UNIVERSITI PUTRA MALAYSIA

EVALUATION ON SUBCRITICAL WATER EXTRACTION OF PHENOLIC COMPOUNDS FROM CHLORELLA SP. MICROALGAE

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By

SITI MAISURAH BINTI ZAKARIA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

EVALUATION ON SUBCRITICAL WATER EXTRACTION OF PHENOLIC COMPOUNDS FROM *CHORELLA* SP. MICROALGAE

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Chlorella sp. microalgae are considered to be an important functional food and a source of nutrients due to their abundance and positive health effects. This study focused on utilization of green solvent as alternative to replace chemical solvent that conventionally used to extract phenolic compounds from microalgae for food and health purposes. Subcritical water extraction (SWE) is the best alternative for this purpose. The use of pressurised hot water and high temperature during the subcritical phase allows the dielectric constant and polarity of water to be modified, which then contributes to an improved extraction process. The extraction of phenolic compounds was studied at different temperatures (100-300°C), times (5-30 min) and solid loading (5-30 wt.%). Results from SWE experiments have shown that this technique has recovered a higher value of phenolic compounds (58.45 mgGAE/g) and antioxidant activity (67.03%) with a lower extraction time (up to 20 min) when compared to conventional soxhlet method using methanol with only 36.46 mgGAE/g phenolic content and 54.83% of antioxidant activity. The release of phenolic compounds into the subcritical water resulted in a mixture of phenolic acids (ferulic, caffeic and ρ-coumaric acids), and these acids showed significant antioxidant properties. The results showed the acid release began during the initial heating up stage at higher temperature. Using response surface methodology, the optimal operating conditions for the extraction process were found; 5 min at 163 °C with 20 wt.% of solid loading resulting in products with 58.73 mgGAE/g total phenolic content and 68.5% antioxidant activity. The phenolic content was also highly correlated (R^2 = 0.935) with the antioxidant capacity. Kinetic studies have revealed that the extraction from *Chlorella* sp. microalgae using subcritical water treatment followed first order kinetics and the extraction was dependent on temperature. As a result of the increasing temperature, a rising trend in the estimated values of the mass transfer coefficient, *k,* was observed. The activation energy, Ea, was calculated as 11.146 kJ/mol for the extraction treatment. The value of thermodynamic activation parameters ($\Delta S^{\neq}, \Delta H^{\neq}, \Delta G^{\neq}$) are affected by increasing the water temperature, which related to an increase

of extraction activity at higher temperature. The findings from this study demonstrated that *Chlorella* sp. extracts possessed a strong antioxidant activity. They also highlighted the potential application for subcritical water, as a green approach, that is able to produce high quality extracts from *Chlorella* sp. as a source of natural bioactive compounds for the food and pharmaceutical industries.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN KE ATAS PENGEKSTRAKAN AIR SUBKRITIKAL BAGI SEBATIAN FENOLIK DARIPADA MIKROALGA *CHLORELLA* SP.

Oleh

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Chlorella sp. mikroalga dipertimbangkan sebagai makanan pelbagai fungsi dan sumber nutrien penting diatas faktor kuantitinya yang banyak dan kesan positif kepada kesihatan. Kajian ini memberi fokus kepada penggunaan pelarut hijau sebagai alternatif untuk menggantikan pelarut kimia yang digunakan secara konvensional untuk mengekstrak sebatian fenolik daripada mikroalga untuk kegunaan makanan dan kesihatan. Pengekstrakan dengan air subkritikal (SWE) adalah alternatif terbaik untuk tujuan ini. Penggunaan air bertekanan dan suhu tinggi dalam fasa subkritikal membolehkan pemalar dielektrik dan polariti bagi air untuk diubah, yang menyumbang kepada proses pengekstrakan yang lebih baik. Pengekstrakan sebatian fenolik dikaji pada suhu (100-300°C), masa (5-30 min) dan kandungan pepejal (5-30wt.%) yang berbeza. Keputusan daripada eksperimen menggunakan SWE menunjukkan bahawa teknik ini berjaya mendapat nilai sebatian fenolik (58.45 mgGAE/g) dan aktiviti antioksidan (67.03%) yang lebih tinggi dengan masa yang lebih singkat (sehingga 20 min) berbanding proses pengekstrakan konvensional soxhlet menggunakan metanol dengan hanya 36.46 mgGAE/g kandungan fenolik dan 54.83% aktiviti antioksidan. Pengekstrakan sebatian fenolik dalam air subkritikal mengandungi campuran asid fenolik (asid ferulik, kafeik dan ρ-kumarik) yang menunjukkan sifat antioksidan yang penting. Keputusan menunjukkan pengekstrakan bermula semasa pemanasan awal pada suhu yang tinggi. Dengan menggunakan metodologi permukaan tindak balas, nilai optimum untuk proses pengekstrakan adalah 5 min pada suhu 163 ° C dengan 20% kandungan pepejal, yang memberikan produk dengan 58.73 mgGAE/g kandungan fenolik dan 68.5% aktiviti antioksidan. Kandungan fenolik juga sangat berkorelasi (R^2 = 0.935) dengan kapasiti antioksidan. Kajian kinetik mendedahkan pengekstrakan daripada mikroalga *Chlorella* sp. menggunakan air subkritikal didapati mengikut aturan kinetik pertama dan bergantung kepada suhu. Tenaga pengaktifan (Ea) dikira sebanyak 11.146 kJ/mol untuk keseluruhan proses pengekstrakan. Nilai bagi parameter pengaktifan termodinamik ($\Delta S^{\neq}, \Delta H^{\neq}, \Delta G^{\neq}$) adalah dipengaruhi oleh peningkatan suhu air dan ia berkait dengan peningkatan aktiviti

pengekstrakan pada suhu yang lebih tinggi. Hasil kajian menunjukkan bahawa ekstrak *Chlorella* sp. mempunyai aktiviti antioksidan yang kuat. Ia juga menekankan potensi penggunaan teknologi air subkritikal sebagai pendekatan hijau untuk menghasilkan ekstrak berkualiti tinggi daripada *Chlorella* sp. sebagai sumber sebatian bioaktif semulajadi untuk industri makanan dan farmaseutikal.

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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 Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

SWE	subcritical water extraction
RSM	response surface methodology
HPLC	high performance liquid chromatography
UV-VIS	ultraviolet–visible
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
MTT	3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
ANOVA	analysis of variance
ROS	reactive oxygen species
DNA	deoxyribonucleic acid
UV	ultraviolet
SEM	scanning electron microscopy
FRAP	ferric reducing ability of plasma
GAE	gallic acid equivalent
TAE	tannic acid equivalent
DOE	design of experiments
CCD	central composite design
min	minutes
R^2	coefficient of determination
F-value	fisher variance
SS	sum of the square
DF	degree of freedom
V	variance
p	number of model parameter
σ^2	residual mean square
n	number of experiment
t	time
C_L	concentrations of compounds in the liquid phase
C_{Le}	concentrations of compounds at equilibrium
k	mass transfer coefficient
Y_t	yield of compounds in the liquid phase
Y_{Le}	yield of compounds at equilibrium
R	universal gas constant
T	absolute temperature
A	pre-exponential factor or frequency factor
Ea	activation energy
J	joule
N	Avogadro's constant;
h	Planck's constant;
$\Delta S^{\neq}$	activation entropy (J/mol K);
$\Delta H^{\neq}$	activation enthalpy (kJ/mol);
$\Delta G^{\neq}$	activation free energy or Gibbs energy (kJ/mol).

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Development of new functional ingredients from natural resources became one of the main areas to focus on in the fields of food science and pharmaceuticals (Joana Gil-Chávez et al., 2013; Vieira da Silva et al., 2016). The focus on these areas has been driven by the increase in the planetary human population and the excessive demand for healthy and nutritive products particularly for use as food or in pharmaceuticals (West and Zubeck, 2012). Bioactive compounds can be applied as natural preservatives across food degradation and also as functional ingredients for health enhancement (Joana Gil-Chávez et al., 2013). The compounds have been naturally incorporated in limited quantities and are exist as mixtures in extracts which have involved long and labour-intensive purification procedures (Lam, 2007). Therefore, in order to overcome these limitations, there has been a move towards the advancement of better technologies that will enhance the process of screening and the production of bioactive compounds from natural sources.

The production of microalgae has been increased significantly due to the demand for its beneficial compounds and nutritive contents. Microalgae have been used in various applications; food, food additives, aquaculture, colourants, cosmetics, pharmaceuticals, and nutraceuticals (Liang et al., 2004). In 1970s, the world energy crisis has driven to the recognition of algae as the sources of renewable and sustainable biofuels production, inciting the analysis of microalgae as a new topic of study for fuels and other beneficial products (Paul Abishek et al., 2014). In 1960s, the first large-scale culture of the algae has extended to new areas, such as food and feed, biofuels, and biopharmaceuticals. Algal extracts contained natural products and has been used as cosmetics and medicinal products (Plaza et al., 2009). By estimation, about US$ 1.25 × 10^9 generated each year by 5000 metric tons of dry algal biomass processed for bioproducts application (Pulz and Gross, 2004).

In Malaysia, extensive coastline surrounded by numerous islands offer variety of perfect habitats for the practicability of algae mass cultivation. In total, marine algae in Malaysia now stands at 375 specific and intra-specific taxa with reference to the regular collections and documentations of algae strains can be dated back to the year of 1859 (Phang, 2006). In addition, Malaysian algae research mainly focused on identification of native microalgae strains, utilized in wastewater treatment, bioindicators of heavy metal pollution and control of mosquito breeding. The fundamental research in algae studies have shown in many publications of checklists and monographs that documented the microalgae diversity in Malaysia (Renganathan and Mohd, 2016).

The microalgae such as *Arthrospira* (*Spirulina*), *Chaetoceros*, *Chlorella, Dunaliella* and *Isochrysis* are the dominating genera in commercial scale cultivation (Lee, 1997). Surveys on ecological of microalgae were recorded on few aspects including distribution, zonation, water quality, frequency in different places in Malaysia. This feature determine competitive advantage under the local geographical, climatic and ecological conditions that these algae species have large potential for offering new bioactive compounds, chemicals, materials. Presently, 31 countries and territories are recorded with algae production, and 99.6% of global cultivated algae production comes only from eight countries and Malaysia produced 1.1% (207,900 tons) of cultivated microalgae (Renganathan and Mohd, 2016). Figure 1.1 shows the microalgal production in Malaysia by comparison to major algae producing countries.

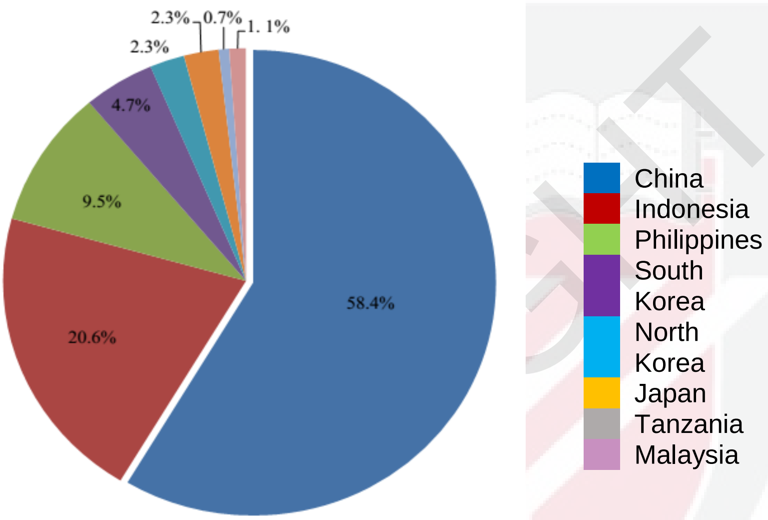

Figure 1.1: Annual microalgal production in Malaysia by comparison to major algae producing countries (adapted from Renganathan and Mohd, 2016)

Chlorella sp*.* are unicellular green microalgae found in many aquatic systems and have been extensively used as a food supplement and health food. This particular microalgae is considered as a valuable and important functional food and source of nutrients in many fields due to its abundance and positive health effects. Extracts of *Chlorella* sp*.* have been documented as possessing diverse antitumor properties (Konishi et al., 1985), performing as antioxidants (Miranda et al., 2001) and demonstrating antimicrobial activity (Hasegawa et al., 1989). *Chlorella* sp. is able to decrease blood pressure, lower cholesterol levels, accelerate wound healing, and improve the immune system (West and Zubeck, 2012). *Chlorella* sp. is also a potential natural source of antioxidant. However, there have been few reports on extracting phenolic compounds from *Chlorella* sp. and evaluating their antioxidant activities.

Phenolic compounds comprise a major group in algae and plants. They are secondary metabolites that are broadly allocated and show an abundant structural diversity (Balasundram et al., 2006). The compounds occur as glycones or glycosides, monomers or constituting well polymerised structures and as free or matrix-bound compounds (Balasundram et al., 2006; Umar Lule and Xia, 2005). Phenolics, in general, have proved that the beneficial health effects of plants and microalgae, and their contribution to the antioxidant capability within the human diet, is much higher than that of vitamins. A combination of free-radical scavenging and epidemiological studies have shown that there were an inverse relationship between the intake of phenolic compounds, in particular, and the incidence of certain diseases and malignancies (Rice-Evans et al., 1997). There are few studies which researched the effect of phenolic intake and their role in the prevention or control of various diseases including different cancers and other diseases such as diabetes, heart diseases and asthma (Knekt et al., 2002).

Subcritical water extraction (SWE) employs high temperatures and pressure to maintain water in its liquid phase, accomplishing safe, “green” and fast extractions. High temperatures alter the dielectric constant of the water which is the solvent. As a result, it is possible to modify the polarity of water and obtain selective extractions. SWE is, therefore, an advantageous option for obtaining extracts from microalgae functional compounds and which is also compatible with food and health regulations (Rodríguez-Meizoso et al., 2010). The conditions under which SWE operates mean that the water is kept in a liquid state during the entire extraction procedure. The SWE technique offers several important advantages compared to other traditional extraction methods; it is faster, usually generates higher yields, and the need of solvents can be considerably reduced (Huie, 2002).

1.2 Problem Background

Chlorella sp. is the most widely cultivated species of microalgae that is possible for the human diet. However, very little is known about the properties and benefits of the microalgae’s phenolic content. Previous studies have revealed that *Chlorella* sp. had the highest total phenolic content when compared to a few samples of various other strains of microalgae (Hajimahmoodi et al., 2010). The reports also presented the information that phenolic compounds were major contributors to the capacity of microalgae as an antioxidant. In addition, a limiting factor to the commercialisation of microalgae is the low concentration yield (Joana Gil-Chávez et al., 2013). Therefore, it is necessary to develop an effective extraction method to retrieve phenolic compounds from *Chlorella* sp. microalgae.

Microalgae is the microorganism that composed of a nucleus, starch grains, chloroplasts and mitochondria surrounded by a cell wall. The rigidity of cell wall maintains the integrity of the cell and is primarily a protection against invaders and harsh environment. Before extraction process, microalgae cell disruption process (eg. high-pressure homogenization method) is needed to facilitate a better diffusion of microalgal compounds into the extraction solvent. Disintegration of intact cells throughout cell disruption contribute to the liberation and released of intracellular compounds from the cellular structures to the surrounding medium. (Halim et al., 2012). Therefore, the next step which is extraction involves direct interaction between the eluting extraction solvent with these compounds without penetrating into the cell wall structures.

Most of the studies on the extraction of phenolic compounds use organic solvents such as methanol, ethanol and acetone to extract the compounds from various materials (Klejdus et al., 2009; Suárez et al., 2010; Sun et al., 2015). Moreover, various extraction methods have been developed for extracting bioactive compounds; such as microwave, ultrasonic and soxhlet (Dey and Rathod, 2013; Hasmida et al., 2014). However, these techniques often suffer from low extraction yields, lengthy processing steps, and they use toxic organic solvents. Since SWE employs less purity of chemical solvent, and as a promising green process, it is a credible choice for the extraction of phenolic compounds from *Chlorella* sp. microalgae. Moreover, there have been no reported studies on the recovery of phenolic compounds from *Chlorella* sp. using SWE.

The functional and biological properties (antioxidant activity) of *Chlorella* sp. extracts depend on its phenolic composition. However, the information regarding the identification and quantification of phenolic composition in microalgae is limited (Cifuentes et al., 2006; Jaime et al., 2005; Klejdus et al., 2009). Therefore, it is also necessary to investigate and identify the major phenolic acids in the extracts and how their concentration changed during SWE.

As the dielectric constant of water is decreased by increasing the temperature during SWE, it is essential to recognise the variability of dielectric constants for different types of compounds and, for this study, phenolic compounds. Careful management of parameters, such as time and temperature, is extremely important to enhance the extraction process and accodingly improve the economic feasibility of the process (Azmir et al., 2013). Hence, rigorous quantification of the extraction kinetics is crucial in order to maintain the optimum characteristics of water and enable a satisfactory extraction process.

1.3 Hypothesis

The SWE process involves the application of water under high pressure to maintain its liquid state, at temperatures that are above the boiling point of water (100 - 374°C). As the temperature rises, the solubility of compounds is increased, which also raises the mass transfer and decreases the viscosity and the surface tension. These changes facilitate better penetration of solvent in the matrix (Ong et al., 2006). Generally, the dielectric constant of a solvent decreases when the temperature is raised which indicates that the polarity of a solvent can be tuned using temperature alteration (Ibañez et al., 2012). For water as a solvent, the dielectric constant is around 80 at 25^{0}C and it can decrease to 55 at 100^{0}C and 27 at 250^{0}C (Uematsu and Franck, 1980), which is closer to the dielectric constant of organic solvent such as methanol and ethanol. Therefore, depending on the applied temperature, the extraction is capable of selectively extracting various classes of compounds. The selectivity of SWE enables the manipulation of the composition of the extract by changing the operating parameters (Fernández-Pérez et al., 2000; Herrero et al., 2006). Therefore, SWE is feasibly expected to be able to extract certain compounds; this study aimed to extract phenolic compounds from *Chlorella* sp. microalgae. Furthermore, the temperature applied and time taken during SWE can greatly influence the extraction yield of phenolic compounds (Kashif et al., 2017). As a result, a kinetics study was found to be necessary in order to evaluate the factors which affect the extraction rate.

1.4 Research Objectives

The research was carried out according to the following objectives:-

i) to evaluate the effects of SWE process condition on the extraction of phenolic compounds from *Chlorella* sp. and its antioxidant activity.
ii) to optimize the operating conditions of SWE; extraction temperature, time and solid loading as a function of the total phenolic content and antioxidant activity.
iii) to investigate the kinetics and thermodynamics parameters for SWE of phenolic compounds from *Chlorella* sp.

1.5 Research Scope

Chlorella sp. microalgae are a remarkable source of phenolic compounds that also possess strong antioxidant activity. This study was aimed at the extraction of phenolic compounds from *Chlorella* sp. microalgae using a green extraction solvent treatment known as SWE.

Conventional soxhlet extraction was performed using three different solvents (ethanol, methanol and water) in order to evaluate the performances of the organic solvent and ability of water to extract the phenolic compounds from *Chlorella* sp. Extraction by water in a subcritical condition was conducted by varying the process parameters. The extractions were monitored by measuring the total phenolic content using the Folin–Ciocalteu method and the antioxidant activity using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

The extracts from all experiments were characterised in terms of their phenolic acid composition. High Performance Liquid Chromatography (HPLC) analysis was applied to determine the major phenolic acid extracted from *Chlorella* sp. and how the concentration of the acid was changed during the extraction. These compounds are believed to be the main cause of antioxidant activity in the extracts.

SWE optimization of phenolic compounds from *Chlorella* sp. is performed with various extraction parameters, such as temperature (°C), time (min) and solid loading (wt. %). This study focused on maximising the recovery of phenolic compounds and the antioxidant activity of the extracts. Response surface methodology (RSM) was then employed to find out the optimal operating conditions for the extraction process.

The data from SWE experiments were used to establish the kinetics of the extraction process and, therefore, understand the factors affecting the extraction rate. The control mechanism for the extraction of phenolic compounds from *Chlorella* sp. was mass transfer, and the kinetics was derived from mass transfer rate equation. A convective mass transfer model was applied to simulate the extraction process and estimate the kinetics parameters. The extraction activation energy was determined using the Arrhenius equation. Thermodynamics activation parameters for this process including activation Gibbs energy, activation enthalpy, and activation entropy are determined utilizing transition state theory.

1.6 Significance of the study

This research has shown that extracts with high phenolic content and high antioxidant activity can be recovered from *Chlorella* sp. microalgae using the SWE treatment. A better insight into the extraction mechanism is able to contribute to a higher product yield. In addition, factors affecting the extraction rates can be identified and have contributed to the development of an effective method for recovery of phenolic compounds using safe and green solvent. In addition, Malaysia offer variety of ideal habitats for the feasibility of microalgae mass cultivation. The finding of this research will be of value to the food and pharmaceutical industries in exploiting the abundant microalgae resources in Malaysia as a renewable source of phenolic and various bioactive compounds.

1.7 Organisation of thesis

This thesis consists of five chapters. It starts with chapter one that provides an outline of the overall research study including the introduction of microalgae as the source of bioactive compounds and current scenario of bioactive compound extraction including SWE. The problem statement reveals the importance of this research study. The objectives of this research project were then formulated to address the needs on development of phenolic compounds from microalgae through the approach of SWE. This is followed by the thesis organisation that highlights the content for each chapter.

Chapter two gives an overall review on various research works that reported in the literatures which includes the properties of microalgae as desirable sources for bioactive compounds and SWE process as one of the most applicable extraction method. The current status and application of SWE are also presented followed by the review on the effects of SWE as the green technique on the quality of the extracts including antioxidant and antimicrobial activities. Statistical analysis by response surface methodology (RSM) and kinetics and thermodynamics studies on the extraction process were also included in this chapter.

Experimental materials and methodology were presented in chapter three. This chapter describes the details on the overall flow of research works, chemicals used and the experimental methods in conducting this research. The extraction process, phenolic compounds determination, and compound analysis were described in this chapter. Is it also included with statistical method and kinetics and thermodynamics studies of SWE.

Chapter four presents the detail discussion on the results obtained in the research work. This chapter has been divided according to the stages of this research work. Five main section were presents in Chapter 4, which includes (1) the extraction of phenolic compounds from *Chlorella sp.* by water and organic solvents using classical soxhlet extraction method, (2) the study on all parameters involved in SWE namely temperature, time and solid loading, (3) the analysis on phenolic compounds composition in the extracts, (4) the process optimization using RSM and (5) the extraction kinetics on each selected temperature, calculation of activation energy and determination on its thermodynamic activation parameters.

Chapter five provides the conclusions on the results obtained in this particular research study. It concludes the overall research work and gives some recommendations for future studies.

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LIST OF PUBLICATIONS

This research produces three manuscripts for publication listed as following:

Siti Maisurah Zakaria and Siti Mazlina Mustapa Kamal, 2016. Subcritical water extraction of bioactive compounds from plants and algae: Applications in pharmaceutical and food ingredients. Food Engineering Reviews, 8(1), pp.23-34.

Siti Maisurah Zakaria, Siti Mazlina Mustapa Kamal, Mohd. Razif Harun, Rozita Omar and Shamsul Izhar Siajam, 2017. Extraction of antioxidants from *Chlorell*a sp. using subcritical water treatment. IOP Conference Series: Materials Science and Engineering, 206(1).

Siti Maisurah Zakaria, Siti Mazlina Mustapa Kamal, Mohd. Razif Harun, Rozita Omar and Shamsul Izhar Siajam, 2017. Subcritical water technology for extraction of phenolic compounds from *Chlorella* sp. microalgae and assessment on its antioxidant activity. Molecules, 22(7), p.1105.

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