EXTRACTION OF SQUALENE FROM AQUILARIA MALACCENSIS LEAVES USING SUPERCRITICAL CARBON DIOXIDE

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...To Ramli, Norhayati, Wan Muhammad Hanif, Wan Muhammad Syazwan,
Wam Muhammad Amirudin and Wan Nurul Asmimi...

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ABSTRACT

Supercritical carbon dioxide (SC-CO_2) and Soxhlet extraction were performed on *Aquilaria malaccensis* leaves. The focus of this research is to investigate the effect of temperature and pressure of SC-CO_2 conditions on extract yield, squalene composition and solubility of extract. In addition, the comparison in terms of extract yield and squalene composition was also done with Soxhlet extraction. The final objective is to optimize the SC-CO_2 extraction of *Aquilaria malaccensis* leaves assisted by a co-solvent using the response surface methodology (RSM). The effects of SC-CO_2 at various pressures and temperatures were investigated on extraction yield and squalene concentration in *Aquilaria malaccensis* leaves extracts. This research was conducted at temperature and pressure of 45 °C to 75 °C and 10 MPa to 30 MPa, respectively, and assisted by 2% ethanol as a co-solvent. The effect of variables and the optimum conditions of extraction yield, squalene concentration, and solubility of extract were examined in the RSM. In comparison, the Soxhlet extraction produced the highest extraction yield (45.66%), whereas SC-CO_2 gave the highest squalene concentration (14.83 wt%). The optimization of SC-CO_2 extraction adequately fits the second-order polynomial model with the coefficient of determination, R^2 of 0.9452 (extraction yield), 0.8685 (squalene concentration) and 0.7770 (solubility of extract) at 95% confidence level. The predicted values of optimum yield of extract (12.2655%), squalene concentration (12.0295%) and solubility of extract (0.0138611) were obtained at 63.66 °C and 30 MPa during 1 hour of extraction. Thus, the extraction of squalene from *Aquilaria malaccensis* leaves using SC-CO_2 was improved in terms of quality and purity of extract compared to the Soxhlet extraction.

ABSTRAK

Pengekstrakan karbon dioksida lampau genting (SC-CO_2) dan pengekstrakan Soxhlet telah dijalankan terhadap daun *Aquilaria malaccenesis*. Fokus penyelidikan ini adalah untuk menyiasat kesan suhu dan tekanan keadaan SC-CO_2 terhadap hasil ekstrak, komposisi skualen dan kelarutan ekstrak. Sebagai tambahan, perbandingan dari segi hasil ekstrak dan komposisi skualen telah dijalankan dengan pengekstrakan Soxhlet. Objektif terakhir pula adalah untuk mengoptimumkan pengekstrakan SC-CO_2 terhadap daun *Aquilaria malaccensis* dengan dibantu oleh pelarut tambahan menggunakan kaedah tindak balas permukaan (RSM). Kesan SC-CO_2 pada beberapa tekanan dan suhu telah disiasat terhadap hasil ekstrak dan kepekatan skualen di dalam ekstrak daun *Aquilaria malaccensis.* Kajian ini telah dijalankan pada suhu dan tekanan masing-masing ialah 45 °C hingga 75 °C dan 10 MPa hingga 30 MPa dengan dibantu oleh 2% etanol sebagai pelarut tambahan. Kesan pembolehubah dan keadaan optimum hasil ekstrak, kepekatan skualen dan kelarutan ekstrak ditentukan di dalam RSM. Secara perbandingan, pengekstrakan Soxhlet menghasilkan ekstrak yang lebih tinggi (45.66%) manakala SC-CO_2 pula menghasilkan kepekatan skualen yang lebih tinggi (14.83 wt%). Pengoptimuman pengekstrakan SC-CO_2 sesuai dengan model polinomial peringkat kedua berserta pekali penentuan, R^2 bernilai 0.9452 (hasil ekstrak), 0.8685 (kepekatan skualen) dan 0.7770 (kelarutan ekstrak) pada tahap keyakinan 95%. Nilai ramalan optimum bagi hasil ekstrak (12.2655%), kepekatan skualen (12.0295%) dan kelarutan ekstrak (0.0138611) diperoleh pada 63.66 °C dan 30 MPa selama 1 jam pengekstrakan dijalankan. Oleh itu, pengekstrakan skualen daripada daun *Aquilaria malaccensis* menggunakan kaedah SC-CO_2 telah ditambah baik dari segi kualiti dan ketulenan ekstrak berbanding kaedah Soxhlet.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AARD	-	Average absolute relative deviation
ABTS	-	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANN	-	Artificial Neural Network
ANOVA	-	Analysis of variance
ASE	-	Accelerated solvent extraction
CCD	-	Central composite design
CITES	-	the Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO_2	-	Carbon dioxide
DPPH	-	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DW	-	Dry weight
EGCG	-	Epigallocatechin gallate
ESI	-	Electrospray ionization
FASE	-	Fatty acid steryl esters
FFA	-	Free fatty acids
FFD	-	Full factorial design
FOPDT	-	First order plus dead time
FTIR	-	Fourier-transform infrared spectroscopy
GAE	-	Gallic acid equivalent
GC	-	Gas chromatography
GC-MS	-	Gas chromatography-mass spectrometry
GP	-	Growth phase
HPLC	-	High performance liquid chromatography
IC_{50}	-	Inhibitory concentration at 50%
IR	-	Infrared
MAE	-	Microwave assisted extraction
NMR	-	Nuclear magnetic resonance
PCB	-	Polychlorinated biphenyls
PFAD	-	Palm fatty acid distillate
PORIM	-	Palm Oil Research Institute of Malaysia

PR	-	Peng-Robinson
QE	-	Quercetin equivalent
RK	-	Redlich-Kwong
RSM	-	Response surface methodology
SC-CO_2	-	Supercritical carbon dioxide
SCF	-	Supercritical fluid
SF	-	Solvent-to-feed
SFE	-	Supercritical fluid extraction
SODD	-	Soybean oil deodorizer distillate
SRK	-	Soave Redlich-Kwong
SWE	-	Subcritical water extraction
TAG	-	Triacylglycerols
TQ	-	Thymoquinone

LIST OF SYMBOLS

μ	-	Micro
D	-	Diffusivity
E	-	Error
f	-	Flow rate
f	-	Flow rate of CO_2
K	-	Kelvin
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
M_c %	-	Percentage of moisture content
Mt	-	Total amount of diffusing substance which has entered the sheet at specific time
M∞	-	Corresponding quantity after infinite time
P	-	Number of terms in model
P	-	Pressure
P_c	-	Critical Pressure
R^2	-	Regression coefficient
r	-	Radius of sphere
T	-	Temperature
T	-	Total
t	-	Time
t	-	Extraction time
T_c	-	Critical Temperature
$V_{co\text{-}colvent}$	-	Volume of co-solvent injected
W_{oil}	-	Weight of extracted yield
$W_{t,i}$	-	Weight of sample before extraction
$W_{t,f}$	-	Weight of sample after extraction
X_1	-	Pressure
X_2	-	Temperature
Y	-	Global oil yield

Y_1	-	Extract yield
Y_2	-	Squalene concentration
Y_3	-	Solubility

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Aquilaria is genera of tropical trees that produces a valuable resinous wood called agarwood. The aromatic properties of agarwood when burned or distilled are extraordinary and there is high demand for the resinous wood to make incense, perfume and as traditional medicine (Blanchette, 2015). The history of trading agarwood internationally started as early as the thirteenth century, with India as one of the origins of agarwood for overseas market. Ordinarily, agarwood is exported in bulk quantities. The primary legal exporters of wild sourced agarwood today are Indonesia and Malaysia. Between 2005 and 2013, Indonesia reported exporting almost 7,000 tons of wild sourced agarwood (UNODC, 2016).

The genus *Aquilaria* of the Thymelaeaceae (Daphne family) consists of commonly rapid grown trees originate in sea-level tropical forests. In the South and Southeast Asia, from the bottoms of the Himalayas (Bhutan) and northern India, over Myanmar, Indochina, Thailand, Malaysia, the Philippines and Indonesia to Papua New Guinea, the tree growing occurs naturally. For different kind of species, they can be found growing on rocky, precipitous, and bare slopes, and in areas that experience hot, and dry season. This type of plant provides an important source of income for local populations who harvest the scented agarwood to sell everywhere (Gratzfeld and Tan, 2008).

Flavors and fragrances represent a consistent and substantial portion of the world natural product market. Essential oils characterize aromatic plants used in the pharmaceutical, food and fragrance industries. Essential oils contain monoterpene and sesquiterpene hydrocarbons and oxygenated compounds (alcohols, aldehydes, ketones, acids, phenols, oxides, lactones, ethers and esters), which are responsible for the characteristic odors and flavors (Capuzzo *et al.,* 2013). With regards to the beneficial phytochemicals in medicinal plants and the shift towards natural products in pharmaceuticals and cosmeceuticals industry, the research on medicinal plants particularly are as important as the research on conventional drugs (Azwanida, 2015).

Squalene can be discovered in certain fish oils, exclusively shark liver oil, in high quantities and some vegetable oils in surprisingly smaller amounts. Human sebum also contains 13% squalene as one in all its primary constituents. Attention in squalene has been raised after its characterization in shark liver oil which is used as a traditional medication for decades. Characterization of liver oil of deep-sea shark (*Echinorhinus brucus*) revealed the presence of palmitic acid (15%), oleic acid (12%), stearic acid (8%), docosahexaenoic acid (DHA) (18%), and eicosapentaenoic acid (EPA) (16%). It was also found to be a good source of squalene (38.5%) and fat-soluble vitamins such as A, D, and K (vitamin A: 17.08mg/100 g of oil, vitamin D: 15.04mg/100 g oil, and vitamin K: 11.45mg/100 g oil) (Venugopal *et al*., 2016). Numerous studies revealed results that validate certain bioactivities for squalene. Recently, anticancer, antioxidant, drug carrier, detoxifier, skin hydrating, and palliative activities of these ingredients have been stated both in animal models and *in vitro* environments. Conferring to encouraging results from recent studies, squalene is considered a significant substance for practical and clinical uses with a massive potential in the nutraceutical and pharmaceutical industries (Kim and Karadeniz, 2012).

In previous years, conventional separation processes such as evaporation, water extraction, and steam distillation have been used to extract bioactive compounds of medicinal plants. However, the use of these methods does not assure the safety of extracted product Additionally, the desired product gained was not completely extracted as it did not achieve the optimum separation condition.

Currently, in order to improve product yields and quality of certain materials used, many innovative technologies have been developed such as microwave assisted extraction (MAE), accelerated solvent extraction (ASE), subcritical water extraction (SWE), ultrasound extraction (Sonication), supercritical fluid extraction (SFE) and phytonics process. As for this study, it will be focused on the supercritical fluid extraction technology using carbon dioxide as solvent. For comparison purpose, Soxhlet extraction method will be carried out besides the SFE method.

Supercritical fluid extraction (SFE) is a substitute method in sample preparation to lessen the use of organic solvents and escalate production. The factors to be considered are pressure temperature, modifier addition, sample volume, analytes collection, flow rate and pressure control and restrictors. There are many benefits in using carbon dioxide as the extracting fluid not only due to its encouraging physical properties, –; carbon dioxide is also economical, safe and copious. Due to its gas-like low viscosity and high diffusivity, a supercritical fluid (SCF), when used as a solvent, can easily penetrate plant materials with a rapid mass transfer rate. In addition, the density of a SCF can be altered by adjusting the pressure and temperature, hence SCF density is said to be tuneable (Khaw *et al.,* 2017). Besides, the extraction of product at low temperature can avoid damages from heat and for some organic solvents; carbon dioxide does not have solvent remains and also practices natural extraction procedure.

Generally, supercritical carbon dioxide (SC-CO_2) extraction apparatus consists of carbon dioxide cylinder, carbon dioxide pump, modifier pump, oven, extraction vessel, yield collector, thermocouple, chiller, pressure gauge, and back pressure regulator. The system to be employed for SFE must contain a pump for the solvent, a pressure cell to contain the sample, a means of maintaining pressure in the system, and a collecting vessel. The liquid, that is, carbon dioxide (CO_2) is pumped to a heating zone, where it is heated to supercritical conditions and, as a supercritical fluid, has some properties of a gas and some of a liquid. It then passes into the extraction vessel; and behaving as a gas; it can rapidly diffuse into the solid matrix and, as a liquid, dissolve a large quantity of lipids in the material to be extracted. The dissolved material is removed from the extraction cell into a separator at lower pressure, and the extracted material settles out. The CO_2 can then be cooled, recompressed, and recycled or discharged to the atmosphere. These instruments are finding ever-greater use, as they alleviate many of the problems associated with the use and disposal of organic solvents, in terms of time and costs as well as environmental impact. Extractions can be performed in static, dynamic, or recirculating mode. During static extraction, the cell is filled with the supercritical fluid, pressurized, and allowed to equilibrate. In the dynamic mode, the fluid is run continuously through the cell, and in the recirculating mode, the same fluid is repeatedly pumped in the cell before being pumped out to the collection vial. During static extraction, the cell is filled with the supercritical fluid, pressurized, and allowed to equilibrate. In the dynamic mode, the fluid is run continuously through the cell, and in the recirculating mode, the same fluid is repeatedly pumped in the cell before being pumped out to the collection vial (Donato *et al.*, 2013).

Another technique of extraction applied in this study is Soxhlet extraction. Soxhlet extraction is one of the most used conventional methods in separation processes. This process was studied to compare between new extraction technology and conventional extraction technology. During Soxhlet extraction, the vapors produced from the solvent were from the round-bottomed flask. The vapors then passed through the thimble and condensed in the condenser at the top. The extraction occurs when condensed solvent made contact with the *Aquilaria malaccensis* leaves in the thimble. When the condensation reaches the maximum level of the thimble, it travels back into the reservoir through a siphon, carrying extracted solutes into the bulk liquid at the bottom of the Soxhlet extractor.

1.2 Problem Statement

The extraction of squalene for healthcare products nowadays is primarily from deep-sea shark liver oil. It is not sustainable as deep-sea sharks tend to have slow growth rate, late sexual maturity, longer lifespan and lower metabolic rates, which resulted to longer turnover times, causing less productive populations. Protection of marine ecology as well as high processing expenses is currently the main hindrance for large production of squalene from a restricted animal source. Furthermore, the unpleasant fish odor and taste of shark liver oil as well as environmental pollutants such as dioxins, polychlorinated biphenyls (PCBs), and heavy metals in shark liver limit the use of shark liver oil for human products.

Nowadays, the leaves of *Aquilaria malaccensis* tree are being discarded as waste due to large consumption of the barks and woods. As the leaves are good sources of natural antioxidants and bioactive compounds, it can be utilized and used as ingredients for healthcare products. Currently, the discovery of squalene compound in plant extract is mainly from olive oil, palm fatty acid distillate (PFAD) and amaranth seed oil. Leaves which are considered as waste, are rich of active compound namely squalene, can revert the excess waste of leaves into valuable source of pharmaceutical products. However, there is no confirmation on the quantity of the squalene contained in the materials.

SFE is a relatively new technology for commercial separation process. It is an advanced and non-conventional separation technique that has many advantages such as green extraction, high quality, low operating cost and selectivity. Small amount of co-solvents may be added to modify the polarity and solvent strength of the primary supercritical fluid to increase the solute solubility and to minimize operating costs in the continuous extraction process. Besides, SFE is an intensive method and requires no other processes to produce high quality yield.

In order to discover squalene compound quantitatively, Soxhlet extraction and supercritical carbon dioxide extraction (SC-CO_2) were conducted on the material and Gas Chromatography analysis was done to recognize the compound presence in it. Optimization by using Response Surface Methodology was done on the SC-CO_2 extraction at different operating conditions which are pressure and temperature, that may affect the quality and yield of the extract.

1.3 Objectives of Research

The objectives of this research are:

i. To investigate the effect of temperature and pressure of SC-CO_2 conditions on extract yield, squalene composition and solubility of *Aquilaria* *malaccensis* leaves and compare with Soxhlet extraction in terms of extract yield and squalene composition.

ii. To optimize the SC-CO_2 extraction of oil yield, squalene composition and solubility by using Response Surface Methodology (RSM).

1.4 Scopes of Research

The research scope is to study the effect of supercritical fluid extraction parameters on *Aquilaria malaccensis* leaves where the average particle size (277 µm, 427 µm, and 600 µm), flow rate (5.3, 8.5, and 10.6 mL/min), moisture content, extraction time, type and amount of modifier were kept constant. Type of modifier used was ethanol due to its high polarity; while the amount of modifier used is 10 mL. Modifier was introduced to the sample prior to the extraction process. All these five parameters were kept constant to ensure accuracy of the experiment result. Meanwhile, the varied parameters are extraction temperature and pressure which have dominant effects on the extraction yield.

In order to determine the effect of independent variables, the selected operating temperature and pressure of extraction process ranges from 45 °C to 75 °C and 10 MPa to 30 MPa, respectively. Extraction temperature was kept above 45 °C to ensure that the carbon dioxide is maintained in supercritical condition and below 75 °C as extraction above that will affect the amount of extracted squalene. Extraction below 10 MPa will yield a lesser amount of extracted squalene and is difficult to handle and analyze. Pressure less than 30 MPa was selected due to the maximum operating capacity of the equipment. The existence of cross over region can be observed by selecting these wide arrays of pressure and temperature.

The quantification of squalene extract from SC-CO_2 extraction and Soxhlet extraction was determined by using Gas Chromatography (GC) by overlaying the peak obtained with squalene standard.

Finally, the data obtained from the analysis of *Aquilaria malaccensis* leaves was evaluated using statistical approach. Software called Design-Expert 6.0.4 was used to evaluate those data. The evaluation was based on the F-test which compares the statistically calculated F and the F value that has been tabulated from statistical data.

1.5 Significance of Research

i. Squalene obtained from *Aquilaria malaccensis* leaves would be the alternative source from shark liver oil.
ii. The data obtained from this research on squalene extraction from *Aquilaria malaccensis* leaves by using SC-CO_2 assisted by co-solvent is very advantageous to be implemented in the pharmaceutical and nutraceutical industries.
iii. The optimum condition of squalene concentration established from the optimization process using RSM can be proposed and used as a reference to any pilot and industrial scale projects for scaling-up purposes.

1.6 Thesis Outline and Organization

This thesis was structured and organized in five chapters. The introduction of this research was written in Chapter 1. This chapter comprises the background study about supercritical fluid extraction and *Aquilaria malaccensis* plant. In addition, the chapter also presents the problem statement, which is the research starting point and the reason for conducting this research. Chapter 1 also includes the objectives and scopes of the study.

Chapter 2 describes the background of *Aquilaria malaccensis* plant and the fundamental principles of supercritical fluid extraction. Besides, this chapter includes the properties and advantages of supercritical fluid extraction in terms of its application for plants and herbs, as well as mathematical modelling and optimization process.

Chapter 3 presents the method for the experimental extraction work. The method commences with the determination of moisture content. This chapter also presents the method of SC-CO_2 and Soxhlet extraction. The chapter includes the determination of average particle sizes, flow rates, and extraction time and it also describes the method for analysis of squalene concentration.

Chapter 4 discusses the results obtained from the experimental work. This chapter discusses the mathematical modelling used for particle size determination, comparison of SC-CO_2 with Soxhlet extraction on the percentage of extracted yield, and squalene concentration. Discussions on experimental data, followed by the optimization of yield extract and squalene concentration are also included in this chapter.

Conclusions and recommendations are discussed in Chapter 5. It concludes the findings in this research and future research prospect. Recommendations are also provided for future research idea and improvement.

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