



IN VITRO ANTIOXIDANT ACTIVITIES AND GAS
CHROMATOGRAPHY-MASS SPECTROMETRY
(GC-MS) ANALYSIS OF PHENOLICS IN EXTRACTS
OF *Dialium indum* var. *indum* L. FRUIT

BY

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(Pharmaceutical Chemistry)

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ABSTRACT

Chronic diseases are prevalent in modern population nowadays and wild fruits have been studied to be good sources of antioxidants, which can be related to reduced risk of developing these diseases. The fruit of *Dialium indum* var. *indum* L. or locally known as ‘keranji’ from the family Fabaceae is one of the wild fruits of Malaysia. This study investigated the antioxidant content, *in vitro* antioxidant activities, and phenolic composition of *D. indum* var. *indum* L. fruit. The proximate content of antioxidants in the exocarp, mesocarp, and seed crude extracts and fractions of *D. indum* var. *indum* L. fruit was measured using the total phenolics content (TPC), total flavonoids content (TFC), and cupric ion reducing antioxidant capacity (CUPRAC) assays, and their *in vitro* antioxidant activities were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and linoleic acid peroxidation inhibition assays. Gas-chromatography-mass spectrometry (GC-MS) analysis was carried out for antioxidant active extracts and fractions obtained from column chromatography fractionation of antioxidant active extract of *D. indum* var. *indum* L. fruit part. Of the twelve different extracts of *D. indum* fruit parts, exocarp dichloromethane fraction (EDF) and seed methanol fraction (SMF) showed the highest TPC, TFC, and CUPRAC, and the strongest *in vitro* antioxidant activities as assessed by DPPH radical scavenging and linoleic acid peroxidation inhibition assays. TPC of the various extracts was found to have positive correlation with CUPRAC, indicating the usefulness of CUPRAC as a method for proximate analysis of antioxidants content. GC-MS analysis of trimethylsilyl (TMS)-derivatized EDF showed that its phenolic antioxidants content was higher in comparison with TMS-derivatized SMF, with detection of nine phenolic antioxidants, namely vanillic acid, syringic acid, ferulic acid, isoferulic acid, sinapic acid, vanillin, syringic aldehyde, *p*-hydroxybenzaldehyde, and coniferyl aldehyde in the TMS-derivatized EDF. Fractionation of EDF using normal phase column chromatography yielded five antioxidant-rich polar subfractions labelled as Di-21, Di-22, Di-24, Di-25, and Di-26, which showed stronger DPPH radical scavenging activities than EDF. Further GC-MS analysis of these five TMS-derivatized subfractions detected the presence of other four phenolic antioxidants, identified as *p*-hydroxybenzoic acid, homovanillic acid, *p*-coumaric acid, and sinapic aldehyde. Observation of sinapic and vanillic acid content in EDF and its five subfractions with their DPPH radical scavenging activities suggested the synergistic effect of sinapic acid and antagonistic effect of vanillic acid with their DPPH radical scavenging activities. Identification of thirteen phenolics in EDF evidenced that the exocarp of *D. indum* fruit is a good source of antioxidants, which may protect the body against deleterious effects of oxidative stress that can cause development of chronic diseases.

خلاصة البحث

الأمراض المزمنة منتشرة هذه الأيام في الشعوب المتقدمة، وبما أن لدى الثمار البرية إمكانية لتكون مصدرا جيدا لمضادات الأكسدة فهي مهمة للتقليل من احتمالات الإصابة بهذه الأمراض. ثمار شجرة الدياليوم إنوم (*Dialium indum var. indum*) (L.) من فصيلة البقوليات، أو كما تعرف محليا باسم كيرينجي، هي أحد الثمار البرية الموجودة في ماليزيا. حقق هذا البحث في محتوى مضادات الأكسدة، ونشاطات مضادات الأكسدة للخبري، والمكونات الفينولية للكيرينجي. تم تقييم محتوى مضادات الأكسدة التقريبي في قشرة، ولب، وبذرة ثمرة الكيرينجي باستخدام اختبارات الكشف عن كل من المحتوى الكلي للفينول (TPC)، والمحتوى الكلي للفلافونويد (TFC)، والنشاط المضاد للأكسدة للأيون النحاسي للمختزل (CUPRAC)، وبالإضافة إلى نشاطاتها المضادة للأكسدة مخبريا باستخدام نشاط الكسح الجذري باستخدام بركيل الهايدرزيل ثنائي الفينيل (DPPH)، وتفاعل البيروكسيدز المثبط للحامض الدهني. تم التعرف على المركبات الكيميائية النباتية في مستخلصات وأجزاء ثمرة الكيرينجي ذي النشاط المضاد للأكسدة العالي باستخدام تحليل للطيف الكتلي-الكروماتوجرافيا (GC-MS) والمتجدة من تجزئة مضادات الأكسدة باستخدام الكروماتوجرافيا العمودية. من بين اثنتي عشرة مستخلصا مختلفا لثمرة الكيرينجي، أظهر جزء الكلوروليثان للقسور (EDF) وجزء لثانول للنبور (SMF) أعلى كمية في ال TPC، و TFC، و CUPRAC، وأقوى نشاط مخبري ضد الأكسدة باستخدام البركيل هايدرزيل ثنائي الفينيل (DPPH)، وتفاعل البيروكسيدز المثبط للحامض الدهني. اكتشف أن ال TPC للمستخلصات المتعددة كان على علاقة إيجابية بال CUPRAC، مما يشير إلى فائدة ال CUPRAC كطريقة لتحليل تقريبي لمحتوى مضادات الأكسدة. أظهر تحليل للطيف الكتلي-الكروماتوجرافيا (GC-MS) لثلاثي ميثيل سيليل (TMS) المشتق من ال ECD أن محتواه من مضادات الأكسدة الفينولية كان أعلى مقارنة بال SMF المشتق من ال TMS مع الكشف عن تسعة مضادات أكسدة فينولية، وهي: حمض الفانيل، وحمض السيرينج، وحمض الفيروليك، وحمض الأيسوفيروليك، وحمض السنائيك، والفانيلين، والألديهيد السيرينجي، هيدروكسي بيتز ألديهيد، وألديهيد الكونيفيريل، وذلك في ال EDF المشتق من TMS. أنتجت تجزئة ال ECD الغنية بمضادات الأكسدة الفينولية باستخدام عمود الوسط العادي للكروماتوجرافيا خمسة أجزاء غنية بمضادات الأكسدة القطبية، وسميت بـ Di-21، و Di-22، و Di-24، و Di-25، و Di-26، والتي أظهرت أقوى نشاط كسح لل DPPH مقارنة بال ECD. كشف التحليل الإضافي باستخدام GC-MS لمشتقات ال TMS الخمسة وجود أربعة مضادات إضافية للأكسدة، وهي حمض البنزويك الكاربوك-سيللي، وحمض الهوموفانيليك، وحمض الكوماريك، وألديهيد السنائيك. أشارت ملاحظة محتوى حامض الفانيليك وحامض السينائيك في ال EDF وفي مشتقاتها الخمسة وبين نشاط كسح لل DPPH الخاص بها النشاط التضافري لحامض السينائيك والتأثير العدائي لحامض الفانيليك مع أنشطة كسح لل DPPH الخاص بهما. أثبت اكتشاف هذه المركبات النباتية الكيميائية الثلاثة عشر في ال EDF أن قشور ثمرة الكيرينجي هي مصدر جيد لمضادات الأكسدة والتي قد تساهم في حماية الجسم من الآثار السلبية للضغط التأكسدي المسببة لتشكيل الأمراض المزمنة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Chemistry).

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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.

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TABLE OF CONTENTS

Abstract.....	ii
Abstract in Arabic.....	iii
Approval Page.....	iv
Declaration.....	v
Copyright Page.....	vi
Acknowledgements.....	vii
List of Tables.....	xi
List of Figures.....	xii
CHAPTER ONE: INTRODUCTION.....	1
1.1 Research Background.....	1
1.2 Problem Statement.....	2
1.3 Research Objectives.....	3
1.4 Research Hypothesis.....	3
1.5 Significance Of Study.....	3
CHAPTER TWO: LITERATURE REVIEW.....	4
2.1 Antioxidants and Their Health Benefits.....	4
2.2 Phenolics and Phenolic Acids.....	6
2.3 Determination of Total Phenolics Content (TPC).....	11
2.4 Determination of Total Flavonoids Content (TFC).....	11
2.5 Cupric Ion Reducing Antioxidant Capacity (CUPRAC) Assay.....	12
2.6 DPPH Radical Scavenging Assay.....	12
2.7 Linoleic Acid Peroxidation Inhibition Assay.....	13
2.8 GC-MS Analysis of Plant Extracts.....	13
2.9 <i>Dialium indum</i> var. <i>indum</i> L.....	14
2.10 Uses of <i>Dialium</i> Species.....	16
2.11 Nutritional and Phytochemical Content of <i>Dialium</i> Species.....	18
2.12 Antioxidant and Other Biological Activities of <i>Dialium</i> Species.....	25
CHAPTER THREE: METHODOLOGY.....	27
3.1 Extracts Preparation from Plant Materials.....	27
3.1.1 Collection of Plant Materials.....	27
3.1.2 Samples Preparation.....	27
3.1.3 Crude Extracts and Fractions Preparation.....	28
3.2 Proximate Analysis and <i>In vitro</i> Antioxidant Assays.....	29
3.2.1 Total Phenolics Content (TPC).....	29
3.2.2 Total Flavonoids Content (TFC).....	30
3.2.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC).....	31
3.2.4 DPPH Radical Scavenging Activity.....	32
3.2.5 Linoleic Acid Peroxidation Inhibition.....	33
3.3 Calculations and Statistical Analysis.....	35
3.4 Samples Selection for GC-MS Analysis.....	35
3.5 Chromatographic Techniques.....	35
3.5.1 Thin Layer Chromatography (TLC).....	35

3.5.1.1 Analytical TLC	35
3.5.2 Column Chromatography.....	37
3.6 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis	38
3.6.1.1 Sample Derivatization	38
3.6.1.2 GC-MS Instrumentation and Conditions.....	39
3.7 Flowchart of Methodology	40
CHAPTER FOUR: RESULTS AND OBSERVATIONS.....	41
4.1 Extraction Yield and Organoleptic Properties.....	41
4.2 Proximate Analysis of Antioxidants Content	41
4.2.1 Total Phenolics Content (TPC).....	41
4.2.2 Total Flavonoids Content (TFC).....	43
4.2.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC).....	45
4.3 <i>In vitro</i> Antioxidant Activities.....	46
4.3.1 DPPH Radical Scavenging Activity	46
4.3.2 Linoleic Acid Peroxidation Inhibition	47
4.4 Samples Selection for GC-MS Analysis.....	51
4.5 GC-MS Analysis of <i>D. indum</i> var. <i>indum</i> L. Seed Methanol Fraction (SMF) and Exocarp DCM Fraction (EDF).....	52
4.6 Fractionation of <i>D. indum</i> var. <i>indum</i> L. Exocarp DCM Fraction (EDF).....	61
4.6.1 DPPH Radical Scavenging Activities of Selected <i>D. indum</i> var. <i>indum</i> L. EDF Subfractions.....	62
4.6.2 GC-MS Analysis of Antioxidant-rich Subfractions of EDF.....	62
CHAPTER FIVE: DISCUSSION.....	67
5.1 Phytochemicals Extraction from <i>D. indum</i> var. <i>indum</i> L. Fruit Parts	67
5.2 Total Phenolics Content (TPC), Total Flavonoids Content (TFC), and Cupric Ion Reducing Antioxidant Capacity (CUPRAC).....	68
5.2.1 Total Phenolics Content (TPC).....	68
5.2.2 Total Flavonoids Content (TFC).....	69
5.2.3 Cupric Ion Reducing Antioxidant Capacity (CUPRAC).....	70
5.3 <i>In vitro</i> Antioxidant Activities.....	71
5.3.1 DPPH Radical Scavenging Activity	71
5.3.2 Linoleic Acid Peroxidation Inhibition	72
5.4 GC-MS Analysis of <i>D. indum</i> var. <i>indum</i> L. Exocarp DCM Fraction (EDF) and Seed Methanol Fraction (SMF)	73
5.5 Fractionation of EDF	79
5.5.1 Antioxidant Activities and GC-MS Analysis of Antioxidant- rich Subfractions of EDF.....	80
CHAPTER SIX: CONCLUSION	85
REFERENCES.....	86

APPENDIX I:	PUBLICATIONS FOR SYMPOSIUM/ CONFERENCE.....	97
APPENDIX II:	PREPARATION OF CHEMICALS	100
APPENDIX III:	CALIBRATION CURVES FOR TPC AND TFC.....	101
APPENDIX IV:	CALIBRATION CURVES FOR CUPRAC AND DPPH ASSAY	102
APPENDIX V:	DETERMINATION OF IC₅₀ VALUES USING GRAPH 4.4.2	103
APPENDIX VI:	CONDITION PARAMETERS OF GC-MS ANALYSIS.....	104
APPENDIX VII:	TLC ANTIOXIDANT BIOAUTOGRAMS OF EDF SUBFRACTIONS.....	105
APPENDIX VIII:	NIST2014 LIBRARY SEARCH REPORT.....	106
APPENDIX IX:	ELECTRON IONIZATION MASS SPECTRA OF TMS-DERIVATIZED PHENOLICS	107

LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
2.1	Structures of Phenolic Acids	10
2.2	Taxonomical Rank of <i>Dialium indum</i> var. <i>indum</i> L.	11
4.1	Percentage Yield and Organoleptic Properties of <i>Dialium indum</i> var. <i>indum</i> Crude Extracts and Fractions	42
4.2	Total Phenolics Content (TPC) of the Various Crude Extracts and Fractions	43
4.3	Total Flavonoids Content (TFC) of the Various Crude Extracts and Fractions	44
4.4	Cupric Ion Reducing Antioxidant Capacity (CUPRAC) of the Various Crude Extracts and Fractions	45
4.5	DPPH Radical Scavenging Activity of the Various Crude Extracts, Fractions, and Quercetin	49
4.6	Linoleic Acid Peroxidation Inhibition of the Various Crude Extracts, Fractions, and Standards	50
4.7	The Ranking of EDF and EHF for the Proximate Analysis of Antioxidants Content and <i>In vitro</i> Antioxidant Assays	52
4.8	Identified Compounds in <i>Dialium indum</i> var. <i>indum</i> L. Seed Methanol Fraction (SMF) by GC-MS Analysis	56
4.9	Identified Compounds in <i>Dialium indum</i> var. <i>indum</i> L. Exocarp DCM Fraction (EDF) by GC-MS Analysis	58
4.10	TMS-derivatized Phenolics Detected in <i>Dialium indum</i> var. <i>indum</i> L. Exocarp DCM Fraction (EDF)	59
4.11	TMS-derivatized Phenolics Detected in <i>Dialium indum</i> var. <i>indum</i> L. EDF Subfractions	65
4.12	Area Percentage of Phenolics in <i>Dialium indum</i> var. <i>indum</i> L. EDF and Its Subfractions	66
5.1	Fragmentation Patterns of TMS-derivatized Phenolics Detected in EDF Subfractions	82

LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
2.1	Classification of Phenolic Antioxidants	8
2.2	Synthetic Phenolic Antioxidants	9
2.3	Fruits of Keranji Papan (left), Keranji Emplawak (middle), and Keranji Masam (right)	17
2.4	Some Phytochemicals from <i>Dialium</i> Species	21
3.1	<i>Dialium indum</i> var. <i>indum</i> Fruit Anatomy	27
3.2	Flowchart of Tests and Methods Used in the Current Study	40
4.1	Total Ion Current (TIC) Chromatogram of <i>Dialium indum</i> var. <i>indum</i> Seed Methanol Fraction (SMF)	55
4.2	Total Ion Current (TIC) Chromatogram of <i>Dialium indum</i> var. <i>indum</i> Exocarp DCM Fraction (EDF)	57
4.3	TLC Profiles of EDF	64

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Research on antioxidant properties of plants has increased over the years due to the discovery of the role of oxidants, known as reactive oxygen species (ROS), in the process of ageing and pathogenesis of chronic diseases such as cancer, cardiovascular diseases, and various metabolic disorders. These diseases are believed to be caused by oxidative stress, which is an imbalance between ROS and antioxidant defences in the body due to overproduction of ROS and depletion of antioxidants level (Gomes, Fernandes, & Lima, 2005). The oxidative stress disrupts cellular functions, thus causing various undesirable pathological conditions to the human body (Sindhi *et al.*, 2013).

Antioxidants are compounds that protect macromolecules like carbohydrates, proteins, fat, and nucleic acids from damaging effect of ROS by reducing oxidative stress (Procházková, Boušová, & Wilhelmová, 2011). Under normal conditions, antioxidant enzymes produced by cells maintain the low production of ROS in the body. In addition to this, various plants had been proven to contain antioxidants in the form of vitamins, carotenoids and phenolic compounds that possess a myriad of biological properties, such as anti-atherosclerotic, anti-carcinogenic, anti-inflammatory, and modulate the gut health by maintaining the gut microbiota (Oroian & Escriche, 2015). These antioxidants are vital for the human body to restore the balance that has been perturbed by oxidative stress.

Malaysia is one of the countries in the world that has the most complex tropical rainforest ecosystems, owing to combination of its tropical maritime climate and the

variation of its geographical, edaphic and climatic features (Guan, 1992). *Dialium indum* var. *indum* L. is an edible fruit-bearing tree that dwells the tropical rainforest of Malaysia. The local name of the tree's fruit 'keranji' has gained popularity among Malaysian population as it is mentioned in a folk poem, namely pantun, where children are politely criticized for being lazy in learning things.

The fruit of *D. indum* is sold at markets by the locals and its sweet-sour dried flesh is eaten as seasonal sweets. Other than that, information regarding the fruit's other uses among Malaysian population is lacking. Hence, this study was conducted to investigate the proximate antioxidant content, *in vitro* antioxidant activities, and phenolic compounds in Malaysian *D. indum* fruit parts, which include the seed, exocarp (skin) and mesocarp (flesh).

1.2 PROBLEM STATEMENT

Wild fruits have been studied to be good sources of antioxidants, which can positively affect the health of human body systems and reduce the risk of developing chronic diseases (Bhatt, Rawat, Badhani, & Rawal, 2017; Guimarães *et al.*, 2013, 2014). To date however, the use of *D. indum* is limited to consumption of its fruit's pulp as sweets while the exocarp and seed of the fruit, which could be good sources of antioxidants, are usually discarded and considered as waste products. Hence, research on this plant can serve as a platform to explore its health benefits to human population in the context of its proximate antioxidant content and *in vitro* antioxidant activities.

1.3 RESEARCH OBJECTIVES

The study aimed to achieve the following objectives:

- 1) To evaluate the proximate content of antioxidants and *in vitro* antioxidant activities of various extracts of *Dialium indum* var. *indum* L. fruit parts.
- 2) To identify the phytochemicals in the selected antioxidant active extracts of *D. indum* var. *indum* L. fruit parts using gas-chromatography-mass spectrometry (GC-MS) analysis.
- 3) To perform column chromatography fractionation of antioxidant active extract of *D. indum* var. *indum* L. fruit part for further analysis of the phenolics.

1.4 RESEARCH HYPOTHESIS

The extract of *D. indum* var. *indum* L. fruit contains phenolic compounds, which significantly contribute to its *in vitro* antioxidant activities.

1.5 SIGNIFICANCE OF STUDY

Determination of phenolic content and *in vitro* antioxidant activities of extracts of *D. indum* var. *indum* L. fruit parts scientifically provide the evidence for its goodness as a source of antioxidants. Besides, recognition of *D. indum* var. *indum* L. as a fruit with strong *in vitro* antioxidant activity can serve as starting point for further research and development for future commercialization.

CHAPTER TWO

LITERATURE REVIEW

2.1 ANTIOXIDANTS AND THEIR HEALTH BENEFITS

Antioxidants are a large group of compounds, consisting of two major groups based on their chemical structures, which are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants can be further categorized into primary and secondary antioxidant enzymes. Primary antioxidant enzymes such as superoxide dismutase and catalase directly react with the free radicals to stabilize them, thus inhibiting the cascade of oxidative damage in the human body systems. Superoxide dismutase exerts antioxidant effect by conversion of superoxide anions into hydrogen peroxide, which is then converted to the non-harmful molecules of water and oxygen gas by catalase (Rahman, 2007).

On the other hand, secondary antioxidant enzymes inhibit the harmful effects of oxidative stress indirectly by supporting generation of active endogenous antioxidants, which are the real scavengers of free radicals present in the internal environment. Examples include glutathione reductase and glucose-6-phosphate dehydrogenase (G6PD) (Carocho & Ferreira, 2013). Glutathione reductase for example, recycles glutathione (an endogenous antioxidant) so that it can continue stabilizing more free radicals in the body (Ratnam, Ankola, Bhardwaj, Sahana, & Kumar, 2006). Glucose-6-phosphate dehydrogenase on the other hand, is involved in regeneration of nicotinamide adenine dinucleotide phosphate (NADPH), an important source of reductive power for antioxidative defence (Mailloux & Harper, 2010).

Another class of antioxidants is non-enzymatic antioxidants, where vitamins, minerals, cofactors, organosulfur compounds, nitrogen non-protein compounds, carotenoids, and polyphenols are grouped together. It is also important to note that most non-enzymatic antioxidants are exogenous, which means they are obtained from the outside of the body, especially the diet. Some examples of exogenous non-enzymatic antioxidants are vitamin C, E, and K (vitamins), zinc and selenium (minerals), allyl sulphide (organosulfur compound), carotenes and xanthophylls (carotenoids), and phenolic acids and flavonoids (phenolics) (Carocho & Ferreira, 2013).

Plant phenolics have been extensively studied due to their antioxidant properties that may be related to the prevention of modern era diseases like chronic inflammation, cardiovascular diseases, cancer, and diabetes (Acosta-Estrada, Gutiérrez-Urbe, & Serna-Saldívar, 2014). Studies have also shown that some phenolics play significant roles in the regulation of brain's immune system, which is vital for the prevention of age-related cognitive and neurones degeneration in both animals and humans through various physiological functions (Spencer, Vafeiadou, Williams, & Vauzour, 2012).

Populations with plant phenolics-rich diet (diet rich in fruits and vegetables) were shown to have lower rates of cancer (Kushi *et al.*, 2012). A study found that there was correlation between dietary patterns and colorectal adenoma and cancer risk, where higher consumption of fruits and vegetables appeared to be protective against colorectal adenoma and cancer incidence (Miller, Lesko, Muscat, Lazarus, & Hartman, 2010). Besides, increasing consumption of foods that are rich in antioxidants was suggested to be one of the approaches to improve the health of the population (Kongkachuichai, Charoensiri, Yakoh, Kringkasemsee, & Insung, 2015).

Plant phenolics also have been applied in chemoprevention of skin cancer. Chemoprevention is a study approach to prevent or delay the development of skin

cancer, by means of investigating the effects of phytochemicals on targeted sites that are responsible for tumor promotion. Plant phenolics such as resveratrol and (-)-epigallocatechin-3-gallate (EGCG) have shown positive effects *in vitro* and *in vivo* on cellular targets involved in UVB-induced carcinogenesis (Oca, Pearlman, Mcclees, Strickland, & Afaq, 2016).

These evidences showed that antioxidants, specifically phenolics, can help in maintenance of good health of human's body systems. Numerous studies led to the discovery of numerous phenolic antioxidants. What compounds comprise the phenolic antioxidants?

2.2 PHENOLICS AND PHENOLIC ACIDS

Based on their origin, phenolic antioxidants can be classified as synthetic and natural, as shown in Figure 2.1 (Shahidi & Ambigaipalan, 2015). Synthetic phenolics are widely utilized in preservation of consumer products (foodstuffs and cosmetics), especially those that are lipid-containing due to their excellent performance in preventing or delaying the onset of lipid oxidation (Sindhi *et al.*, 2013). As the term 'synthetic' implies, there are structural modifications to obtain the desired features. For example, addition of propyl group to gallic acid produces propyl gallate (1) (Figure 2.2), which is more lipophilic than gallic acid. These synthetic antioxidants are commonly used in combination with each other due to the synergistic effect with greater overall antioxidative potency than the contribution of each individual compound (Shahidi & Ambigaipalan, 2015).

The position and number of substituents (hydroxyl, methoxy, methyl groups, and others) of the molecules affect the antioxidant activities because these functional groups exhibit strong resonance and inductive effects, enabling charge delocalization in

the radicals formed by the antioxidant molecules (Jeremić *et al.*, 2017). Butylated hydroxyanisole (BHA) (2), butylated hydroxytoluene (BHT) (3), and tertiary-butyl hydroquinone (TBHQ) (4) are hindered phenolics, where the phenolic ring contains tertiary-butyl groups (Yehye *et al.*, 2015). This results in higher solubility in lipophilic media (higher log *P*). However, the presence of two tertiary-butyl groups in BHT has rendered the molecule to be less antioxidative in comparison with BHA. This is because these substituents provide greater steric hindrance to the hydroxyl group, which is responsible for the antioxidant activity of phenolics (Nanditha & Prabhasankar, 2010). The high pKa values are largely due to the phenolic group, making them soluble in alkaline media of pH 10.

The second class of phenolic antioxidants is natural phenolics. The distinctive features of natural phenolics are the simple phenolic acids and more complex structures of aromatic compounds termed polyphenolics. They are produced abundantly by plants as secondary metabolites that play diverse functions for the plants' survival. In plants, these phenolics are allelochemicals that affect permeability of plant cells' membrane, uptake of nutrients, activities of enzymes, photosynthesis, and respiration (Iannucci, Fragasso, Platani, & Papa, 2013). Simple phenolic acids are one of the most common plant phenolics as they can be found in free, bound, and conjugated forms. Phenolic acids are one of the components of cell wall's structure (Acosta-Estrada *et al.*, 2014). They are bound to the cell wall polymers via ester linkage (Iannucci *et al.*, 2013). Different distribution of phenolic acids in various plants has been described to be influenced by several factors such as genotype, environment in which the plants grow, and genotype-environment interactions (Navas-Lopez *et al.*, 2014).

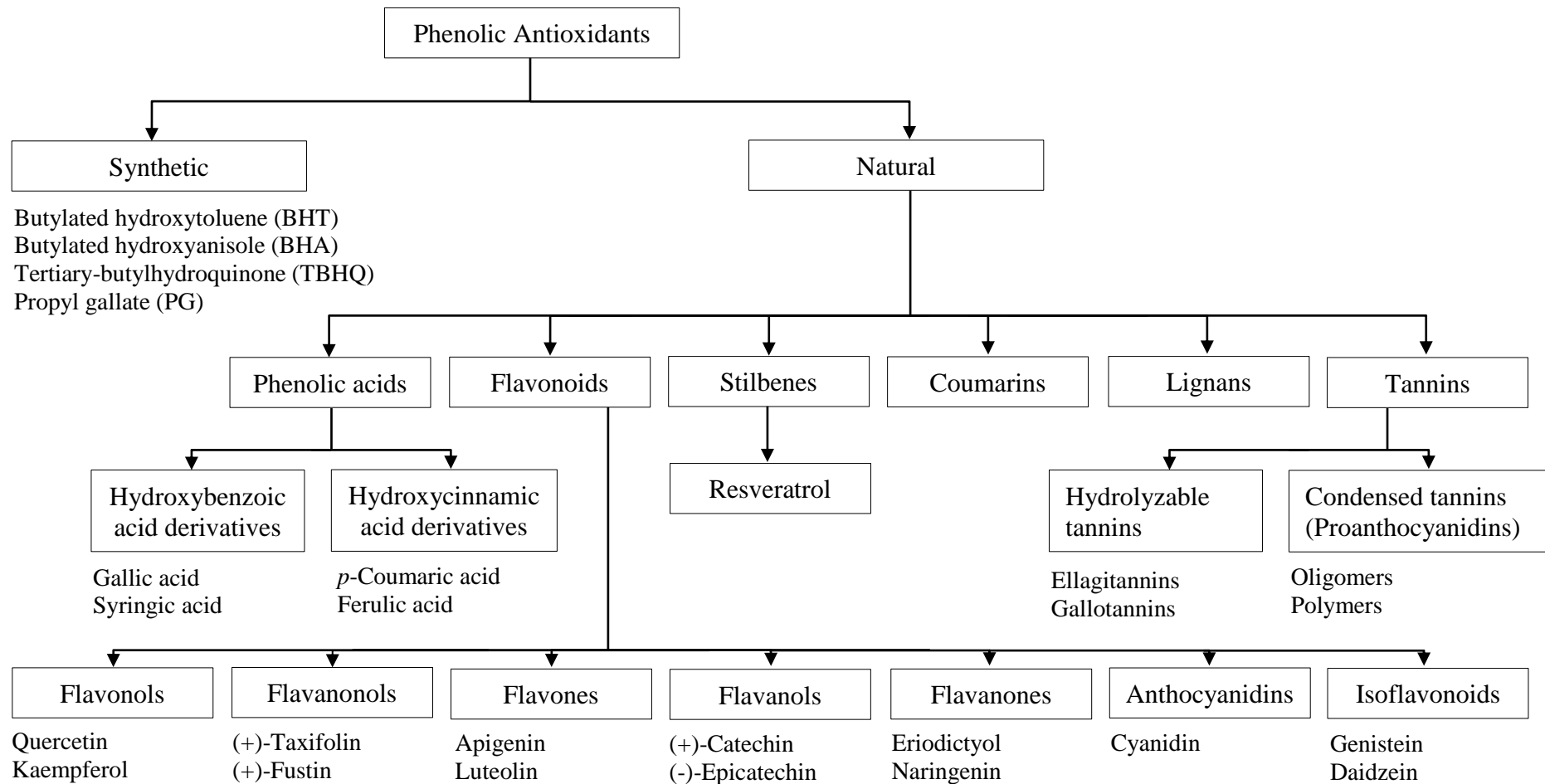
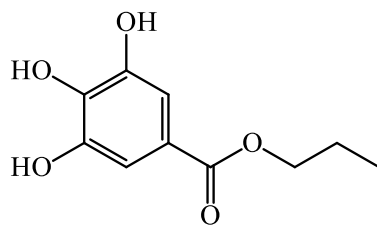
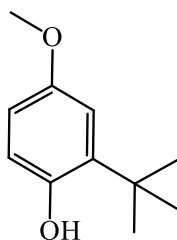


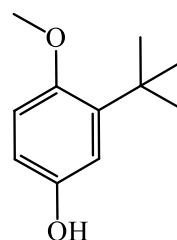
Figure 2.1 Classification of Phenolic Antioxidants
Source: (Shahidi & Ambigaipalan, 2015)



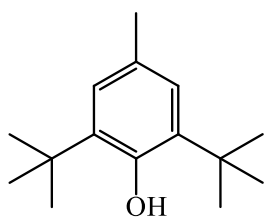
Propyl gallate
(1)



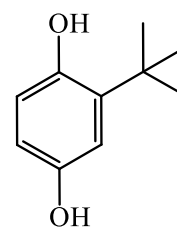
3-Tertiary-butyl-4-hydroxyanisole (90%)
Isomer I
(2)



2-Tertiary-butyl-4-hydroxyanisole (10%)
Isomer II
(2)



3,5-Di-tertiary-butyl-4-hydroxytoluene
(3)



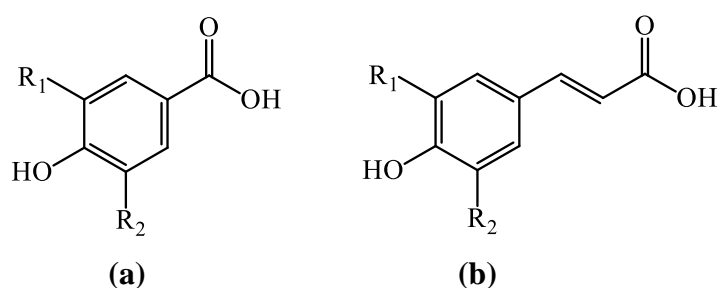
2-Tertiary-butylhydroquinone
(4)

Figure 2.2 Synthetic Phenolic Antioxidants

The various polyphenolics in plants were synthesised via phenylpropanoid biosynthetic pathway. It controls up to 40% of organic compounds circulating in the biosphere. The phenylpropanoid pathway is linked to the shikimate pathway, which produces aromatic amino acids (phenylalanine, tyrosine, and tryptophan) from central carbon metabolisms. Phenylalanine ammonia lyase acts as the entry-point enzyme for the phenylpropanoid pathway, resulting in the synthesis of *trans*-cinnamic acid (*t*-CA). The *t*-CA produced is then further transformed into many other phenolics (Zhang & Liu, 2015).

Biosynthesis of phenolic acids occurs via the shikimate pathway from L-phenylalanine or L-tyrosine. Phenolic acids can be classified into two categories based on the molecules they are derived from, namely hydroxybenzoic and hydroxycinnamic acid derivatives, as shown in Table 2.1. Deamination, hydroxylation, and methylation are the main three reactions involved in the formation of these various phenolic acids.

Table 2.1 Structures of Phenolic Acids
Source: (Heleno, Martins, Queiroz, & Ferreira, 2015)



Substitution		<i>para</i> -Hydroxybenzoic acid derivatives (a)	<i>para</i> -Hydroxycinnamic acid derivatives (b)
R ₁	R ₂		
H	OH	Protocatechuic acid (5)	Caffeic acid (9)
OCH ₃	H	Vanillic acid (6)	Ferulic acid (10)
OCH ₃	OCH ₃	Syringic acid (7)	Sinapic acid (11)
OH	OH	Gallic acid (8)	-

2.3 DETERMINATION OF TOTAL PHENOLICS CONTENT (TPC)

TPC is an electron transfer-based assay that employs yellow-colored Folin-Ciocalteu (FC) reagent, which consists of a mixture of the phosphomolybdic and phosphotungstic acid with oxidation number of +6. After being reduced by a phenolic group, a blue-colored coordination complex was formed with the mean oxidation number of molybdenum and tungsten ions being between +5 and +6. The saturated sodium carbonate solution is added since the reaction only occurs under basic condition. It is a sensitive and precise assay, but lacks specificity because the FC reagent can also be reduced by many nonphenolic compounds such as ascorbic acid and copper (I) ion (Agbor, Vinson, & Donnelly, 2014; Huang, Boxin, & Prior, 2005).

The use of basic medium in this assay also contributes to its drawback since in basic medium, the dissociation of the phenolic group's proton occurs and this would enhance a sample's reducing capacity, as can be seen in higher values for TPC result in comparison with other *in vitro* antioxidant assays. Besides, the use of basic medium does not reflect the real chemical behaviour of phenolics at the body's physiological pH (Apak *et al.*, 2013). The drawbacks of the TPC assay merit the utilization of other *in vitro* antioxidant assays to complement the evidence on antioxidant content and activities of tested samples.

2.4 DETERMINATION OF TOTAL FLAVONOIDS CONTENT (TFC)

TFC is another colorimetric assay based on the relative measurement of the concentration of acid stable aluminium-flavonoid complexes formed after the reaction of the aluminium ions with the ketone or hydroxyl groups of the flavonoids (Jauhari *et al.*, 2013). It is an assay specific for quantification of flavones (for examples: chrysin, apigenin, and luteolin) and flavonols (for examples: quercetin, rutin, and morin).

Besides, it is noteworthy that the aluminium ions react differently with the flavonoids that do not have the C2-C3 double bond (flavanones and flavanonols) (Chia-Chi Chang, Yang, Wen, & Chern, 2002).

2.5 CUPRIC ION REDUCING ANTIOXIDANT CAPACITY (CUPRAC) ASSAY

CUPRAC assay is an electron transfer-based assay developed in the analytical chemistry laboratories of Istanbul University, Turkey. It measures the extent of reduction of light blue-colored bis(neocuproine)copper(II) chelate to yellow-orange-colored bis(neocuproine)copper(I) chelate by the antioxidants. The bis(neocuproine)copper(II) chelate is formed by reaction of copper(II) chloride with 2,9-dimethyl-1,10-phenanthroline (neocuproine) in ammonium acetate buffer of pH 7.0 (Ozyurek, Guclu, & Apak, 2011). The CUPRAC assay was carried out in this study because of its advantages over other *in vitro* antioxidant assays. The bis(neocuproine)copper chelate in the assay has lower redox potential ($E^\circ = 0.60$ V) than ABTS ($E^\circ = 0.68$ V) and FRAP ($E^\circ = 0.70$ V), hence, it does not oxidize simple sugars and citric acid present in the sample. Besides, the reagent is more photostable and easily accessible by antioxidants compared with DPPH radical. Other than that, the redox reaction in CUPRAC assay occurs at nearly physiological pH (pH 7.0 of ammonium acetate buffer) as opposed to the acidic medium of FRAP (pH 3.6) or basic medium of TPC assay (pH 10.0) (Apak *et al.*, 2013).

2.6 DPPH RADICAL SCAVENGING ASSAY

DPPH radical scavenging activity assay involves the measurement of reduction of a purple-colored nitrogen-containing radical namely 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl to yellow-colored hydrazine by the phenolic antioxidants. The