EXTRACTION OF FUCOXANTHIN FROM SELECTED MALAYSIAN BROWN SEAWEEDS AND ITS EFFECTS ON 3T3-L1 ADIPOCYTE CELLS

BY

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ABSTRACT

Obesity (BMI >30 kg/m^2) is becoming a major public problem as it affects over 400 million of the population. Despite advances in the development of more effective weight loss drugs, safety and dependence remains an issue. This study aims to establish the antiobesity potential of Malaysian brown seaweeds *Sargassum oligocystum, Padina australis* and *Dictytota dichotoma.* Fucoxanthin has been described as the main bioactive compound in brown seaweed exerting antiobesity effect. Three extraction parameters were optimized in order to retain maximum fucoxanthin content in the extract: solvent, particle size and method of extraction. Optimal solvent and particle size for extraction across all seaweeds was acetone and particles sieved through 500µm mechanical sieve. *S. oligocystum* and *P. australis* were best extracted using Soxhlet procedures, while *D. dichotoma* was best extracted using maceration. The fucoxanthin content was analyzed using HPLC equipment. The highest amount of fucoxanthin present in the seaweeds was found in *Sargassum oligocystum* (754.8 mg/g dry weight), followed by *D. dichotoma* (142.9 mg/g) and finally *P. australis* extracts (91.58 mg/g). The extract showing best antiadipogenic effect on the preadipocytes was SAE (lipid accumulation equivalent to 0.170 ± 0.034 A), followed by DAE (0.196 ± 0.082 A) and finally PAE (0.203 ± 0.047 A). SAE had the best proadipolytic effect on mature lipid cells (31.86 ± 17.56 mg/ml glycerol release), followed by PAE (30.54 ± 15.41 mg/ml) and finally DAE (28.12 ± 11.36 mg/ml). Statistical analysis using one- way ANOVA indicated that the differences were not significant ($p>0.05$). Overall, antiobesity activity levels did not coincide with fucoxanthin levels present in the extract, indicating the presence of various compounds in the extract that exert diverse effects on 3T3-L1 adipocytes. The study describes fucoxanthin content of the Malaysian seaweeds and its optimal extraction parameters. Furthermore, it indicates the adipogenic and adipolytic potential of the extracts as antiobesity agents and the potential for seaweed extracts to be further developed as a safe and effective natural alternative for the management of obesity.

خلاصة البحث

أصبحت السمنة المفرطة (مؤشر كتلة الجسم أو BMI أعلى من 30 كجم/م^2) من المشاكل الإجتماعية الكبرى والتي تؤثر على حوالي 400 مليون نسمة من التعداد السكاني. على الرغم من التقدم العلمي في تطوير عقاقير تخفيض الوزن فلا زالت أمور السلامة والإدمان من المشاكل المتعلقة بها. هدفت هذه الدراسة إلى إثبات الخواص المضادة للسمنة للأعشاب البحرية البنية الماليزية، وهي من نوع سارجسم أوليقوسستم، وبادينا أوستراليس، وديكتيوتا دايكوتوما. تم التعرف على أن مركب فوكوزانتين هو المركب النشط حيويا الأساسي في الأعشاب البحرية البنية المتميزة بالخواص المضادة بالسمنة. تم تحسين مؤشرات عملية الاستخلاص للحفاظ على أعلى نسبة من الفوكوزانتين في المستخلصات، وهي نوع المذيب، حجم الذرة، وطريقة الاستخلاص. نوع المذيب وحجم الذرة المناسبين لاستخلاص الأعشاب البحرية كان الأسيتون، والذرات المصفاة خلال مصفاة من قياس 500 مكرومتر. أفضل طريقة لاستخلاص سارجسم أوليقوسستم وبادينا أوستراليس كان بواسطة السوكسليت، والديكتيوتا دايكوتوما بواسطة النقع. تم حساب كمية الفوكوزانتين بواسطة جهاز التحليل الكروماتوجرافي العالي الكفاءة والذي وجد بأعلى كمية في سارجسم أوليقوسستم (الوزن الجاف 754.8 مغ/غ)، ومن ثم الديكتيوتا دايكوتوما (142.9 مغ/غ)، وأخيرا وبادينا أوستراليس (91.58 مغ/غ). أفضل نشاط ضد السمنة على الخلايا الماقبل الدهنية كان من قبل السارجسم أوليقوسستم (تراكم الدهون المعادل لـ 0.170±0.034 A)، ومن ثم الديكتيوتا دايكوتوما (0.196±0.082 A) وأخيرا البادينا أوستراليس (0.203±0.047 A). أظهر السارجسم أوليقوسستم أفضل تحلل للخلايا الماقبل الدهنية وعلى الخلايا الدهنية المكتملة (31.86±17.56 مغ/مل كمية إطلاق الجلسرول)، ومن ثم البادينا أوستراليس (30.54±15.41 مغ/مل)، وأخيرا الديكتيوتا دايكوتوما (28.12±11.36 مغ/مل). أظهر تحليل التباين الأحادي الأنوفا أن الفروق ليست مهمة نسبيا (p>0.05). بشكل عام، مستويات النشاط المضاد للسمنة لم تتناسب مع مستويات الفوكوزانتين المتواجدة في المستخلصات، مما يؤشر إلى أن هنالك العديد من المركبات الأخرى المؤثرة بشكل مختلف على خلايا 3T3-L1 الدهنية. وصفت الدراسة محتوى الفوكوزانتين في الأعشاب البحرية الماليزية وطرق استخلاصها المثلى. بالإضافة إلى ذلك، أشارت هذه الدراسة إلى الخواص المكونة للشحوم والخواص المضادة للشحوم للمستخلصات المحتملة كمركبات مضادة للسمنة واحتمال تطوير هذه الأعشاب في كبدائل طبيعية وآمنة لتنظيم السمنة.

APPROVAL PAGE

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LIST OF EQUATIONS

LIST OF SYMBOLS

µm	Micrometer
cm	Centimeter
cm^2	Centimeter squared
g	Grams
h	Hours
kg	Kilograms
Kg/m^2	Kilograms per square meter
m	Meters
m^2	Square meters
mg/g	Milligram per gram
min	Minutes
ml	Milliliters
ml/min	Milliliters per minute
mm	Millimeter
nm	Nanometer
Å	Angstrom
Ac	Acetone
CO_2	Carbon dioxide
D0	Day zero
D2	Day two
D4	Day four
D7	Day seven
pH	Potential hydrogen

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
ANOVA	Analysis of Variance
ATCC	American Type Cell Culture
BMI	Body Mass Index
CEBP	CCAT enhancer binding protein
CAMP	Cyclic adenosine monophosphate
CREB	Cyclic response element binding
DAE	*D. dichotoma* acetone extract
DMX	Dexamethasone
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
et al.	Et alia
EtOH	Ethanol
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FFA	Free fatty acids
Fx	Fucoxanthin
HPLC	High performance liquid chromatography
IBMX	Isobutylmethylxanthine
IGF1	Insulin growth factor 1
LLE	Liquid- liquid extraction

M	Mean
MDI	Methylisobutylxanthine, dexamethasone, insulin
MeOH	Methanol
Mets	Metabolic syndrome
mRNA	Messenger ribonucleic acid
ORO	Oil red O
PAE	*P. australis* acetone extract
PBS	Phosphate buffered saline
PPAR-ɣ	Peroxisome proliferator activated receptor gamma
PUFA	Polyunsaturated fatty acids
RAPD	Random amplified polymorphic DNA
RP	Reverse phase
Rt	Retention time
SD	Standard deviation
SLE	Solid liquid extraction
SPSS	Statistical Package for Social Sciences
TNF-α	Tumor Necrosis Factor alpha
UCP	Uncoupling protein
UK	United Kingdom
UV	Ultraviolet
WAT	White adipose tissue
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE RESEARCH

Obesity is fast becoming the next preventable epidemic in the world, with over 11.6 billion of the population being overweight (BMI > 25 kg/m^2), and 400 million more obese (BMI > 30 kg/m^2) (WHO, 2013). Prevalence has been rising, including in developing and underdeveloped countries, with numbers forecasted to increase by 33 % in 2030 (Finkelstein, Khavjou, Thompson, Trogdon, Pan, Sherry, Dietz; 2012). This trend is worrying as obesity has been reported as a risk factor for several diseases including high blood pressure, diabetes, cardiovascular disease, several cancers, disabilities and death (WHO, 2013; Reilly, Methven, Mcdowell, Hacking, Alexander, Stewart, Kelnar; 2003). The healthcare costs associated with the treatment of these diseases has also seen a worrying rise. In 2006 alone, healthcare expenditure was estimated at 100 billion dollars per year and is expected to rise to approximately 500 billion dollars per year in 2030 (WHO, 2013; Finkelstein et al., 2012). It has been reported that the in addition to the increased risk for metabolic disease, quality of life of obese/overweight patients is lower than the average individual, owing to the discrimination, stigma and prejudice they face (Puhl & Heuer, 2009; Jia & Lubetkin, 2005). All these factors combined have resulted in obesity becoming a serious global healthcare issue.

Several methods are prescribed in the management of obesity, including mild lifestyle changes, to the use of antiobesity drugs, to invasive surgery in chronic cases. It has been reported that diet and exercise do not result in sustained weight loss

(Douketis, Macie, Thabane, Williamson; 2005); therefore pharmacotherapy remains an interesting option for the management of obesity. However, despite advances in the development of more effective weight loss drugs that result in sustained, meaningful weight loss, safety and dependence remains an issue. Amidst approval for new weight loss drugs such as Contrave® in 2014, regulatory bodies such as the Food and Drug Administration (FDA) have warned that such drugs have the potential for abuse, and increase the risk of serious suicidal thoughts, seizures and hypertension in some patients (FDA, 2014). Natural products have long been an alternative to drugs in the treatment of diseases (Newman & Cragg, 2012). This makes the search for effective and safe weight loss prescriptions sourced from natural products an attractive pursuit for the management and treatment of obesity, as previous studies have reported (Vasudeva, Yadav, Sharma, 2012; Han, Kimura, Okuda, 2005; Mohamed, Ibrahim, Elkhayat, El Dine, 2014; Yun, 2010).

Malaysia is blessed with high biodiversity on land and in the sea which may be exploited to discover new natural products. This study focuses on three brown seaweeds harvested from Malaysian waters *Sargassum oligocystum, Padina australis* and *Dictyota dichotoma*. The main bioactive compound reportedly contributing to antiobesity activity in brown seaweeds is the carotenoid fucoxanthin (Maeda et al., 2005, 2006). This study aimed to establish the antiobesity potential of selected Malaysian brown seaweeds *Sargassum oligocystum, Padina australis* and *Dictytota dichotoma* via their action on adipocyte differentiation (adipogenesis) and metabolism (adipolysis) and to optimize extraction parameters to retain maximum fucoxanthin content in the extract.

1.2 SCOPE AND OBJECTIVES

The study focused on the anti-obesity potential of selected Malaysian brown seaweeds *Sargassum oligocystum, Padina australis* and *Dictyota dichotoma* harvested from the west coast of Port Dickson via adipogenesis and adipolysis. Optimization steps for high extraction yields of the target compound, fucoxanthin were also studied. The specific objectives were:

- To determine optimal solvent, particle size and method of extraction of brown seaweeds to achieve high yields of fucoxanthin
- To determine the effect of fucoxanthin-rich extracts of seaweed on adipogenesis in 3T3-L1 cells
- To determine the effect of fucoxanthin-rich extracts of seaweeds on adipolysis in 3T3-L1 cells
- To link between fucoxanthin content and antiobesity activity present in the seaweed extracts

1.3 HYPOTHESIS

Optimal parameters for high retention of fucoxanthin in the seaweed would be different according to the nature of the seaweed. Malaysian brown seaweed extracts containing fucoxanthin would exert anti-adipogenic and pro-adipolytic activities towards 3T3-L1 cells.

1.4 BRIEF EXPLANATION ON THE FLOW OF EXPERIMENTS

Briefly, the study consisted of two major parts: firstly, optimizing the extraction process of the brown seaweeds to ensure high retention of fucoxanthin and secondly,

applying the extracts to 3T3-L1 fat cells to establish the adipogenic and adipolytic effects on the locally sourced brown seaweeds.

Fucoxanthin is the main compound reported to be responsible for anti-adipogenic activity in brown seaweeds, as reported by previous studies (Maeda, Hosokawa, Sashima, Takahashi, Kawada, Miyashita; 2006; Maeda, Hosokawa, Sashima, Funayama, Miyashita; 2005). Therefore, an important consideration during extraction is to ensure that fucoxanthin is retained in the extract during processing. In the first part of this study, important parameters were controlled to optimize extraction: solvent, particle size used and extraction method. Up to three solvents with different degrees of polarity were used: acetone, methanol and ethanol. Two methods were used for extraction: Soxhlet extraction and room temperature maceration (26 °C ± 2 °C). Different particle sizes were achieved after grinding by sieving the dried plant powder and passed through different mechanical sieve sizes: 125 µm, 500 µm and 1000 µm (1 mm). Fucoxanthin in the extracts was quantified using HPLC. The results were tabulated and analysed using SPSS. Extracts containing the highest fucoxanthin content from each type of brown seaweed was selected for the second part of the study.

The second part of the study involved investigating the effect of the extracts from different Malaysian brown seaweeds on 3T3-L1 cells. 3T3-L1 cells are well-established models for understanding lipid metabolism. Adipogenesis assay was carried out using an adipogenesis assay kit. Extracts were applied during the differentiation stage to study the effect of extracts on preadipocyte differentiation. The cells were stained with Oil Red O and cell differentiation was quantified using a microplate reader. Meanwhile, adipolysis assay was carried out on differentiated cells to study the effect of the extracts on release of triglycerides from the cells. The

preadipocytes were allowed to mature before application of extracts. Glycerol release in the cell media was quantified using a microplate reader. Suitable concentrations of extracts for the study were determined using MTT assay. Cells were treated at different concentrations of the extract to determine the cutoff point to ensure cell viability. A general flowchart of the entire research is presented in Figure 1.1.

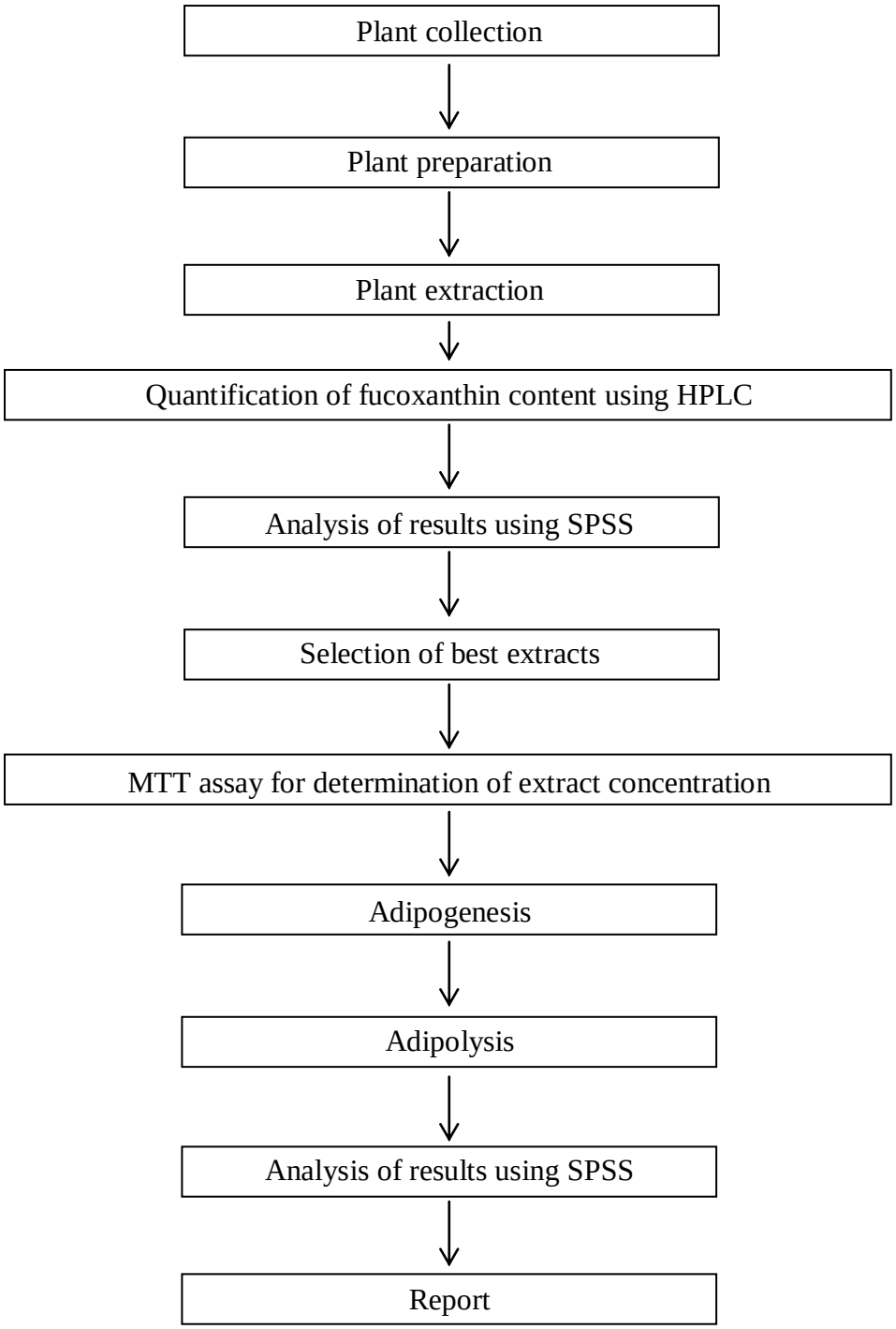

Figure 1.1 General flow chart of experiment