UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF LIPASE-CATALYZED *Nigella sativa* L. OIL-BASED FERULATE ESTERS PRODUCTION AND THEIR ANTIOXIDANT ACTIVITY

FARIHIN BINTI AZHARI

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FARIHIN BINTI AZHARI

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Chairman : Uswatun Hasanah binti Zaidan, PhD
Institute : Halal Products Research Institute

Nowadays, the generation of novel product from natural substance has received tremendous attention due to the health benefits. *Nigella sativa* L. seed, also recognized as black cumin is a medicinal plant that possesses miraculous power of healing due to the abilities to cure various ailments excluding death. However, it is oily and prone to oxidative degradation. Ethyl ferulate was added to provide an antioxidative shield against lipid oxidation. The ester exhibits special features such as non-toxic, outstanding moisturizing action and good solubility characteristics but most importantly the absence of oily texture. In addition, the ester may consist of valuable characteristics from both the *Nigella sativa* oil (NSO) and ethyl ferulate (EF). In this study, long chain ferulate esters (LCFEs) were produced by transesterification of NSO with EF in the presence of *Rhizomucor miehei* immobilized lipase (Lipozyme RM IM). The production conditions were optimized by rotatable central composite design (RCCD) from response surface methodology (RSM) attaining maximum esters conversion of 49.87% under lipase dosage of 67 mg, temperature of 56°C, substrate ratio (NSO: EF) of 4.4: 1 (w/w) and time of 4 hours. The attained conversion value was in agreement with the predicted value of 48.82%. The produced LCFEs were characterized by fourier transform-infrared (FT-IR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC). FT-IR spectrum of LCFEs showed two common bands of ester group: C=O and C-O strong bands at 1763-1653 cm^{-1} and 1115-1100 cm^{-1}, respectively. Long chain of aliphatic ester component was also present indicated by an absorption band at the range of 1480-1365 cm^{-1} region. The GC-MS analysis of LCFEs revealed that remaining EF (32.41%) and decreased amount of fatty acids (50.64%) were identified in comparison with substrates EF and NSO, correspondingly which confirmed the production of LCFEs. The fatty acids found in LCFEs including oleic acid and essential fatty acid such as linoleic acid. TLC showed the presence of LCFEs at retention factor, R_f = 0.64 showing the lowest polarity compared to NSO (R_f = 0.52) and

EF (R_f = 0.28). Moreover, the antioxidant activity of LCFEs was analyzed by three different methods: 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and β-carotene bleaching (BCB). The produced LCFEs exhibited outstanding antioxidant property due to their high percentage inhibition (83.37 ± 3.42-85.88 ± 0.12%) as well as low IC_{50} value (47.79 ± 3.04-114.33 ± 3.95 µg/mL). Thus, LCFEs are potential source of natural antioxidant and can be used as alternative ingredient in cosmeceutical product.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PENGOPTIMUMAN PENGHASILAN PEMANGKINAN-LIPASE ESTER FERULAT-BERASASKAN MINYAK *Nigella sativa* L. DAN AKTIVITI ANTIOKSIDAN

Oleh

FARIHIN BINTI AZHARI

Ogos 2019

Pengerusi : Uswatun Hasanah binti Zaidan, PhD
Institut : Penyelidikan Produk Halal

Pada masa kini, penghasilan produk baru daripada bahan semula jadi telah mendapat perhatian orang ramai kerana kelebihannya untuk kesihatan. Biji *Nigella sativa* L., juga dikenali sebagai jintan hitam ialah tumbuhan perubatan yang mempunyai kuasa penyembuhan ajaib kerana kebolehannya untuk merawat pelbagai penyakit kecuali mati. Walau bagaimanapun, sifatnya berminyak dan mudah mengalami degradasi oksidatif. Ferulat etil ditambahkan kepadanya untuk memberi perlindungan oksidatif yang menghalang pengoksidaan minyak. Ester mempamerkan ciri-ciri istimewa seperti tidak beracun, tindakan pelembap yang bagus dan sifat mudah larut tetapi ciri paling penting ialah bebas daripada tekstur berminyak. Selain itu, ester mungkin mengandungi ciri-ciri berharga daripada kedua-dua minyak *Nigella sativa* (NSO) dan ferulat etil (EF). Dalam penyelidikan ini, rantaian panjang ester ferulat (LCFEs) telah dihasilkan dengan transesterifikasi NSO dan EF dengan kehadiran penyekatgerakan lipase *Rhizomucor miehei* (Lipozyme RM IM). Keadaan penghasilan dioptimumkan dengan menggunakan reka bentuk komposit pusat berputar (RCCD) daripada kaedah rangsangan permukaan (RSM) mencapai pembentukan ester maksimum sebanyak 49.87% dengan dos lipase sebanyak 67 mg, suhu 56°C, nisbah substrat (NSO: EF) 4.4: 1 (w/w) dan masa 4 jam. Nilai pertukaran yang dicapai dalam persetujuan dengan nilai ramalan sebanyak 48.82%. LCFEs terhasil dicirikan dengan menggunakan spektroskopi inframerah-transformasi fourier (FT-IR), kromatografi gas-spektrometri jisim (GC-MS) dan kromatografi lapisan nipis (TLC). Spektrum FT-IR daripada LCFEs menunjukkan dua jalur biasa daripada kumpulan ester: jalur kuat C=O dan C-O di mana masing-masing pada 1763-1653 cm^{-1} dan 1115-1100 cm^{-1}. Rantaian panjang daripada sebatian ester alifatik juga hadir ditunjukkan dengan jalur serapan pada julat 1480-1365 cm^{-1}. Analisis GC-MS daripada LCFEs mendedahkan bahawa baki EF (32.41%) dan pengurangan jumlah asid lemak (50.64%) telah dikenalpasti berbanding dengan substrat EF dan NSO, yang mengesahkan penghasilan LCFEs. Asid lemak yang dijumpai

dalam LCFEs termasuk asid oleik dan asid lemak penting seperti asid linoleik. TLC menunjukkan kehadiran LCFEs pada faktor pengekalan, R_f = 0.64 menunjukkan kekutuban paling rendah berbanding dengan NSO (R_f = 0.52) dan EF (R_f = 0.28). Selain itu, aktiviti antioksidan daripada LCFEs dianalisis dengan menggunakan tiga kaedah berbeza: 2,2-Dwifenil-1-pikrilhidrazil (DPPH), 2,2'-azino-bis(asid 3-etilbenzotiazolin-6-sulfonik) (ABTS) dan pelunturan β-karotena (BCB). LCFEs terhasil mempamerkan ciri antioksidan yang bagus disebabkan oleh peratus perencatan mereka yang tinggi (83.37 ± 3.42-85.88 ± 0.12%) dan juga nilai IC_{50} yang rendah (47.79 ± 3.04-114.33 ± 3.95%). Oleh itu, LCFEs berpotensi sebagai antioksidan semula jadi dan boleh digunakan sebagai ramuan alternatif dalam produk kosmeseutikal.

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I certify that a Thesis Examination Committee has met on 23 August 2019 to conduct the final examination of Farihin binti Azhari on her thesis entitled "Optimization of Lipase-Catalyzed *Nigella sativa* L. Oil-Based Ferulate Esters Production and Their Antioxidant Activity" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

Members of the Thesis Examination Committee were as follows:

Siti Nurbaya binti Oslan, PhD
Senior Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd Shukuri bin Mohamad Ali, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Juliana bt Jumal, PhD
Associate Professor
Faculty of Science and Technology
Universiti Sains Islam Malaysia
Malaysia
(External Examiner)

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

Date: 10 October 2019

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:

Uswatun Hasanah binti Zaidan, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Syahida binti Ahmad, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Siti Salwa binti Abd. Gani, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

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

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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Signature: _____
Name of Chairman of Supervisory Committee: Assoc. Prof. Dr. Uswatun Hasanah binti Zaidan

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Syahida binti Ahmad

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Siti Salwa binti Abd. Gani

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

2FI	interactive system
A_c	absorbance of control
A_s	absorbance of sample
ABTS	2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ANOVA	analysis of variance
ATR	attenuated total reflection
BCB	β-carotene bleaching
C.V.	coefficient of variance
d.f.	degree of freedom
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH•	2,2-diphenyl-1-picrylhydrazyl free radical
DR	degradation rate
EC	enzyme commision
EF	ethyl ferulate
ESR	electron spin resonance
F	Fisher
FT-IR	fourier transform-infrared
GC-MS	gas chromatography-mass spectrometry
IC_{50}	50% inhibition concentration
iNOS	inducible nitric oxide synthase
LCFE	long chain ferulate ester
Lipozyme RM IM	*Rhizomucor miehei* immobilized lipase
log P	partition coefficient
LOO•	peroxyl radical

m/z	mass-to-charge ratio
N	number of replicates
NIST	National Institute of Standards and Technology
Nm	Nanometer
NMR	nuclear magnetic resonance
NSO	*Nigella sativa* oil
OFAT	one-factor-at-a-time
P	Probability
R^2	coefficient of determination
RCCD	rotatable central composite design
R_f	retention factor
ROS	reactive oxygen species
Rpm	revolutions per minute
RSM	response surface methodology
SFA	saturated fatty acid
SPSS	Statistical Package for the Social Sciences
SS	sum of the square
TLC	thin layer chromatography
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UFA	unsaturated fatty acid

CHAPTER 1

INTRODUCTION

1.1 Research Background

Nowadays, the interest for natural substance containing a valuable function to human has greatly increased and there is an extensive development of pharmaceutical and nutraceutical commodities produced from natural substances. Furthermore, the natural substance is used to improve the function of a certain food or food component for the consumer that has high awareness in wellbeing. The primary attention is focused on natural substance containing great antioxidant action because oxidative stress stimulated by various factors has been the significant contribution to diverse pathological diseases including aging, heart disease, cancer and inflammation (Roby, 2017).

Nigella sativa L. oil has a prolonged record of folklore application in Arabian and Indian communities as medicine and food. Current studies on *Nigella sativa* oil showed that it has numerous properties including antioxidant, antimicrobial, antihepatotoxic and anticancer activities. In addition, it can be applied externally as local anesthetic and antiseptic (Ahmad *et al.*, 2013). Nickavar *et al.* (2003) performed chemical analysis on *Nigella sativa* oil to discover the active components of the oil, where the eight significant fatty acids were found. The most abundant component was linoleic acid (55.6%) while palmitic acid (12.5%) was the least abundant component.

Long chain ferulate ester (LCFE) has received tremendous attention because of its potent antioxidant capability with outstanding behavior on the skin. LCFE is derivative of ethyl ferulate that is available in plant species. Remarkable attribute due to structural arrangements of methoxy and hydroxyl groups bound to benzene ring are able to destroy free radical and carboxyl group consisting of a nearby unsaturated double bond that can offer alternative attack areas for free radicals. Accordingly, this component and its derivatives have been stated to possess diverse physiological roles such as antioxidant, anticancer, antimicrobial, anti-thrombotic and anti-inflammatory actions (Pei *et al.*, 2015).

LCFE can be produced by chemical or enzymatic approach. The application of common chemical catalyzed procedure requires high energy and constantly causes various challenges such as deterioration of ester, corrosion of appliance and risk in managing hazardous chemical. On the contrary, the application of enzymatic approach presents a moderate reaction process and is an acknowledged “greener” approach of LCFE production (Mohamad *et al.*, 2015). Lipase is one of the frequently utilized enzymes in catalysis and it is vastly utilized due to its great specificity in action, making sure that solely the desired product will be formed (Kumar *et al.*, 2016). Moreover, the utilization of

immobilized lipase is able to tolerate high temperature and prevent thermal deterioration of ester (Skoronski *et al.*, 2014). Therefore, LCFEs were generated by transesterification of *Nigella sativa* oil with ethyl ferulate in the presence of immobilized lipase.

For the enzymatic approach, research upon the optimization of the process to elevate the operation efficacy is truly essential. Response surface methodology (RSM) is a proficient program for assessing the consequence of various factors impacting the outcomes by limiting the quantity of experimental run and expense. Few researchers had implemented RSM in the lipase-catalyzed production of LCFEs (Radzi *et al.*, 2014; Yang *et al.*, 2012). In this present study, RSM was used to optimize the condition of the transesterification process in order to achieve the greatest LCFEs yield. The impacts of four synthesis parameters (lipase dosage, temperature, substrate ratio and time) on the outcome yield were assessed. Moreover, the formed LCFEs were assumed to portray good antiradical potential that can function as promising antioxidant in a lipophilic environment. Thus, the antioxidant potential of formed LCFEs was also analyzed to assist possible application.

1.2 Problem Statement

Nonetheless, the disadvantages of *Nigella sativa* oil are frequently correlated to its greasy texture and high susceptibility to oxidation which constrict its application for industrial utilization. *Nigella sativa* oil is easily oxidized because it contains a high amount of unsaturated double bonds which could develop unwanted off-flavors and destruction of endogenous antioxidants leading to reduced nutritional value and the existence of harmful oxidation products. According to Roby (2017), oxidation of plant oil can be prevented by adding antioxidants such as ethyl ferulate. The combination of *Nigella sativa* oil with ethyl ferulate leads to the generation of LCFEs which stabilize *Nigella sativa* oil and prevent it from oxidation. The esters formed have remarkable properties due to the mixed health benefits of *Nigella sativa* oil and ethyl ferulate. In addition, ester has drawn the interest of industry across the past years because of its great solubility characteristic, remarkable emollient action and non-toxicity, yet most importantly does not possess the greasy texture. Ester is extensively utilized as antifoaming substance, polish, lubricant and plasticizer and as a raw component in pharmaceutical and cosmetic commodities (Abdullah *et al.*, 2019).

1.3 Objectives

The main objective of this present study was to optimize the enzymatic production of esters using a multivariate statistical method and the assessment of their antioxidant activities. Therefore, the study was performed corresponding to the following specific approaches:

1. To synthesize long chain ferulate esters (LCFEs) via transesterification of *Nigella sativa* oil and ethyl ferulate catalyzed by immobilized lipase and characterize the substrates and their LCFEs by fourier transform-infrared (FT-IR) spectroscopy, gas chromatography-mass spectrometry (GC-MS) and thin layer chromatography (TLC).
2. To optimize the parameters of transesterification reaction using response surface methodology (RSM) for high LCFEs yield.
3. To evaluate the antioxidant potentials of substrates and their LCFEs by 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and β-carotene bleaching (BCB) assays.

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BIODATA OF STUDENT

Farihin binti Azhari was born in Subang Jaya, Selangor on the 8th June 1991. She had her primary education at Sekolah Kebangsaan Hicom, Selangor and continued her secondary education at Sekolah Kebangsaan USJ 23, Selangor followed by Maktab Rendah Sains Mara Alor Gajah, Melaka. After completing her Sijil Pelajaran Malaysia in 2008, she continued her study at Matriculation level at International Islamic University Malaysia (IIUM), Selangor. Two years later, she continued her study at IIUM, Kuantan (2010-2015). Thereafter, she enrolled in the Master of Science program at Halal Products Research Institute, UPM.

LIST OF PUBLICATIONS

Research Papers

Farihin Azhari, Uswatun Hasanah Zaidan, Syahida Ahmad and Siti Salwa Abdul Gani. (2019). Optimization of *Nigella sativa* oil-based ferulate ester synthesis using response surface methodology. *Malaysian Applied Biology*, 48(2): 27-31.

Conferences/Exhibitions

Farihin Azhari, Uswatun Hasanah Zaidan, Syahida Ahmad and Siti Salwa Abdul Gani. Optimization of lipase-catalyzed synthesis of *Nigella sativa* oil-based ferulate ester by response surface methodology. *International Conference on Biochemistry, Molecular Biology and Biotechnology (ICBMBB) 2018*, 15th – 16th August 2018, Selangor, Malaysia.

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