeaxanthin and violaxanthin. Comparison of different extracts revealed clear differences in the metabolite profiles and the partial least square (PLS) model indicated that the carotenoids were significantly associated with the tested

bioactivities. The results suggested $CHCl_3$ and acetone extracts of *C. calcitrans* showed the abundance of high-value metabolites as markers for antioxidant and anti-inflammatory activities. The findings from this research may serve as a benchmark for future extraction processes particularly in recovering antioxidant and anti-inflammatory metabolites derived from diatom. These metabolites can be important active ingredients for medicinal preparation, functional foods, and cosmeceutical and nutraceutical applications.

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

PEMPROFILAN METABOLOM CIRI ANTIOKSIDAN DAN ANTI-RADANG DALAM EKSTRAK MIKROALGA DIATOM *Chaetoceros calcitrans* MENGGUNAKAN GABUNGAN NMR DAN UHPLC-MS DENGAN ANALISIS KEMOMETRIK

Oleh

AWANIS BINTI AZIZAN

April 2019

Pengerusi : Professor Madya Faridah Abas, PhD
Institut : Biosains

Chaetoceros calcitrans merupakan sejenis diatom mikroalga yang kaya dengan asid amino, lipid, asid lemak, pigmen semulajadi yang dikenalpasti mempunyai potensi sebagai sumber penting antioksidan dan anti-radang semula jadi. Walau bagaimanapun, hanya sedikit maklumat yang diketahui mengenai metabolom dan kemampuan antioksidasi dan anti-radang oleh mikroalga tempatan, *C. calcitrans*. Objektif utama kajian ini adalah untuk menilai metabolit yang menyumbang terhadap aktiviti antioksida (DPPH*), aktiviti rencatan nitrik oksida (NO) dan juga jumlah kandungan fenolik (TPC) bagi *C. calcitrans* yang diekstrak dengan beberapa pelarut berkutub yang berbeza seperti 70% etanol, metanol, heksana, aseton, dan klorofom dengan menggunakan pendekatan multi-platfom metabolomik. Resonans magnet nukleus (NMR) digabungkan dengan analisis data multivariat (MVDA) telah digunakan untuk memprofil metabolomik dan kuantifikasi relatif terhadap ekstrak. Pengesahan lanjut mengenai metabolit yang dikenal pasti dan dikuantifikasi telah dilakukan dengan menggunakan kromatografi cecair prestasi tinggi-spektrometri jisim (UHPLC-MS). Keputusan menunjukkan ekstrak aseton dan klorofom ($CHCl_3$) untuk sampel *C. calcitrans* memiliki tahap TPC yang tertinggi sebanyak 30.79 and 25.41 mg GAE/g dw setiap satu. Kedua ekstrak ini juga telah mempamerkan aktiviti antioksida yang sederhana melalui aktiviti pemerangkap radikal bebas DPPH sebanyak 43.01 and 35.03% pada kepekatan 333 µg/ml. Tambahan pula, ekstrak $CHCl_3$ telah merencatkan pembebasan NO dari dalam sel RAW 264 yang diaktifkan oleh LPS dengan nilai IC_{50} sebanyak 3.46 µg/ml. Dua puluh sembilan metabolit telah dikenal pasti melalui analisis NMR dari ekstrak *C. calcitrans* yang terdiri daripada 6 asid lemak, kolestrol, 11 asid amino, 2 gula dan 1 gula-beralkohol, 6 karotenoid dan 2 klorofil. Struktur sebatian juga disahkan dengan menggunakan spektrometri jisim bergandingan. Metabolit sekunder utama yang telah dikenal pasti adalah karotenoid antaranya fucoxanthin, lutein, astaxanthin, canthaxanthin, zeaxanthin and violaxanthin. Perbandingan dengan ekstrak yang berbeza mendedahkan perbezaan

ketara profil metabolit dan model analisa separa persegi (PLS) menunjukkan karotenoid mempunyai kaitan yang signifikan dengan bioaktiviti yang dikaji. Keputusan kajian ini mencadangkan ekstrak $CHCl_3$ dan aseton untuk sampel *C. calcitrans* mempunyai kelimpahan metabolit yang bernilai tinggi yang bertindak sebagai penanda yang bertanggungjawab ke atas aktiviti antioksidan dan anti-radang. Hasil kajian dari penyelidikan ini boleh dijadikan penanda aras untuk proses pengekstrakan pada masa akan datang terutamanya dalam perolehan semula metabolit antioksidan dan anti-radang yang berasal dari diatom. Metabolit ini juga boleh dijadikan bahan aktif penting untuk diaplikasikan di dalam proses penyediaan ubat, makanan fungsian, kosmeseutikal dan nutraseutikal.

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This thesis was 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 were as follows:

Faridah Abas, PhD
Associate Professor
Institute Bioscience
Universiti Putra Malaysia
(Chairman)

Khozirah Shaari, PhD
Professor
Institute Bioscience
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Signature: _____

Name of Member of Supervisory Committee: Professor Dr. Khozirah Shaari

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a.u	Arbitrary unit
amu	Atomic mass unit
AA	Arachidonic acid
ABTS	2,2-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid)
ACN	Acetonitrile
Ac	Acetone extract
ALA	α-linolenic acid
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
API	Atmospheric ionization
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BPC	Base Peak Chromatogram
C40	40 carbon atoms
CCl_4	Carbon tetrachloride
$(CD_3)_2CO$	Acetone-d6, Deuterated acetone
$CHCl_3$	Chloroform extract
Chl *a*	Chlorophyll *a*
Chl *c*	Chlorophyll *c*
CO_2	Carbon dioxide
CODA	Component Detection Algorithm
COX	Cyclooxygenase

CS	Calibration standard
CUPRAC	Cupric reducing antioxidant capacity
d	Doublet
DAD	Diode array detector
DA	Domoic acid
dd	Doublet of doublet
DPPH	2,2-diphenyl-1-picrylhydrazyl free radicals
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Sodium 2,2-dimethylsilapentane sulphonate
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EPS	Exopolysaccharides
EtOH	Ethanol extract
ET	Electron transfer
ESI	Electrospray ionization
FA	Fatty acids
FBS	Fetal bovine serum
FC	Follin-Ciocalteau
FRAP	Ferric reducing antioxidant power
FTC	Ferric thiocyanate
GAE	Gallic acid equivalent

HCD	High-collisional dissociation
GABA	γ-Amino butyric acid
GLA	γ-Linolenic acid
H_3PO_4	Phosphoric acid
HAT	Hydrogen atom transfer
HCA	Hierarchal cluster analysis
HCN	Hydrogen cyanide
Hex	Hexane extract
HESI	Heat electrospray ionization
HMBC	Heteronuclear Multiple Bond Correlation
HMDB	Human metabolome databases
HSQC	Heteronuclear single quantum correlation
HPLC	High performance liquid chromatography
HRAM	High resolution accurate mass
IFN-γ	Interferon gamma
iNOS	Inducible nitric oxide synthase
IL	Interleukins
J	Coupling Constant
J-res	*J*-resolved
kV	kiloVolt
LC	Liquid chromatography
LC/MS	Liquid chromatography/mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation

LOX	Lipoxygenase
LOOH	Lipid hydroperoxides
LPS	lipopolysacharides
m	Multiplet
m/z	Mass-to-charge ratio
MeOH	Methanol extract
MS	Mass spectrometry
MTT	3-(4,5-dimethythiazol2-yl)-2,5-diphenyl tetrazolium bromide
MVDA	Multivariate data analysis
N.D	Not determined
Na_2CO_3	Sodium carbonate
NCE	Normalized collision energy
NMR	Nuclear magnetic resonance
NO	Nitric oxide
ORAC	Oxygen radical absorption capacity
OPLS-DA	Orthogonal partial least square discrimination analysis
PBS	Phosphate buffered saline
PCA	Principle component analysis
PLS	Partial least squares
PLS-DA	Partial least squares–discriminant analysis
PNP	p-nitrophenol
PNPG	p-nitrophenyl-α-D-glucopyranose
ppm	Part per million
PGs	Prostaglandins

PUFAs	Polyunsaturated fatty acids
QTOF	Quadruple-time of flight
RNS	Reactive nitrogen species
Rs	Resolution
RSD	Relative standard deviation
RT	Retention times
ROS	Reactive oxygen species
S/N	Signal to noise ratio
s	Singlet
SD	Standard deviation
SIM	Single ion monitoring
SIMCA	Soft independent modelling in class analogues
t	Triplet
t_R	Retention time
TAG	Triacylglycerols
TBA	Thiobarbituric acid
TEAC	Trolox equivalent antioxidant capacity
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TNF-a	Tumor necrosis factor alpha
TMS	Tetramethylsilane
TOF	Time of flight
TPC	Total phenolic content
TSP	Sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4

Tukey's-HSD	Tukey's honest significant difference
TW	Time warping
UHPLC-MS	Ultra-high performance liquid chromatography mass spectrometry
UHPLC-MS/MS	Ultra-high performance liquid chromatography tandem mass spectrometry analysis
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VIP	Variable importance in the projection
WHO	World Health Organization
δ	Chemical shift in ppm
1D	One-dimensional
^{1}H	Proton
2D	Two-dimensional
^{13}C	Carbon-13
O_2^-	Superoxide anion
•OH	Hydroxyl
ROO•	Peroxyl
RO•	Alkaloyl radicals
H_2O_2	Hydrogen peroxide
$^{1}O_2$	singlet oxygen

CHAPTER 1

INTRODUCTION

1.1 Background

Oxygen free radicals known as reactive oxygen species (ROS) formed through oxygen poisoning and radiation injury lead to may deleterious effect (Devasagayam et al., 2004). It is increasingly reported that this kind of oxygen free radical plays a key role in approximately hundreds of oxidative stress disorders including cancer, inflammatory bowel disease, cardiovascular diseases, Alzheimer's disease, rheumatoid arthritis, stroke and septic shock (Halliwell, 1996; Wiseman and Halliwell, 1996). Therefore, removal of the harmful radicals exists are indispensable, via antioxidants defence system that capable of blocking the generation of ROS. Since then, most biomedical research has been circulating on free radical chemistry, oxidative pathology, and leading to translate the knowledge from the laboratory into new-drugs discovery.

In recent years, the limited land for growing terrestrial crops, worrying levels of global warming and enlarging numbers of the human population, resulting in the urgent need for sustainable biomass resources from marine ecology (Gaurav et al., 2017). Hence, over the past years have witnessed that marine organisms have received increasing attention from researchers in the various fields of industries to meet the demands. Marine organisms including marine algal species are considered as "barely tapped source" (Stengel and Connan, 2015). They have dominated many kinds of marine environment from oceans, seas and also in the coastal areas (Kim, 2015). Many therapeutic metabolites derived from marine algae have responsible to aid human being in curing diseases and reducing aches, simultaneously, improved human life quality (Kim, 2015). Furthermore, these valuable metabolites including natural pigments, phycobiliproteins, lipids, polyunsaturated fatty acids (PUFAs), polyphenols and polysaccharides also have other enormous potentials such as nutritive feed for mariculture animal species, as ingredients in cosmeceutical products, as lipid-based bioproducts and also as an alternative power sources (biofuels) (Gouveia et al., 2010).

Owing to the variety of their pharmacological properties including antiradical and anti-inflammatory, microalgae carotenoids have been considered as a source of therapeutic metabolites to oxidative stress besides their role in photosynthesis and protection against UV solar radiation (Gong and Bassi, 2016; El Gamal, 2010; Zuluaga et al., 2017). Antioxidant activity of microalgae is frequently measured by several assays including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) (Goiris et al., 2012; Foo et al., 2015; Foo et al., 2017), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Goiris et al., 2012; Foo et al., 2017) ferric reducing antioxidant power (FRAP) and thiobarbituric acid (TBA) (Pangestuti and Kim, 2011). Carotenoids not only function as scavengers, but they also have ability to modulate the macrophages function as secretory of a vast array of mediators and cytokines including nitric oxide (NO), prostaglandins (PGs), tumour

necrosis factor alpha (TNF-a), interleukins (IL), lipoxygenase (LOX) and cyclooxygenases (COX) (Pangestuti and Kim, 2011). Although microalgae-derived carotenoids have promising anti-inflammatory activities, little research has been performed on this activity and only a few studies were reported on microalgae (Pangestuti and Kim, 2011).

Chaetoceros calcitrans is a microscopic and fast growth microalga diatom which offers higher production of potent biological sources. It is known as de novo producers of antioxidants and immune stimulants metabolites such as carotenoids, lipids, polyunsaturated fatty acids (PUFAs) and vitamins (Salas-Leiva and Dupré, 2011; Foo et al., 2015). This species is frequently used as food source for feeding the maricultered animal species including bivalve molluscs (etc. mussels), echinoderms (e.g. copepods), crustaceans (e.g. penaeid shrimps) and also zooplankton (e.g. brine shrimp *Artemia)* (Becker, 2004). Although it was claimed as essential food sources for marine animal studies, its application in human consumption still remains elusive.

To develop effective medications for oxidative stress disorders, a better understanding of the metabolic changes caused by stress conditions through metabolomics tools predominantly based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) may facilitate in finding of potential biomarkers for early detection of abnormalities associated with chronic oxidative diseases (Andrisic et al., 2018). Although MS sensitivity in detecting metabolites is much higher (femtomolar to attomolar) compared to NMR as an analytical tool, we believe that the weaknesses of NMR are the strengths of MS spectrometry (Veenstra, 2012). Since NMR and MS have easy sample preparation steps, and capable in detecting of primary and secondary metabolites, these approaches were often selected in previous studies of plant extracts for understanding the dynamical processes regarding interacting biomolecules involved in antioxidant activity. At present, the complete profile and metabolic network of this diatom *C. calcitrans* have not been fully characterized. A sound understanding of the *C. calcitrans* metabolome will further contribute to give a holistic overview of its system biology, lead to new applications and promote this diatom as attractive prolific producers of bioactive metabolites.

1.2 Problem statement

Chaetoceros calcitrans is being widely used to provide a direct source of nutrition for marine fish and shellfish aquaculture study especially as an alternative to conventional feed. Only a few bioactivities related to this species have been reported including antioxidant (Foo et al., 2015) and anti-cancer (Nigjeh et al., 2013). Even though the presence of carotenoids, chlorophylls, phenolic, amino acids, sterol, fatty acids, oxylipins and lipids had been reported in this diatom, comprehensive metabolic identification and correlation for the metabolites that could contribute to the claimed bioactivities have not much explored.

The inherent metabolic variation in the microalgae is associated by the post-harvest handling, including extraction process. Owing to great economic stakes for bioactive production from microalgae, researchers and scientific community have targeted to find efficient and economical ways to recover the metabolites. Besides, extraction is a pivotal step for assessing the deeply inaccessible metabolites from the complex matrix of a given system like microalgae. It was crucial to recovering all classes of metabolites at one time of extraction due to the substantial level of complexity of microalga metabolites. Therefore, extracting the metabolite in an efficient way should be investigated and optimized.

1.3 Scope and Objectives

The main goal of this study is to profile the chemical scaffolds of *C. calcitrans* extracts using NMR and UHPLC-MS metabolomics approaches that allow efficient and accurate identification of the wide spectrum of interesting compounds resulting from the effect of different solvent extractions. Consequently, correlation of the metabolite profile of *C. calcitrans* extracts obtained from both high power metabolomic tools with the antioxidant and anti-inflammatory activities including DPPH free radical scavenging and nitric oxide (NO) inhibitory activities was performed. Also, the correlation will provide information on the effects of type of solvents as a parameter to improve the extraction efficiency of bioactive metabolites from *C. calcitrans*. In this thesis, the reports are presented and discussed in three parts. First part of the work aimed to screen the biological activities of *C. calcitrans* extracted using five different solvent polarities for antioxidant and NO inhibitory activities (Chapter 4, part 4.1). Subsequently, different crude extracts of *C. calcitrans* will be further characterized, quantified and then correlated their biological activities using NMR (Chapter 4, part 4.2) and method development for UHPLC-MS followed by the UHPLC-MS-based metabolomics approaches (Chapter 4, part 4.3 and part 4.4). Lastly, an attempt was made to quantify the compounds in the active extract by using UHPLC-MS.

The specific objectives for this study are:

1. To screen the five different solvent extracts including 70% ethanol, methanol, chloroform, acetone and hexane of *C. calcitrans* for antioxidant, nitric oxide (NO) inhibitory activity and total phenolic content (TPC).
2. To determine the effect of different solvent extractions on the metabolome of *C. calcitrans* and the correlation with antioxidant, nitric oxide (NO) inhibitory activity and total phenolic content (TPC) using NMR based metabolomics.
3. To characterize the different solvent extractions of *C. calcitrans* and correlation with antioxidant and nitric oxide (NO) inhibitory activity using UHPLC-MS-based metabolomics.
4. To quantify the metabolites present in the *C. calcitrans* extract using NMR and UHPLC-MS.

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