

OPTIMIZATION OF  $\beta$ -SITOSTEROL FROM *SWIETENIA MAHAGONI* SEEDS  
EXTRACT USING SUPERCRITICAL CARBON DIOXIDE (SC-CO<sub>2</sub>)  
EXTRACTION AND ITS ANTI-DIABETIC ACTIVITIES

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I dedicate this thesis to my parent for their encouragement and motivation

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## ABSTRACT

*Swietenia mahagoni* is broadly used in traditional remedies. It contains several bioactive compounds such as  $\beta$ -sitosterol. The aim of this study is to investigate the effect of supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction conditions (pressure and temperature) on oil yield and  $\beta$ -sitosterol content from *swietenia mahagoni* seeds and to analyze the anti-diabetic activities of *swietenia mahagoni* seeds extract. *Swietenia mahagoni* seeds extracts were obtained by SC-CO<sub>2</sub> extraction at pressures of 20-30 MPa and temperatures of 40-60 °C. The optimization process was done by a three-level factorial design with a response surface methodology using Statistica software 7.0 (StatSoft, EUA). Quantification of  $\beta$ -sitosterol content was performed by using a high performance liquid chromatography conducted on C18 reversed phase Kinetex Biphenyl column using methanol: acetonitrile (60:40 v/v) as mobile phase. The oil yields at different conditions obtained were analyzed with  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibition assays. One way analysis was used for statistical significance by using Statistica software 7.0 (StatSoft, EUA) and IC<sub>50</sub> (extract concentration causing 50% enzyme inhibitory) was determined by using GraphPad Prism 6.0 software. The experimental data of SC-CO<sub>2</sub> extraction obtained were fitted to a second-order polynomial model and the obtained oil yields were between 1.5-14.5%, while  $\beta$ -sitosterol content obtained were 3.1-9.2 mg/g. The highest inhibition of  $\alpha$ -glucosidase and  $\alpha$ -amylase enzymes were 98.4%  $\pm$  0.2 and 34.9%  $\pm$  1.2, respectively. The best conditions within the studied ranges were 30 MPa and 40 °C to extract  $\beta$ -sitosterol in the highest amount. All conditions for extraction of *swietenia mahagoni* seeds were potent to  $\alpha$ -glucosidase inhibitory assay but showed a weak inhibition toward  $\alpha$ -amylase inhibitory assay. These findings implied that *swietenia mahagoni* seeds extract containing  $\beta$ -sitosterol, could be an effective natural anti-diabetic agent.

## ABSTRAK

*Swietenia mahagoni* secara umumnya digunakan dalam perubatan tradisional. Ia mengandungi beberapa sebatian bioaktif seperti  $\beta$ -sitosterol. Tujuan kajian ini adalah untuk mengkaji kesan keadaan (tekanan dan suhu) pengekstrakan karbon dioksida superkritikal (SC-CO<sub>2</sub>) pada hasil minyak dan kandungan  $\beta$ -sitosterol daripada biji *swietenia mahagoni* dan untuk menganalisis aktiviti anti-diabetik dari ekstrak biji *swietenia mahagoni*. Minyak dari biji *swietenia mahagoni* diperolehi dengan pengekstrakan SC-CO<sub>2</sub> pada tekanan 20-30 MPa dan pada suhu 40-60 °C. Proses pengoptimuman dilakukan dengan menggunakan reka bentuk faktorial tiga peringkat oleh kaedah tindak balas permukaan menggunakan perisian Statistica 7.0 (StatSoft, EUA). Pengkuantitan kandungan  $\beta$ -sitosterol telah dilakukan dengan menggunakan kromatografi cecair berprestasi tinggi yang dijalankan pada kolum C18 fasa berbalik Kinetex Bifenil menggunakan pelarut metanol: asetonitril (60:40 v/v) sebagai fasa bergerak. Kadar hasil minyak yang diperolehi pada keadaan berbeza dikaji dengan analisa perencatan enzim  $\alpha$ -glukosidase dan enzim  $\alpha$ -amilase. Analisa sehalu telah digunakan untuk kegunaan statistik dengan menggunakan perisian Statistica 7.0 (StatSoft, EUA) dan IC<sub>50</sub> (kepekatan ekstrak yang menyebabkan 50% perencatan enzim) telah ditentukan dengan menggunakan perisian GraphPad Prism 6.0. Data eksperimen yang diperolehi dari pengekstrakan SC-CO<sub>2</sub> telah dipadankan dengan model polinomial tertib kedua dan hasil minyak diperolehi adalah 1.5-14.5%, manakala kandungan  $\beta$ -sitosterol diperolehi adalah 3.1-9.2 mg/g. Perencatan tertinggi enzim  $\alpha$ -glukosidase dan enzim  $\alpha$ -amilase masing-masing adalah 98.4%  $\pm$  0.2 dan 34.9%  $\pm$  1.2. Keadaan pengekstrakan yang terbaik adalah pada julat 30 MPa dan 40 °C untuk mengekstrak  $\beta$ -sitosterol dalam jumlah yang paling tinggi. Semua keadaan perahan benih *swietenia mahagoni* menunjukkan perencatan yang kuat pada enzim  $\alpha$ -glukosidase tetapi menunjukkan perencatan yang lemah pada enzim  $\alpha$ -amilase. Penemuan ini menunjukkan bahawa ekstrak biji *swietenia mahagoni* yang mengandungi  $\beta$ -sitosterol, berkesan untuk dijadikan agen anti-diabetik semulajadi.

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**LIST OF ABBREVIATION**

SC-CO <sub>2</sub>	-	Supercritical carbon dioxide
RSM	-	Research surface methodology
CO <sub>2</sub>	-	Carbon dioxide
<i>S. mahagoni</i>	-	<i>Swietenia mahagoni</i>
HPLC	-	High performance liquid chromatography
IC <sub>50</sub>	-	Half maximal inhibitory concentration
CCD	-	Central composite design
BBD	-	Box-Behnken design
ANOVA	-	Analysis of variance
p-value	-	Probability value
NaCO <sub>3</sub>	-	Sodium carbonate
NaNO <sub>2</sub>	-	Sodium nitrite
NaOH	-	Sodium hydroxide
AlCl <sub>3</sub>	-	Aluminium chloride
DOE	-	Design of experiment

**LIST OF SYMBOLS**

$M_c\%$	-	Percentage of moisture content
$m_0$	-	Mass in grams of dish
$m_1$	-	Mass in grams of dish and sample before drying
$m_2$	-	Mass in grams of dish and sample after drying
mm	-	Millimetre
$\mu\text{m}$	-	Micrometre
g	-	Gram
mL	-	Millimetre
MPa	-	Megapascal
K	-	Kelvin
$^{\circ}\text{C}$	-	Degree celcius
$P_c$	-	Critical pressure
$T_c$	-	Critical temperature
$X_i$	-	Variables
$Y_i$	-	Responses
P	-	Density

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## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of The Study

*Swietenia mahagoni* (*S. mahagoni*), also known as ‘tunjuk langit’ in Malaysia is used traditionally to treat various diseases such as diabetes and high blood pressure (Goh *et al.*, 2010). *S. mahagoni* are usually 30 meters or taller (Eid *et al.*, 2013) and its wood, is usually being used for making furniture (Falah *et al.*, 2008). Meanwhile, the bark can be used for natural colorant (Haque *et al.*, 2013). The fruits of *S. mahagoni* are woody and consists of capsules containing winged seeds (Blundell and Gullison, 2003). Whereas, the seed of *S. mahagoni* can be obtained by removing the wing. In addition, *S. mahagoni* seeds have been reported to have various biological activities such as anti-inflammatory activity, anticancer and antitumor activity (Goh and Kadir, 2011) as well as antidiabetic activity (Maiti *et al.*, 2009).

$\beta$ -sitosterol is one of the diversified group of compounds in phytosterols. Phytosterols are well known as plant sterols, one of the vital components of plant membranes (Schuler *et al.*, 1991). The most ample compound in natural sterols is  $\beta$ -sitosterol (Saeidnia, 2014) and it can be found in seeds, nuts, vegetables and fruits. Furthermore,  $\beta$ -sitosterol have been reported to have various pharmacological activities such as anti-inflammatory activity (Loizou *et al.*, 2010), chemopreventive effects (Ju *et al.*, 2004), hypocholesterolemic activity (Miettinen and Gylling, 2002), antioxidant effects (Vivancos and Moreno, 2005) and also antidiabetic effects (Gupta *et al.*, 2011).

Currently, 117 million diabetes cases were reported and expected to rise by 336 million by the end 2030 (Adefegha *et al.*, 2014). Diabetes is a prevalent disease depicted by distorted glucose level in blood also known as hyperglycemia (Kumar *et al.*, 2011). Hyperglycemia can be depicted by the disorder of  $\beta$ -cells, insulin inadequacy and distorted glucose level in blood (American Diabetes Association, 2009). In managing diabetes mellitus, one of the efficacious ways is to delay the glucose level and to achieve this is by the inhibition of carbohydrate-digesting enzymes such as  $\alpha$ -glucosidase and  $\alpha$ -amylase (Kumar *et al.*, 2011). In the final digestive process,  $\alpha$ -glucosidase acts as a vital enzyme in catalyzing the disaccharides and oligosaccharides into glucose (Cheng and Fantus, 2005). Meanwhile,  $\alpha$ -amylase implies in catalyzing starch to disaccharides and oligosaccharides. Thus,  $\alpha$ -glucosidase and  $\alpha$ -amylase inhibitors can be used to delay the release of d-glucose from carbohydrate which also delays the absorption of glucose in the small intestine (Casirola and Ferraris, 2006; Wresdiyati *et al.*, 2015). Hence, reducing the glucose level in blood and repression of postprandial hyperglycemia (PPHG). By retaining the reduction of hyperglycemia, the risk of developing microvascular and macrovascular complications can be reduced (Tas *et al.*, 2005).

*S. mahagoni* seeds are also reported to have various biological activities such as anti-inflammatory activity, anticancer and antitumor activity (Goh and Kadir, 2011) and also antidiabetic activity (Maiti *et al.*, 2009). In Malaysia, the raw seeds have been used for hypertension and diabetes (Balijepalli *et al.*, 2014). Supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction is a separation process of matters using supercritical carbon dioxide as a solvent. Carbon dioxide (CO<sub>2</sub>) is the most frequent solvent used that is environmentally friendly (fairly non-toxic), low cost and can be easily removed from the extract (Machmudah *et al.*, 2007; Krichnavaruk *et al.*, 2008; Xu *et al.*, 2011; Md Salleh, 2012; Viganó *et al.*, 2016). The elimination of CO<sub>2</sub> is easily achieved as CO<sub>2</sub> is in a gas state at room temperature. In addition, CO<sub>2</sub> in the supercritical state is in a moderate critical temperature (31.3°C) and pressure (7.38 MPa). Supercritical state is when gas and liquid are indistinguishable where at this state it is compressible but possesses the density of a liquid. In a word, supercritical CO<sub>2</sub> makes a good solvent due to the gas-like state that is attributed to the low viscosity and high diffusion

coefficient and the liquid-like state that gave the solvating power (Aionicesei *et al.*, 2008).

In this study, the aim is to determine the effect of pressure and temperature of supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction on oil yield and  $\beta$ -sitosterol content from *S. mahagoni* seeds using a response surface methodology (RSM). Furthermore, the evaluation of antidiabetic activity of *Swietenia mahagoni* seed extract from SC-CO<sub>2</sub> extraction will be carried out. The antidiabetic activity of *S. mahagoni* seeds extract was analyzed by an *in vitro* diabetic model (inhibition of carbohydrate-digesting enzymes). Moreover, the correlation of  $\beta$ -sitosterol and the inhibitory activities of  $\alpha$ -glucosidase and  $\alpha$ -amylase were also evaluated.

## 1.2 Problem Statement

According to World Health Organization (WHO), approximately 80% of world population prefer traditional medicine rather than modern approach. Relatable, synthetic oral antidiabetic agents such as acarbose was reported with side effect and failure to reduce diabetes complications (Triggle and Ding, 2014). Therefore, interest for natural oral antidiabetic agent from medicinal plants is in demand. *Swietenia mahagoni* in Malaysia is used traditionally to treat various diseases such as diabetes and high blood pressure (Goh *et al.*, 2010). Previous research on *S. mahagoni* seeds had proven the antidiabetic activity but the conventional methods of extraction were used. Thus, limited study on antidiabetic study using advance extraction method such as supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction was studied.

Recently, attention on the importance of natural compounds from plants and herbs has been reassessing. As a matter of fact, bioactive compounds from plant sources are chemically sensitive and present in low concentration hence supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction is the appropriate extraction method to be used. In this case, thermolabile and non-polar compounds can be extracted by using SC-CO<sub>2</sub> extraction due to the low operating temperature of 30°C without any degradation. The



downside of this technique is it cannot be used to extract polar compound since SC-CO<sub>2</sub> extraction is more suitable to be used in extracting compounds that is non-polar (Vilegas *et al.*, 1997). Previously,  $\beta$ -sitosterol has been extracted from various plants using SC-CO<sub>2</sub> since  $\beta$ -sitosterol is a non-polar compound. Therefore, no co-solvent is needed in the extraction of  $\beta$ -sitosterol using SC-CO<sub>2</sub> extraction.

Furthermore, the extraction of  $\beta$ -sitosterol from various plants using SC-CO<sub>2</sub> extraction had been reported in the extraction of saw palmetto berries (Catchpole *et al.*, 2002), *Vitex agnus castus* fruit (Cossuta *et al.*, 2008) and sea buckthorn seeds (Sajfrtová *et al.*, 2010). Sajfrtová *et al.*, (2010) reported that low temperature as low as 50°C was applied in the extraction of  $\beta$ -sitosterol as the degradation of  $\beta$ -sitosterol exceeds the temperature mentioned. The yield of  $\beta$ -sitosterol increased slightly as pressure increased and the highest yield found from *Vitex agnus castus* fruit was 1.1 mg/g at a pressure of 45 MPa and a temperature of 40°C (Cossuta *et al.*, 2008). This shows that the extraction of  $\beta$ -sitosterol can be manipulated or controlled by pressure and temperature. Pressure and temperature are the most relevant parameters in supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction. In general, quantitative recovery of analytes are influenced by the increase in pressure which leads to the increase in solvent power. Solvent power is described as the solvent density in any given conditions. Significantly, high pressure and moderate temperature are favored by the extraction of  $\beta$ -sitosterol from plants using SC-CO<sub>2</sub>. Therefore, SC-CO<sub>2</sub> extraction was proposed as the method of extraction for *S. mahagoni* seeds.

### 1.3 Research Objectives

The objectives of this study are:

- i. To determine the effect of pressure and temperature of supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction on oil yield and  $\beta$ -sitosterol content from *S. mahagoni* seeds.
- ii. To study the antidiabetic activity of the *Swietenia mahagoni* oil extract.

#### 1.4 Research Scope

The scopes of the research are:

- i. The pre-treatment process involving sample preparation, determination of moisture content and particle size were performed prior to extraction. The determination of flow rate and extraction time used during the extraction was done in order to obtain constant parameters.
- ii. The concentration of  $\beta$ -sitosterol in *S. mahagoni* extract obtained from extraction using SC-CO<sub>2</sub> was studied. A method of high-performance Liquid Chromatography (HPLC) was developed to analyze  $\beta$ -sitosterol quantitatively.
- iii. Optimization of *S. mahagoni* extraction using SC-CO<sub>2</sub> was studied using Response Surface Methodology (RSM). The optimization of *S. mahagoni* extraction was performed at two different parameters, which are pressure (20, 25 and 30 MPa) and temperature (40, 50 and 60°C) using dynamic extraction.
- iv. In vitro study of antidiabetic activity of *S. mahagoni* oil was analysed by  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes inhibition assays.

#### 1.5 Research Contributions

- i. The data obtained from this research on the extraction of  $\beta$ -sitosterol from *S. mahagoni* seeds using SC-CO<sub>2</sub> is very advantageous since no recent publications reported on the determination of  $\beta$ -sitosterol from *S. mahagoni* seeds using SC-CO<sub>2</sub> extraction.
- ii. The optimum condition of  $\beta$ -sitosterol concentration established from the optimization process using response surface methodology can be proposed and used as a reference to the pilot and industrial scale for scaling-up purpose.
- iii. The study of antidiabetic property of *S. mahagoni* seeds using  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes inhibition assays can be contributed in the pharmaceutical and nutraceutical industries.

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## LIST OF PUBLICATIONS

1. Nur Salsabila Md Norodin, Liza Md Salleh, Hartati and Nik Musaadah Mustafa. (2016). Supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction of essential oil from *Swietenia mahagoni* seeds. Presented in Second International Conference on Chemical Engineering (ICCE) at The Jayakarta Suites, Bandung, Indonesia from 26- 27 October 2016.
2. Nur Salsabila Md Norodin, Liza Md Salleh, Siti Machmudah and Nik Musaadah Mustafa. (2016). Extraction of  $\beta$ sitosterol from *Swietenia mahagoni* Seeds by Using Supercritical Carbon Dioxide (SCCO<sub>2</sub>) Extraction. Submitted to Separation Science and Technology in November 2016. *In Review*.