

INVESTIGATION OF ANTICANCER COMPOUNDS
FROM NON-INFECTED AGARWOOD BRANCH
TOWARDS DEVELOPMENT OF BREAST CANCER
THERAPEUTICS

BY

PHIRDAOUS BIN ABBAS

A thesis submitted in fulfilment of the requirement for the
degree of Doctor of Philosophy (Engineering)

Kulliyyah of Engineering
International Islamic University Malaysia

FEBRUARY 2022

ABSTRACT

Agarwood; a dark-aromatic resinous substance released by *Aquilaria* trees upon infection; is one of the most treasured forest valuable resources. The plant materials from the trees were also reported to be utilized traditionally across various communities to fulfil religious, medicinal, and aromatic preparations. There has also been an increase of modern ethnomedicinal reports of agarwood or its plant materials towards various diseases. While a lot of focus has been on the resin itself, less has been focused on the agarwood non-infected branch which is abundant all year round and mostly considered as a waste from agarwood plantation. Our previous work done on *Aquilaria subintegra* non-infected branch has demonstrated profound growth inhibitory effects against Michigan Cancer Foundation-7 (MCF-7) breast cancer cells. Thus, the current study attempted to investigate the underlying cell death mechanisms behind the agarwood branch ethanolic extract (ABEE) against MCF-7 cells as well as enhance the extraction process conditions via one factor at a time analysis (OFAT) and Response Surface Methodology (RSM)-based experimental design; employing the Faced Centered Central Composite Design (FCCCD). The study also employed a bio-guided approach in an attempt to isolate and identify potential active compounds using column chromatography, thin layer chromatography, Gas Chromatography Mass Spectroscopy (GCMS), Fourier Transformed Infrared Spectroscopy (FTIR), Proton (H-NMR) and Carbon-13 (C-NMR) Nuclear Magnetic Resonance Spectroscopy, Ultraviolet-visible spectroscopy (UV) and Liquid Chromatography Mass Spectroscopy (LCMS). The study first replicated the established extraction process conditions to obtain the extract, which was then subjected to flow cytometry analysis and gene expression profiling with pathway analysis via real time Human Cell Death PathwayFinder™ RT² Profiler PCR array. It was observed that the extract caused time-dependent apoptosis-necrosis of MCF-7 cells and significantly affected 48 genes (41 down-regulated and 7 up-regulated genes) indicative of cellular responses towards stimuli of specific apoptotic signals. NF-KB1 gene was the most down-regulated gene (fold change of -26.704) and TRAF2 gene was the most up-regulated gene (fold change of 5.52). Pathway analysis conducted using online KEGG tool suggested that these differentially expressed genes (DEGs) regulated cell death mechanisms via the apoptosis and p53 signalling pathways. Then, through combination of OFAT and FCCCD; an optimal extraction process employing the temperature and solid-liquid ratio at 45 °C and 1:19 (w/v), respectively, with desirable factors; (i) safe temperature (less risk towards thermos-labile compounds) and (ii) economical solvent volume, was obtained. The model predicted ABEE yield of 30.232 ± 0.266 mg/g dried materials (DM) and validation run afforded ABEE at 25.35 ± 1.19 mg/g DM (p -value = 0.007). ABEE obtained from the recommended process conditions showed cytotoxicity effects on MCF-7 cells with IC₅₀ estimate of 3.645 ± 0.099 µg/mL. The extract also affected MCF-7 cell attachment and viability with altered morphology. The bio-guided approach of fractionation and isolation process then led to a plethora of fractions. However, decrease of cytotoxicity was observed after each phase of fractionation suggesting synergism-dependent effect (of the crude extract) which became more apparent towards the end of the fractionation study. Nonetheless, the study isolated and identified a non-active orange-yellow needle-like crystal flavonoid, ABEE-FR4A1; characterized to be 5-hydroxy-7,4'-dimethoxyflavone (13.7 mg, 6.13 %

w/w). The parent fraction (FR 2-3, 2037 mg) and subfraction (FR 3A3, 223.5 mg) exhibited moderate cytotoxic effects (28.52 ± 0.1524 and 23.11 ± 0.1141 $\mu\text{g/mL}$), but inferior to the crude extract. In conclusion, the study had determined the underlying cell death mechanisms of ABEE against MCF-7 cells, optimized the extraction process conditions and identified several compounds with varying cytotoxic effects. These finding would add value to the non-infected agarwood branch that is abundant in agarwood plantations thus help promote the sustainable growth of agarwood industry; and pave the way towards development of locally sourced natural anti-cancer therapeutics.



خلاصة البحث

العود، مادة صمغية عطرية داكنة تفرزها أشجار آكيلاريا عند الإصابة بعدوى؛ هي واحدة من أكثر موارد الغابات قيمة. وتشير التقارير إلى استخدام المواد النباتية من هذه الأشجار بشكل تقليدي عبر المجتمعات المختلفة للأغراض الدينية والطبية والعطرية، وهناك أيضاً المزيد من التقارير الطبية العرقية الحديثة عن خشب العود أو مواد النباتية تجاه أمراض مختلفة. وفي حين كان الكثير من التركيز على المادة الصمغية نفسها، فقد تمّ التركيز بشكل أقلّ على فرع خشب العود غير المصاب والمتوفر بكثرة طيلة العام ويعدّ في الغالب من نفايات مزارع خشب العود. أظهر عملنا السابق الذي تمّ إجراؤه على فرع *Aquilaria subintegra* غير المصاب آثاراً مثبطة للنمو العميق ضد خلايا سرطان الثدي MCF-7. وبالتالي، حاولت الدراسة الحالية التحقيق في آليات موت الخلايا الكامنة وراء المستخلص الإيثانولي لفرع خشب العود (ABEE) ضد خلايا MCF-7 وكذلك تحسين ظروف عملية الاستخراج عبر عملية الاستخلاص عبر التصميم التجريبي القائم على OFAT ومنهجية سطح الاستجابة (RSM) باستخدام التصميم المركب المركزي الموجه (FCCCD). استخدمت الدراسة أيضاً نهجاً موجهاً بيولوجياً في محاولة عزل وتحديد المركبات النشطة المحتملة باستخدام كروماتوجرافيا العمود، كروماتوجرافيا الطبقة الرقيقة، الكروماتوجرافيا الغازية المتصلة بمطياف الكتلة (GCMS)، مطياف فورييه للأشعة تحت الحمراء (FTIR)، التحليل الطيفي بالرنين المغناطيسي النووي: البروتوني (H-NMR) والكربوني- 13 (C-NMR)، التحليل الطيفي للأشعة فوق البنفسجية المرئية (UV) و الكروماتوجرافيا السائل المزود بمقياس طيف الكتلة (LCMS). قامت الدراسة أولاً بتكرار ظروف عملية الاستخلاص المحددة المتعارف عليها للحصول على المستخلص، والذي خضع بعد ذلك لتحليل قياس التدفق الخلوي وتنميط التعبير الجيني مع تحليل المسار الفوري عبر Human Cell Death PathwayFinder™ RT² Profiler PCR. وقد لوحظ أن المستخلص تسبب في نخرٍ وقي لخلايا MCF-7، وأثر المستخلص أيضاً بشكل كبير على 48 جيناً (41 جيناً منظمًا بالإنقاص و 7 جينات منظمّة بالزيادة) مما يدل على الاستجابات الخلوية تجاه محفزات إشارات موت الخلايا المبرمج المحددة. كان جين NF-KB1 هو الجين الأكثر خضوعاً للتنظيم (تغيير بقيمة 26.704-) بينما كان جين TRAF2 هو الجين الأكثر تنظيمًا بالزيادة (تغيير بقيمة 5.52). اقترح تحليل المسار الذي تمّ إجراؤه باستخدام أداة KEGG عبر الإنترنت أن هذه الجينات المعبر عنها تفاضلياً (DEGs) تنظم آليات موت الخلايا عبر مسارات إشارات موت الخلايا المبرمج و p53. بعد ذلك، ومن خلال الجمع بين OFAT و FCCCD؛ تم الحصول على عملية استخلاص مثالية تستخدم درجة حرارة ونسبة المادة السائلة إلى صلبة عند 45 درجة مئوية و 1:19 (وزن / حجم)، على التوالي، مع العوامل المرغوبة؛ تم الحصول على (1) درجة حرارة آمنة (مخاطر أقل تجاه المركبات المتأثرة بالحرارة) و (2) حجم اقتصادي للمذيب. تنبأ النموذج بإنتاجية ABEE تبلغ 30.232 ± 0.266 ملجم/جم من المواد المجففة (DM) وتم التحقق من صحة النموذج بالحصول على ABEE بقيمة 1.19 ± 25.35 ملجم/جم DM (القيمة الاحتمالية $p=0.007$). أظهر ABEE الذي تم الحصول عليه من ظروف

عملية الاستخلاص الموصى بها تأثيرات السمية الخلوية على خلايا سرطان الثدي MCF-7 بقيمة IC_{50} تقديريها 3.645 ± 0.099 ميكروجرام/مل. وأثر المستخلص كذلك على ارتباط خلية MCF-7 ونموها مع تغير في الشكل. ثم أدى النهج الموجه بيولوجيًا لعملية التجزئة والعزل لعدد كبير من الأجزاء. ومع ذلك، لوحظ انخفاض في السمية الخلوية بعد كل مرحلة من مراحل التجزئة مما يشير إلى التأثير المعتمد على التآزر (للمستخلص الخام) والذي أصبح أكثر وضوحًا في نهاية دراسة التجزئة. ومع ذلك، قامت الدراسة بعزل وتحديد فلافونويد بلوري أصفر برتقالي غير نشط يشبه الإبر ، -ABEE FR4A1 ؛ يتميز بكونه بكونه 5-هيدروكسي-4،7-ديميثوكسي فلافون (13.7 ملجم، 6.13% وزن/وزن). وأظهر الجزء الأصلي (FR 2-3، 2037 ملجم) والجزء الفرعي (FR 3A3، 223.5 ملجم) تأثيرات سمية معتدلة للخلايا (0.1524 ± 28.52 و 0.1141 ± 23.11 ميكروجرام/مل) ، ولكنها أدنى في قيمتها من المستخلص الخام. وختاماً، حدّدت الدراسة الآليات الأساسية لمستخلص ABEE ضد خلايا MCF-7، وحسّنت ظروف عملية الاستخلاص وحددت العديد من المركبات ذات تأثيرات سميّة متفاوتة. ستضيف هذه النتائج قيمة إلى فروع خشب العود غير المصاب والمتوفرة بكثرة في مزارع خشب العود، وبالتالي تساعد في تعزيز التنمية المستدامة في صناعة العود ؛ وتمهيد الطرق نحو تطوير علاجات طبيعية مضادة للسرطان من مصادر محلية.

APPROVAL PAGE

The thesis of Phirdaous Bin Abbas has been approved by the following

Yumi Zuhanis Has-Yun Hashim
Supervisor

Hazmah Mohd Salleh
Co-Supervisor

Ma'an Fahmi Rashid Al-Khatib
Co-Supervisor

Saripah Salbiah Syed Abdul Azziz
Co-Supervisor

Noor Illi Binti Mohamad Puad
Internal Examiner

Asmah Awal
External Examiner

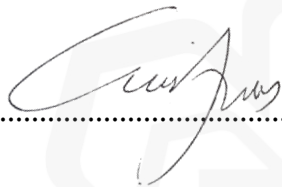
Roslina Othman
Chairperson

DECLARATION

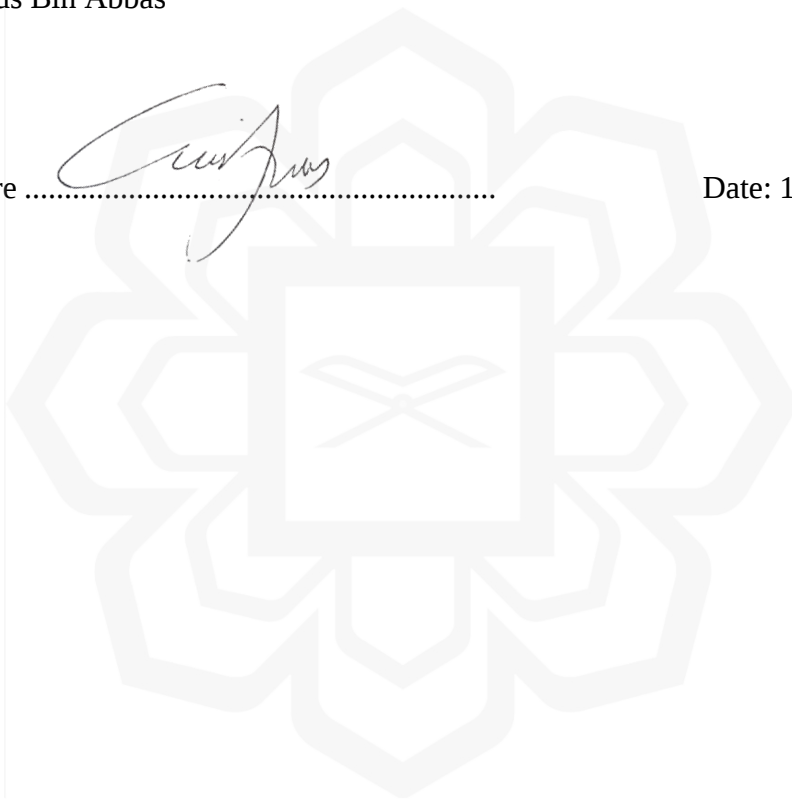
I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Phirdaous Bin Abbas

Signature



Date: 11 February 2022



INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF
FAIR USE OF UNPUBLISHED RESEARCH**

**INVESTIGATION OF ANTICANCER COMPOUNDS FROM
NON-INFECTED AGARWOOD BRANCH TOWARDS
DEVELOPMENT OF BREAST CANCER THERAPEUTICS**

I declare that the copyright holders of this thesis are jointly owned by the student and IIUM.

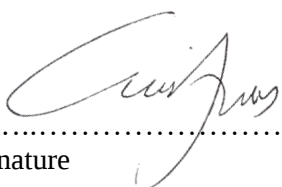
Copyright © 2022 Phirdaous Bin Abbas and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.
2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.
3. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Phirdaous Bin Abbas


.....
Signature

Date: 11 February 2022

ACKNOWLEDGEMENT

In The Name of Allah, the Most Beneficent, the Most Merciful

Praises to Allah S.W.T., The Almighty God for His blessings has enabled me to accomplish the study. This thesis is a result of the long but fruitful process studying at the International Islamic University Malaysia (IIUM). I would like to express the deepest appreciation to my supervisor, Prof. Dr. Yumi Zuhanis Has-Yun Hashim for her guidance, advises, encouragement, patience and valuable assistance that enable me to accomplish my Masters program smoothly and efficiently. I also would like to thank my co-supervisor, Prof. Dr. Hamzah Mohd Salleh, Prof. Dr Ma'an Fahmi Rashid Al-Khatib, and Assoc. Prof. Dr Saripah Salbiah for their vital input, time and support during my study.

I sincerely appreciate the friendship and assistance from all lab mates and lab technicians, Br. Annuar, Br. Aslan, Br. Hafizul, Br. Ezza Faiez, and Sis. Adilla. A special thank you is dedicated to Dr. Amal and Dr. Husna, from the INHART department for their support and attention throughout my study. I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis.

Last but not least, I want to take the opportunity to thank my wife, Norazura Zainal, whose love, patience and company are the strongest motivation. My deepest gratitude to my parents, Abbas Alias and Hasnah Saat, and my sister, Syuhada Abbas, for their unconditional love, patience, willingness and sacrifice for my well-being throughout my life. Without all of you, it would not be possible for me to complete my project and thesis. May Allah bless you all for your kindness and for that, I will forever be grateful.

TABLE OF CONTENTS

Abstract.....	ii
Abstract in Arabic.....	iv
Approval Page.....	vi
Declaration.....	vii
Copyright Page.....	viii
Acknowledgements.....	ix
List of Tables.....	xv
List of Figures.....	xxii
List of Abbreviations.....	xxxvii
CHAPTER ONE: INTRODUCTION.....	1
1.1 Background of the study.....	1
1.2 Problem statement and significant of study.....	4
1.3 Research Philosophy.....	5
1.4 Research Objectives.....	7
1.5 Research Methodology.....	8
1.6 Scope of Study.....	9
1.7 Thesis Organization.....	10
CHAPTER TWO: LITERATURE REVIEW.....	11
2.1 Introduction.....	11
2.2 Cancer.....	12
2.2.1 Development and Causes.....	12
2.2.2 Classification of cancers.....	15
2.2.3 Breast cancer.....	17
2.2.3.1 Breast cancer epidemiology.....	18
2.2.3.2 Breast cancer pathophysiology.....	19
2.2.3.3 Breast cancer stages.....	21
2.2.4 Different cancer treatments.....	22
2.2.5 Natural product and cancer.....	25
2.3 Agarwood.....	26
2.3.1 Agarwood taxonomy and botanical profile.....	28
2.3.2 History of agarwood and trade distribution.....	30
2.3.3 Agarwood formation and current practises.....	34
2.3.4 Agarwood ethnopharmacology and phytochemistry.....	37
2.3.4.1 Ethnopharmacology of agarwood.....	37
2.3.4.2 Phytochemistry of agarwood.....	39
2.3.5 Agarwood current pharmacological activities.....	44
2.3.6 <i>Aquilaria subintegra</i>	51
2.4 Natural product extraction for bioactivity investigation.....	53
2.4.1 Principle and fundamental aspects.....	53
2.4.2 Conventional extraction methods.....	54
2.4.3 Process conditions towards efficient extraction process....	57
2.4.3.1 Extraction temperature effect.....	57
2.4.3.2 Extraction time effect.....	58

2.4.3.3	Agitation speed effect.....	58
2.4.3.4	Solid-liquid (SL) ratio effect.....	59
2.4.4	Experimental design and extraction.....	59
2.4.5	Isolation and purification of natural products.....	61
2.4.5.1	Column chromatography (CC).....	62
2.4.5.2	Thin layer chromatography (TLC).....	64
2.4.5.3	Preparative TLC, washing and recrystallization method.....	66
2.5	Bioactivity analysis and natural product.....	67
2.5.1	<i>In vitro</i> bioassay in natural product analysis.....	67
2.5.1.1	Cell attachment assay.....	67
2.5.1.2	Cell viability assay.....	68
2.5.1.3	Cell cytotoxicity assay.....	69
2.5.2	Flow cytometry in natural product analysis.....	71
2.5.2.1	Apoptosis analysis in flow cytometry.....	72
2.5.3	Pathway analysis and cancer study.....	73
2.5.3.1	Gene expression and cancer study.....	74
2.5.3.2	RT ² profiler PCR array technology.....	76
2.6	Summary.....	78
CHAPTER THREE: RESEARCH METHODOLOGY.....		79
3.1	Introduction.....	79
3.2	Plant material.....	81
3.3	Extraction of agarwood branch.....	82
3.3.1	Phase 1: Extraction of agarwood branch based on previous study.....	82
3.3.2	Phase 2: One factor at a time (OFAT) for process conditions screening.....	83
3.3.2.1	Chemicals and reagents.....	83
3.3.2.2	OFAT experimental setup.....	84
3.3.2.3	Data collection and analysis.....	86
3.3.3	Optimization of process conditions.....	86
3.3.3.1	Chemicals and reagents.....	86
3.3.3.2	Response surface methodology for ABEE extraction.....	87
3.3.3.3	Data collection and analysis.....	88
3.4	General cell culture handling and bioactivity screening.....	88
3.4.1	Chemicals and reagents.....	88
3.4.2	Cell lines.....	89
3.4.3	Reagents preparation.....	89
3.4.3.1	Media preparation.....	89
3.4.3.2	Preparation of phosphate buffered saline (PBS).....	90
3.4.3.3	Preparation of culture medium with 10 % (v/v) fetal bovine serum (FBS).....	90
3.4.4	Cell culture handling techniques.....	90
3.4.4.1	Thawing cryopreserved cells.....	91
3.4.4.2	Adherent cell subculture technique.....	91
3.4.4.3	Cryopreserving adherent cell line.....	92

3.4.4.4	Trypan blue dye exclusion technique for cell quantification.....	93
3.5	Investigation of biological activities.....	94
3.5.1	Cell attachment assay (CAA).....	94
3.5.2	Cell viability assay (CVA).....	95
3.5.3	Cell cytotoxicity assay using Cell Count Reagent SF.....	96
3.5.4	Flow cytometry.....	96
3.5.4.1	Preparation of agarwood branch extract.....	97
3.5.4.2	Chemicals and reagents.....	97
3.5.4.3	Apoptosis analysis using Annexin A5 FITC kit.....	98
3.5.5	Real time PCR array.....	99
3.5.5.1	Preparation of extract.....	99
3.5.5.2	Chemicals and reagents.....	99
3.5.5.3	Cell viability study for RNA extraction.....	100
3.5.5.4	RNA extraction technique via RNeasy® Mini Kit.....	100
3.5.5.5	cDNA synthesis via RT ² First Strand Kit.....	102
3.5.5.6	Real time PCR using RT ² profiler array using Human Cell Death PathwayFinder™ (PAHS-212Z).....	103
3.5.5.7	Data collection and analysis.....	104
3.6	Characterization of extract and fractions.....	105
3.6.1	GCMS analysis.....	105
3.6.2	FTIR analysis.....	106
3.7	Isolation and purification of ABEE using column chromatography..	106
3.7.1	Chemicals and reagents.....	107
3.7.2	Column chromatography setup for natural product isolation.....	107
3.7.3	Column loading and elution techniques.....	109
3.7.4	Thin layer chromatography.....	111
3.7.4.1	<i>p</i> -anisaldehyde-sulphuric acid preparation and procedure.....	111
3.7.4.2	Dragendorff's reagent preparation and procedure.....	112
3.7.4.3	Vanillin-sulphuric reagent preparation and procedure.....	112
3.7.5	Washing and recrystallization technique.....	113
3.7.6	Structural elucidation of purified compounds.....	114
3.7.6.1	GCMS Analysis.....	115
3.7.6.2	FTIR Analysis.....	115
3.7.6.3	LC-MS Analysis.....	115
3.7.6.4	Ultraviolet-visible (UV) analysis.....	116
3.7.6.5	Nuclear Magnetic Resonance (NMR) analysis...	116
CHAPTER FOUR: RESULTS AND DISCUSSIONS.....		117
4.1	Introduction.....	117
4.2	Flow cytometry and gene expression analysis of agarwood branch extract.....	118
4.2.1	Flow cytometry analysis of ABEE16.....	118

4.2.2	Gene expression profiling of ABEE16-treated MCF-7 cells.....	121
4.2.2.1	ABEE16 effect on cell viability and morphology.....	122
4.2.2.2	RNA recovery from ABEE16-treated MCF-7 cells.....	125
4.2.2.3	Real time PCR using RT2 profiler array using Human Cell Death PathwayFinder™ RT² Profiler™ PCR Array (PAHS-212Z).....	126
4.2.2.4	Pathway analysis of DEGs affected in ABEE16-treated MCF-7 cells.....	129
4.2.2.5	Roles of selected genes in apoptotic pathway...	135
4.2.4	Discussion: Apoptotic effect and differential gene expression of ABEE16 treated MCF-7 cells.....	139
4.3	Process conditions optimization for high ABEE yield.....	144
4.3.1	Overview.....	144
4.3.2	Process conditions screening via One Factor at a Time (OFAT) method.....	144
4.3.2.1	Agitation effect on ABEE yield.....	145
4.3.2.2	Extraction time effect on ABEE yield.....	147
4.3.2.3	Solid-liquid ratio effect on ABEE yield.....	149
4.3.2.4	Extraction temperature effect on ABEE yield...	151
4.3.2.5	Discussion on process conditions OFAT.....	153
4.3.3	Optimization of selected process conditions for ABEE extraction.....	155
4.3.3.1	Model prediction and statistical analysis.....	156
4.3.3.2	Response surface analysis.....	157
4.3.3.3	Model validation.....	159
4.3.3.4	<i>In vitro</i> validation of bioactivity of optimized ABEE yield.....	160
4.3.3.5	Characterization of optimized ABEE through GCMS and FTIR analysis.....	168
4.3.3.6	Discussion on optimization of process conditions of ABEE extraction.....	174
4.4	Isolation and purification.....	180
4.4.1	Overview.....	180
4.4.2	First column chromatography of ABEE.....	182
4.4.3	First phase TLC profile and bio-guided assays.....	185
4.4.4	Characterization of active fractions in the first phase CC...	190
4.4.5	Discussion on first column chromatography of ABEE.....	196
4.4.6	Second column chromatography of active first phase subfractions.....	198
4.4.7	Second phase TLC profile and bio-guided assays.....	199
4.4.8	Characterization of active fractions in the second phase CC.....	205
4.4.9	Discussion on second phase column chromatography.....	217
4.4.10	Third column chromatography of active second phase subfractions.....	220
4.4.10.1	Column chromatography for FR 2-3.....	220

4.4.10.2	Column chromatography for FR 2-6.....	222
4.4.10.3	Column chromatography for FR 2-7.....	224
4.4.11	Third phase TLC profile and bio-guided assays.....	226
4.4.11.1	TLC profile and bio-guided assay on subfractions from FR 2-3.....	227
4.4.11.2	TLC profile and bio-guided assay on subfractions from FR 2-6.....	231
4.4.11.3	TLC profile and bio-guided assay on subfractions from FR 2-7.....	234
4.4.12	Characterization of active fractions in third phase CC.....	240
4.4.13	Discussion on third phase column chromatography.....	258
4.4.14	Fourth column chromatography of active third phase subfractions.....	261
4.4.14.1	Column chromatography for FR 3C1-C3.....	261
4.4.14.2	Column chromatography for FR 3C4.....	264
4.4.14.3	Washing and recrystallization of FR 3A3.....	267
4.4.15	Fourth phase TLC profile and bio-guided assays.....	268
4.4.15.1	TLC profile and bio-guided assay on subfractions from FR 3C1-C3.....	269
4.4.15.2	TLC profile and bio-guided assay on subfractions from FR 3C4.....	273
4.4.15.3	TLC profile on FR 4A1.....	278
4.4.16	Characterizations of active fractions in the fourth phase CC.....	280
4.4.17	Discussion on fourth phase column chromatography.....	295
4.4.18	Overall discussion.....	299
CHAPTER FIVE: CONCLUSION AND RECOMMENDATIONS.....		304
5.1	Conclusions.....	304
5.2	Recommendations.....	307
REFERENCES.....		308
LIST OF PUBLICATIONS.....		338
APPENDIX A.....		340
APPENDIX B.....		341
APPENDIX C.....		342

LIST OF TABLES

Table 2.1	Stage of breast cancer as described by Fust, 2015. These stages provide information and reference for treatment and data exchange	22
Table 2.2	Common chemotherapy drugs administered for different types of cancer with different mechanism of action as listed by the American Cancer Society and Waks & Winer (2019)	24
Table 2.3	Reported agarwood name based on different regions	27
Table 2.4	List of accepted names of agarwood tree from the <i>Aquilaria</i> genus	29
Table 2.5	Reported traditional use of <i>Aquilaria</i> spp	34
Table 2.6	Reported ethnomedicinal uses of agarwood in different countries	39
Table 2.7	Reported chemical compounds identified/isolated from agarwood and/or its derivatives	42
Table 2.8	Summary of bioactivity studies conducted on agarwood plant in recent years	45
Table 2.9	Summary of isolated and identified compounds from agarwood leaf with their respective bioactivity data.	49
Table 2.10	List of agarwood studies using extraction procedures. Most studies employed exhaustive-type extraction without using experimental design tools.	61
Table 2.11	Common visualization reagents used in TLC analysis	66
Table 2.12	List of potential bioassay that can be employed to create complementary bioactivity data alongside the compound characterization profiles.	70
Table 2.13	Differences between different types of bioassays that can either be advantages or disadvantages depending on the study preferences.	71

Table 3.1	List of process conditions selected for various plant extraction processes found in literature. These parameter values serve as guide and were modified for the ABEE OFAT analysis in this study.	84
Table 3.2	List of parameter levels for the process conditions screening of agarwood branch extraction via OFAT method	85
Table 3.3	Coded Face Centred Central Composite Design (FCCCD) for optimization of process conditions to obtain maximum ABEE yield (mg/g dried material, DM)	87
Table 3.4	Reverse transcription mix as instructed by the RT ² First Strand Kit	102
Table 3.5	PCR component mixture for Format C 96-well array	103
Table 3.6	Cycling conditions for real-time cycler by Applied Biosystems model StepOnePlus	104
Table 4.2.1	Characterized cell percentage measured using flow cytometry apoptotic cell analysis stained with the DNA fluorescence dye, propidium iodide, and Annexin-V-FITC dye, apoptotic marker dye.	119
Table 4.2.2	Measured RNA absorbance, ratio, and yield extracted from MCF-7 cells. 260/280 ratio is a measure of RNA purity usually ± 2.0 for pure RNA.	125
Table 4.2.3	Ratio, absorbance, and yield of RNA recovery conducted using T-75 cm ² growth flasks	126
Table 4.2.4	List of DEGs affected by 48 hours of ABEE16 treatment with fold change cut off 2 ($p < 0.05$). Among the 48 genes significantly affected by the treatment, 41 genes were down-regulated while 7 genes were up-regulated based on the collected data	127
Table 4.3.1	Mean yield (mg/g DM) for 1:30 sample-solvent ratio obtained after 24 hours of incubation at 45 °C at different agitation speed. Experiment was conducted in triplicate set of runs ($n = 3 \pm \text{s.d.}$).	146
Table 4.3.2	Mean yield (mg/g DM) for 1:30 sample-solvent ratio obtained after selected time extraction at 45 °C and 100 rpm. Experiment was conducted in triplicate set of runs ($n = 3 \pm \text{s.d.}$).	148

Table 4.3.3	Mean yield (mg/g DM) after 24 hours of extraction at 45 °C and 100 rpm using selected solid-liquid ratios. Experiment was conducted in triplicate set of runs (n = 3 ± s.d.).	150
Table 4.3.4	The mean yield (mg/g DM) after 24 hours of extraction at 100 rpm and 1:30 solid-liquid ratio at selected temperature levels. Experiment was conducted in triplicate set of runs (n = 3 ± s.d.).	152
Table 4.3.5	Coded Face Centred Central Composite Design (FCCCD) for optimization of process conditions to obtain maximum ABEE yield (mg/g dried material, DM). A represented temperature with midpoint 55 °C and B represented SL ratio with midpoint 20 both from previous selection and screening section. Responses recorded were based on triplicate experimental sets (n = 3).	155
Table 4.3.6	ANOVA data for the regression model for ABEE extraction.	157
Table 4.3.7	Cell number obtained from trypan blue dye exclusion method for CAA showing 50 % of viability reduction in treated sample. CVA data showed 40 % reduction of viable MCF-7 cells. Experiments were conducted in triplicate (n = 3 ± s.d.).	166
Table 4.3.8	Classification of the identified volatile chemical constituents of ABEE based on functional group.	171
Table 4.3.9	Summary of characteristic of detected peak bands on FTIR spectra for crude ABEE.	173
Table 4.4.1	Subfractions collected from ABEE fractionation. Each fraction was collected, dried and redissolve in minimum amount of DCM for further analysis using TLC method.	184
Table 4.4.2	Five new subfractions derived from the original 19 based on stain spots analysis and R _f data on the TLC test conducted.	187
Table 4.4.3	Non-linear regression analysis results for IC ₅₀ estimation on the first column chromatography.	188
Table 4.4.4	Classification of the identified volatile chemical constituents of FR 1-2 based on functional group, area (%) and confidence measure (Qual).	193

Table 4.4.5	Summary of characteristic of detected peak bands on FTIR spectra for FR 1-2.	195
Table 4.4.6	Fractions collected for the CC on FR 1-2. The process managed to separate 31 fractions gradient-eluted by hexane/DCM/MeOH mobile phases.	199
Table 4.4.7	New subfractions combination based on TLC profile analysis of the original 31 subfractions.	201
Table 4.4.8	Derived IC ₅₀ values from non-linear regression analysis. CCK-8 assay was normalized using control group (1 % v/v DMSO) and duplicate experimental setup was used (n=2 ± s.d) due to scarcity of CCK-8 reagent at the time.	202
Table 4.4.9	Classification of the identified volatile chemical constituents of FR 2-3 based on functional groups, areas (%), and confidence measures (Qual).	206
Table 4.4.10	Summary of characteristic of detected peak bands on FTIR spectra for FR 2-3.	208
Table 4.4.11	Classification of the identified volatile chemical constituents of FR 2-6 based on functional groups, areas (%), and confidence measures (Qual).	209
Table 4.4.12	Summary of characteristic of detected peak bands on FTIR spectra for FR 2-6.	212
Table 4.4.13	Classification of the identified volatile chemical constituents of FR 2-7 based on functional groups, areas (%), and confidence measures (Qual).	214
Table 4.4.14	Summary of characteristic of detected peak bands on FTIR spectra for FR 2-7.	216
Table 4.4.15	Subfractions collected from FR 2-3 eluted using hexane-EA-MeOH mobile phase. Subfractions were collected, dried and redissolve in minimum amount of DCM for further analysis using TLC method.	222
Table 4.4.16	Subfractions collected from FR 2-6 eluted using DCM-EA-MeOH mobile phase. Subfractions were collected, dried and redissolve in minimum amount of DCM for further analysis using TLC method.	224

Table 4.4.17	Subfractions collected from FR 2-7 eluted using hexane-DCM-EA mobile phase with MeOH as final elution solvent for exhaustive rinsing process. Subfractions were collected, dried and redissolve in minimum amount of DCM for further analysis using TLC method.	226
Table 4.4.18	New subfractions combination based on TLC profile analysis of the original 44 subfractions from the CC of FR 2-3.	228
Table 4.4.19	Derived IC ₅₀ values from non-linear regression analysis. CCK-8 assay was normalized using control group (1 % v/v DMSO) and duplicate experimental setup was used (n=2 ± s.d) due to scarcity of CCK-8 reagent at the time.	229
Table 4.4.20	New subfractions combination based on TLC profile analysis of the original 40 subfractions from the CC of FR 2-6	232
Table 4.4.21	Derived IC ₅₀ values from non-linear regression analysis. CCK-8 assay was normalized using control group (1 % v/v DMSO) and triplicate experimental setup was used (n=3 ± s.d).	233
Table 4.4.22	New subfractions combination based on TLC profile p-anisaldehyde analysis of the original 69 subfractions from the CC of FR 2-7.	235
Table 4.4.23	Derived IC ₅₀ values from non-linear regression analysis for 3Cs subfractions. CCK-8 assay was normalized using control group (1 % v/v DMSO) and triplicate experimental setup was used (n=3 ± s.d).	235
Table 4.4.24	Colour stain observed using different staining tests on FR 3C1-C3 and FR 3C4	237
Table 4.4.25	Classification of the identified volatile chemical constituents of FR 3A3 based on functional group, area (%) and confidence measure (Qual).	241
Table 4.4.26	Summary of characteristic of detected peak bands on FTIR spectra for FR 3A3.	244
Table 4.4.27	Classification of the identified volatile chemical constituents of FR 3C1 based on functional group, area (%) and confidence measure (Qual).	246

Table 4.4.28	Summary of characteristic of detected peak bands on FTIR spectra for FR 3C1.	248
Table 4.4.29	Classification of the identified volatile chemical constituents of FR 3C3 based on functional group, area (%) and confidence measure (Qual).	251
Table 4.4.30	Summary of characteristic of detected peak bands on FTIR spectra for FR 3C3.	253
Table 4.4.31	Classification of the identified volatile chemical constituents of FR 3C3 based on functional group, area (%) and confidence measure (Qual).	254
Table 4.4.32	Summary of characteristic of detected peak bands on FTIR spectra for FR 3C4.	257
Table 4.4.33	Colours and R_f values recorded for the FR 3C1-C3 mobile phase screening and selection tests.	263
Table 4.4.34	List of collected 72 FR 4Cs eluted using hex-EA mobile phase. These subfractions were dried, redissolved in minimum amount of DCM for analysis later in the next section.	264
Table 4.4.35	Colours and R_f values recorded for the FR 3C4 mobile phase screening and selection tests.	266
Table 4.4.36	List of collected 48 FR 4Ds eluted using DCM-EA mobile phase. These subfractions were dried, redissolved in minimum amount of DCM for analysis later in the next section.	267
Table 4.4.37	New subfractions combination based on R_f values from TLC profile analysis of the original 72 subfractions from the CC of FR 3C1-C3.	269
Table 4.4.38	Derived IC_{50} values from non-linear regression analysis for FR 4Cs. FR 4C4 showed high cytotoxicity towards both MCF-7 and VERO cells. Other subfractions showed unreliable R^2 values suggesting non-significant effect towards both cell lines. CCK-8 assay was normalized using control group (1 % v/v DMSO) and triplicate experimental setup was used ($n=3 \pm s.d$).	271

Table 4.4.39	New subfractions combination based on R_f values from TLC profile analysis of the original 72 subfractions from the CC of FR 3C1-C3.	274
Table 4.4.40	Derived IC_{50} values from non-linear regression analysis for FR 4Ds. FR 4D3 showed moderate cytotoxicity towards both MCF-7 and VERO cells. Other subfractions were analysed to be ambiguous as well as having unreliable R^2 values suggesting non-significant effect towards both cell lines. CCK-8 assay was normalized using control group (1 % v/v DMSO) and triplicate experimental setup was used ($n=3 \pm s.d$).	276
Table 4.4.41	Classification of the identified volatile chemical constituents of FR 4C4 based on functional group, area (%) and confidence measure (Qual).	281
Table 4.4.42	Summary of characteristic of detected peak bands on FTIR spectra for FR 4C4.	284
Table 4.4.43	Classification of the identified volatile chemical constituents of FR 4D3 based on functional group, area (%) and confidence measure (Qual).	286
Table 4.4.44	Summary of characteristic of detected peak bands on FTIR spectra for FR 4D3.	288
Table 4.4.45	Classification of the identified volatile chemical constituents of FR 4A1 based on functional group, area (%) and confidence measure (Qual).	290
Table 4.4.46	Spectral data of ABEE-FR4A1 1H -NMR (500 MHz, $CDCl_3$) and ^{13}C -NMR (125 MHz, $CDCl_3$) compared to 5- hydroxy-7,4'-dimethoxyflavone.	294
Table 4.4.47	List of all potential active subfractions with their estimated IC_{50} values and R^2 score via the non-linear regression analysis	300

LIST OF FIGURES

Figure 2.1	Cancer statistics in Malaysia as reported by Globocan in 2018. Data obtained from https://gco.iarc.fr/today/data/factsheets/populations/458-malaysia-fact-sheets.pdf	18
Figure 2.2	Representative images of BRCA1 and BRCA2 genes located in the long arms of chromosome 17 and 13, respectively. Image was retrieved from MacroGen:Humanizing Genomics website at https://www.macrogen.com/en/business/diagnosis_service1.php	20
Figure 2.3	<i>Aquilaria</i> tree a) cultivated in Bangi aged around 6 to 7 years with trunk diameter ranging from 20 cm to 40 cm measured 1.3m from the ground. Image was taken in August 2018. b) Wild agarwood tree in Melaka and image was taken in January 2010.	30
Figure 2.4	Representative images of agarwood presence in felled branches of <i>Aquilaria subintegra</i> in Nilai, Negeri Sembilan taken on 28 th February 2019. <i>Aquilaria sinensis</i> chip images are graded according to the system adopted by the local trader in Kuala Kangsar, Perak. Images were taken on 4 th March 2020	32
Figure 2.5	Artificial inoculation of agarwood tree in farm located in Nilai, Negeri Sembilan. The process used agarwood inducer, a biologically formulated combination of bacteria and fungi, loaded into the tree trunk using Chemjet. Images were captured on 11 th April 2018.	35
Figure 2.6	Dark region formation in agarwood tree trunk as a result of modern day induction. Images were taken after a few months induction in an agarwood farm in Nilai, Negeri Sembilan on the 10 th and 28 th of February 2019	37
Figure 2.7	Representative images of <i>Aquilaria subintegra</i> trees locally cultivated in Melaka, Malaysia. Images were captured in October 2018	52
Figure 2.8	Common Soxhlet extraction apparatus setup	56

Figure 2.9	Representative basic chromatographic separation mechanism. Compound elution responded to the type of mobile phase used as well as selection of proper solid/stationary phase.	63
Figure 2.10	Assembled CC apparatus used in this study. Cotton ball and acid-washed sand layer are placed at the bottom of the glass column followed by silica gel slurry. The setup is completed by additional layer of acid-washed sand at the top of silica gel slurry added protection to the packed bed column	64
Figure 2.11	Catalogued PCR array layout embedded with 84 pathway-specific by SABiosciences, QIAGEN. Wells H1 through H5 are embedded with a panel of housekeeping genes (HK1-HK5) for normalization purpose. Genomic DNA Control (GDC) primer high sensitivity set that specifically detects non-transcribed, repetitive genomic DNA is placed in well H6. Wells H7 through H9 are embedded with replicate Reverse Transcription Controls (RTC) that verify the RT reaction efficiency. The replicate Positive PCR Controls (PPC) embedded in wells H10 through H12 is for measuring the overall efficiency of the polymerase chain reaction.	77
Figure 3.1	Overall flow of work according to objectives lined up for this study	80
Figure 3.2	Pre-treated agarwood branch sample used in this study. Sample was cleaned, washed, dried and ground prior to extraction process	81
Figure 3.3	RT2 Profiler PCR array format C layout. Wells A1 to G12 were embedded with different genes for a specific pathway/disease/function. Wells H1 to H5 were embedded with housekeeping genes (ACTB, B2M, HPRT1, GAPDH and RP1P0) for array data normalization. Well H6 was setup as genomic DNA control (GDC), H7 to H9 embedded with replicate reverse transcription controls (RTC), and H10 to H12 embedded with replicate positive PCR controls (PPC). Image was retrieved from RT2 Profiler PCR array handbook available at https://www.qiagen.com/us/resources/resourcedetail?id=f4b13eaa-884f-4357-abe6-1a5f9469bc32&lang=en	103
Figure 3.4	Representative gravity column chromatography setup for separation, isolation and purification purpose in this study. a) Gravity column bed setup for the study purpose with glass column loaded with silica gel 230-400 mesh (38-63 μm) protected by acid washed sand at both end and cotton wool at the exit of the column. b) Representative image of sample loaded onto the column bed. Note that at all time, the solvent	108

level must be above the sample or stationary phase to avoid bed cracking

Figure 3.5	Sample binding/pre-adsorbing process using DCM as the mixing solvent. a) Sample-bound silica was collected from an almost dried mixture and air-dried to complete dryness. b) Completely dried sample-bound silica. c) If mixture was evaporated to a complete dryness by the rotary evaporator, hard crust formation will occur and sample loss cannot be avoided.	110
Figure 3.6	Preparative TLC flow conducted for sample/fraction in order to isolate the potential major compound/s. The process includes a) TLC profiling, b) scraping off confirmed location of major compound/s, c) collection of compound-bound silica flake and d) eluting out the isolated compound from the silica flake using selected solvent through a simple glass dropper experimental setup	114
Figure 4.2.1	Apoptotic cells analysis using Annexin-V-FITC staining through flow cytometry. Scatter plots correspond to 24, 48 and 72 hours of ABEE16-treated with the 72 hours control MCF-7 cells showing a small increase in apoptotic cells at 72 hours ABEE16 treatment (1.80 %) compared to 72 hours control (0.05 %). Necrotic characterized cells were observed to increase in a time-dependent manner in which 5.50 %, 8.21 %, and 54.48 % cells measured at 24, 48, and 72 hours, respectively	120
Figure 4.2.2	Cell viability at 24, 48 and 72 hours of treatment of 8 µg/ml ABEE16 compared to control (0.1 % DMSO v/v). Highest viability reduction was recorded at 72 hours at 74.35 % compared to control. Results are based on three independent experiments ($n = 3 \pm \text{s.d.}$).	123
Figure 4.2.3	Representative images of MCF-7 cells population density in this investigation. Control group a) 24 hours, b) 48 hours, and c) 72 hours showed normal cell morphology with increasing cell population density. ABEE16-treated MCF-7 cells showed abnormal morphology in all time point with decreasing cell population density	124
Figure 4.2.4	Bar chart representing ABEE16-treated DEGs with fold change normalized against control set with fold regulation threshold set up at 2 and p-value < 0.05. Highest fold change analyzed was TRAF2 genes up-regulated by 5.52 fold while the lowest was NFKB1 down-regulated by 26.704 fold	127