UNIVERSITI PUTRA MALAYSIA

TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS ISOLATED FROM LEAVES OF SELECTED CITRUS SPECIES

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS ISOLATED FROM LEAVES OF SELECTED CITRUS SPECIES

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November 2006

Chairman: **Associate Professor Umi Kalsom Yusuf, PhD**

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Water and 80% methanol crude extracts from the leaves of *Citrus aurantifolia, C. hystrix, C. grandis,* and *Citrofortunella microcarpa* were investigated for their total phenolic content and its relationship with total antioxidant activity. The total phenolic content was determined using a Folin-Ciocalteu reagent whereas determination of total antioxidant activity was carried out using a ***β***-carotene bleaching method. Among the citrus leaves extracts, the highest total phenolic content was found from the water extract of *Citrofortunella microcarpa* whereas the water extract of *C. maxima* and *Citrofortunella microcarpa* gave the highest total antioxidant activity and was comparable with the antioxidant activity of marker BHT. However, no clear correlation was found between the total phenolic content and the total antioxidant activity of citrus leaves in both crude extract. Eleven flavonoid components were isolated and identified from the 80% methanolic extract of the leaves of *Citrus aurantifolia, C. hystrix, C. grandis* and

Citrofortunella microcarpa. The isolation and purification of all flavonoid components were carried out using standard paper chromatography technique. The isolated components were identified basis of their spectral analysis with shift reagents, R_f values, colour reactions under UV and UV+NH_3, hydrolysis with acid and co-chromatogram with standard markers. The successfully isolated flavonoids were consisted from the group of flavone, flavone *C*-glycoside and flavonol. The methylated and glycosylated flavonoids were also isolated. The antioxidant capacity of isolated flavonoids was measured by two assays; a ***β***-carotene bleaching method and a DPPH scavenging method. Statistical analysis showed that the total antioxidant and free radical scavenging activity of the isolated flavonoids was significantly low compared to the markers. Among the isolated flavonoids, results showed that at the 60^{th} minute, quercetin 4'-*O*-glucoside exhibited the highest total antioxidant activity. Quercetin 4'-*O*-glucoside also showed a significantly higher free radical scavenging activity in DPPH scavenging method than the other isolated flavonoids. The lowest activities in both assays were exhibited by vitexin 7-*O*-arabinoside and isovitexin.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

JUMLAH KANDUNGAN FENOLIK DAN AKTIVITI ANTIOKSIDAN FLAVONOID YANG DIPENCILKAN DARIPADA DAUN CITRUS TERPILIH

Oleh

SALFARINA BINTI RAMLI

November 2006

Pengerusi: **Profesor Madya Umi kalsom Yusuf, PhD**

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Jumlah kandungan fenolik dan aktiviti antioksidan daripada ekstrak mentah air dan 80% metanol daun *Citrus aurantifolia, C. hystrix, C. grandis,* dan *Citrofortunella microcarpa* telah dikaji. Penentuan jumlah kandungan fenolik dilakukan menggunakan bahan reagen Folin-Ciocalteu manakala jumlah aktiviti antioksidan dijalankan melalui kaedah pelunturan ***β***-carotene. Daripada kesemua ekstrak mentah daun citrus, jumlah kandungan fenolik yang tertinggi diperolehi daripada ekstrak air daun *Citrofortunella microcarpa*, manakala ekstrak air daun *C. maxima* dan *Citrofortunella microcarpa* menunjukkan aktiviti antioksidan yang paling tinggi dan setara dengan aktiviti antioksidan penunjuk BHT. Walaubagaimanapun, tiada hubungan jelas yang dapat mengaitkan antara nilai aktiviti antioksidan dengan jumlah kandungan fenolik ekstrak ektraks daun citrus tersebut. Sebelas komponen flavonoid telah berjaya diasingkan daripada ekstrak 80% metanol daun *Citrus aurantifolia, C. hystrix, C. grandis* dan *Citrofortunella microcarpa*. Pemencilan dan penulenan komponen flavonoid dilakukan menggunakan teknik kertas kromatografi. Pengenalpastian komponen flavonoid dibuat melalui analisis spektrum menggunakan bahan reagen, perubahan warnanya di bawah sinaran UV dan UV+NH_3,

hidrolisis asid serta perbandingan ko-kromatografi dengan bahan penanda piawai. Komponen flavonoid yang telah dipencilkan terdiri daripada kumpulan flavone, flavone C-glycoside dan flavonol. Komponen flavonoid yang terikat dengan kumpulan metal dan gula juga berjaya dipencilkan. Kapasiti antioksidan bagi komponen flavonoid yang telah dipencilkan diuji melalui dua kaedah; pelunturan ***β***-carotene dan kebolehan menghalang tindakan radikal bebas DPPH. Daripada ujian statistik yang dijalankan didapati aktiviti antioksidan dan tindakan menghalang radikal bebas komponen flavonoid yang telah dipencilkan adalah lebih rendah berbanding aktiviti penunjuk. Hasil kajian menunjukkan pada minit ke 60, *quercetin 4'-O-glucoside* menunjukkan aktiviti antioksidan yang tertinggi. *Quercetin 4'-O-glucoside* juga merupakan penghalang radikal bebas yang paling baik berbanding komponen flavonoid yang lain. Aktiviti yang terendah dalam kedua dua kaedah ujian antioksidan telah ditunjukkan oleh *vitexin 7-O-arabinoside* dan *isovitexin*.

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This thesis 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 are as follows:

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Date: 12 APRIL 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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SALFARINA BINTI RAMLI

Date:

TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

$AlCl_3$	Aluminium chloride
BAW	n-nutanol:acetic acid:water; 4:1:5
BEW	n-butanol:ethanol:water; 4:1:2:2
BHA	Buthylated hydroxyanisole
BHT	Buthylated hydroxyl toluene
CAW	Chloroform:acetic acid:water; 30:15:2
Forestal	Hydrochloric acid:acetic acid:water; 3:30:10
GAE	Gallic acid
H_3BO_3	Boric acid
HCl	Hydrochloric acid
HoAc	Acetic acid
NaOAc	Natrium acetate
NaOH	Natrium hydroxide
NH_3	Ammonia
Nm	Nanometer
OH	Hydroxyl
OMe	Methyl ether
Sh	Shoulder
TBPW	Toluene:n-buthanol:pyridine:water; 1:5:3:1

CHAPTER 1

INTRODUCTION

Plants synthesize diversity of chemical compound through networks of metabolic pathways. These individual chemicals made by plants called phytochemical, can be found accumulated at every parts of plant as in leaves, fruits, seeds, bark and others. Each phytochemical has different basic chemical properties that in turn gives special function to the plants that synthesized it.

In common usage, many phytochemicals are associated with human benefits. Phenolic compounds are one of phytochemicals that has long history in the human industry as in tanning skin industry, ink manufacturing, in the fining of wines as well as to human health. Previously research showed the occurrence of phenolics in diet as in vegetables and fruits conferred protection against cardiovascular disease and certain forms of cancer (Hertog *et al.*, 1992). Therefore, it was recommended to have at least 400 g or five portions of fruit and vegetables each day to protect against coronary heart disease (CHD), stroke and cancers (Jackson *et al.,* 2005).

Phenolics constitute of compounds with one or more hydroxyl groups (OH group) bounded to an aromatic ring. Numerous categories of these compounds exist, from the simple phenols to the complex structure of condensed tannin. Although phenolics are often classified as secondary metabolites which means not essential for the normal

growth, development or reproduction of plant, numerous studies have been dedicated to the benefits of plant phenolics and its antioxidant properties is among that received the highest attentions.

Phenolic compounds such as flavonoids, catechin and anthocyanin from foods and beverages had conclusively exhibited antioxidant activities (Ghiselli *et al.*, 1998), as a result, screening of antioxidant activity of food and plants extracts frequently accompanied with the total phenolics study. A number of studies reported a significant correlation between the phenolics content of the plant extracts and their antioxidant properties.

Lipid peroxidation and free radicals are major cause and contributor to the oxidation process. Oxidation process has been implicated as commence in cancer and atherosclerosis as well as deterioration of fats and oils in food. An antioxidant has been used to help inhibiting oxidation generally by donating electron and hydrogen to the free radicals (Halliwell and Gutterridge, 1995).

The flavonoids are the most studied phenolics related to the antioxidant ability of phenolics. The ability of flavonoids to act as antioxidant was due to their molecular structures that enable them to act against free radicals. The number of hydroxyl group, presence of aromatic ring, heterocyclic rings, and the other structural groups of flavonoids were factors that affected the antioxidant activity of flavonoids (Rice-Evans *et al.,* 1996).

The action of phenolics especially flavonoids as substrates for oxidation is viewed as beneficial in both food study and human health. The isolation, identification of the active constituents and the study of their antioxidant activities has been carried out extensively. This knowledge will provide an identification of natural antioxidants that can be alternatives to synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which has been debated for their safety in usage.

Research by Benavente-García *et al*. (1997) found that flavonoids as flavanones, flavones, and flavonols present in the citrus fruits have shown powerful antioxidants and free radical scavenger activities. In Malaysia, citrus species are popular as the fruits and leaves are used as flavor in food and native medication. The plants are widely distributed and can be found in any part of Malaysia (Verheij and Coronel, 1992).

However, from the previous researches and presence data, the antioxidant study and the isolation of flavonoids from leaves of citrus plants have never yet been done as data available so far were mainly focusing on the fruits, peels and seeds of citrus which were by-products of citrus juice-making industry.

Therefore, in this study the leaves of four citrus species namely *Citrus aurantifolia, C. hystrix, C. grandis,* and *Citrofortunella microcarpa* were selected. Among the different parts of the plants, leaves deserve special attention owing to it copiousness and availability. Moreover, a study by Hyder *et al*. (2002) reported that leaves and green stems accumulated appreciable amounts of phenolics. Since the leaves of citrus are

abundant in Malaysia, this study could be a preliminary attempt of studying the citrus leaves as an economical natural source of antioxidant.

Thus, the objectives of this study were:

1. to determine the total phenolic content of the citrus leaves in the organic and water extract
2. to study the relationship between total phenolic content and the antioxidant activity of the citrus leaves in both organic and water extract
3. to isolate and identify the flavonoid components from the leaves of selected citrus
4. to evaluate the antioxidant activity of the isolated flavonoid components using two assays; ***β***-carotene bleaching and DPPH radical scavenging.

CHAPTER 2

LITERATURE REVIEW

Phenolic compound

According to Walker (1975), the term phenolic compound embraces a wide range of chemical compounds possessing an aromatic ring bearing one or more hydroxyl groups together with a number of other substituents. Therefore plant constituents that possesses a phenol group; an aromatic ring bonded with hydroxyl groups (OH) are collected as phenolic compound (Figure 2.1).

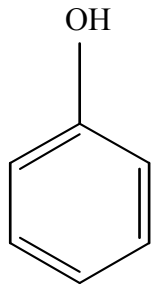


Figure 2.1: Phenol (Source: Walker, 1975)

Numerous plant constituents with phenol group existed. The existence of phenol in nature so diverse; from the simplest to the most complicated structures, thus a classification of phenols is very important specially in studying the structure activity relationship of the compounds. The classifications of phenolics were done according to the number of

carbon in their basic skeleton (Table 2.1). Figure 2.2 and 2.3 showed the classes of phenolic compounds and the number of their basic skeleton.

In nature, phenolic compounds do not occur in a free state but bound to glycosides in the form of esters. The nomenclatures of aglycones (phenolics without sugar group attached) are distinguished from their glycosides form by the insertion of *–id-* into the name of aglycone, for example, cyanin for the glycosylated form and cyanidin for the aglycone (Ribĕreau-gayon, 1972).

Table 2.1: Basic skeleton and classes of phenolics (source: Ribĕreau-gayon, 1972)

No of carbon atoms	Basic skeleton	Class
6	C_6	Simple phenols, benzoquinones,
7	C_6-C_1	Phenolic acids
8	C_6-C_2	Acetophenones, phenylacetic acid
9	C_6-C_3 (phenylpropanoid)	Hydroxycinnamic acid, phenylpropenes, coumarins, isocoumarins
10	C_6-C_4	Naphthoquinones
11	C_6-C_1-C_6	Xanthones
13	C_6-C_2-C_6	Stilbenes
15	C_6-C_3-C_6	Flavonoids, isoflavonoids
18	$(C_6$-$C_3)_2$	Lignan
30	$(C_6$-C_3-$C_6)_2$	Biflavonoids
n	$(C_6$-$C_3)_n$ $(C_6)_n$ $(C_6$-C_3-$C_6)_n$	Lignins Catechol melanin Flavolans (condensed tannin)

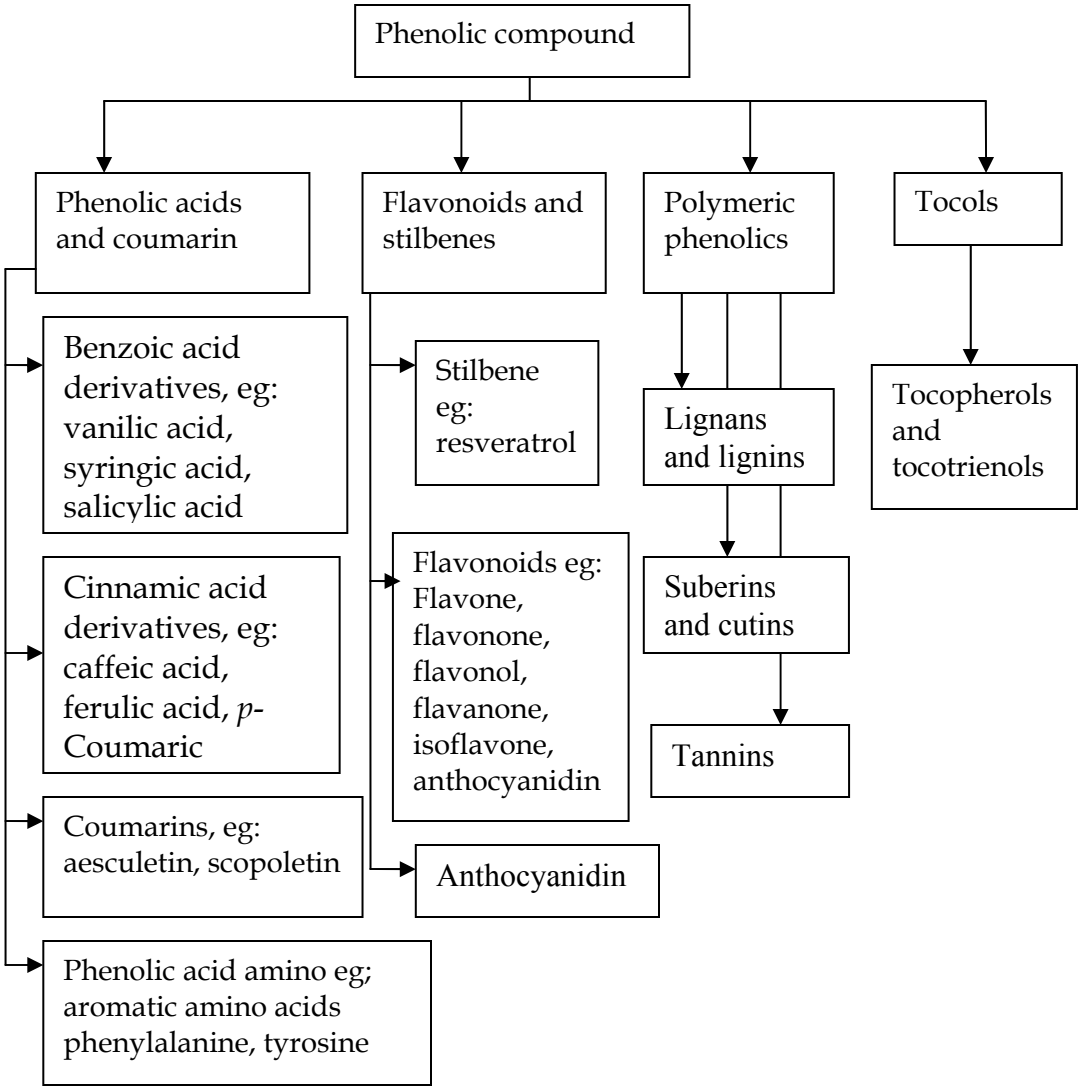
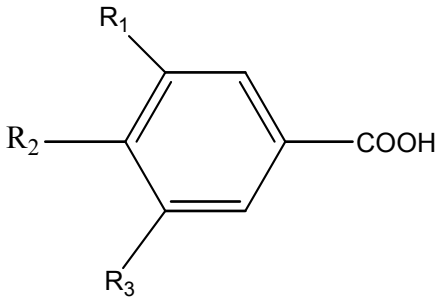
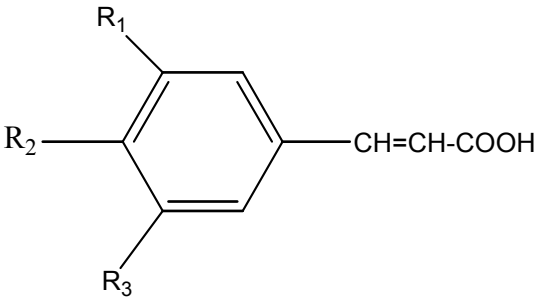


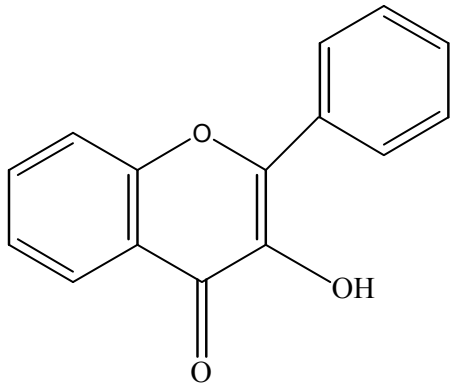
Figure 2.2: classes and examples of phenolic compounds (sources: Shahidi and Naczk (2004) and Walker (1975).



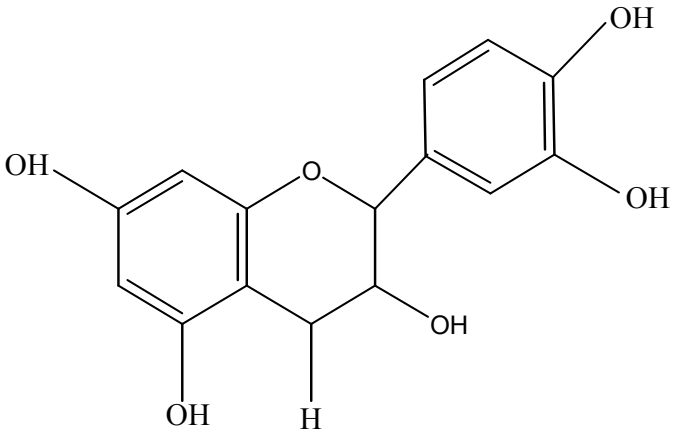
Benzoic acid (C_6-C_1)



Cinnamic acid (C_6-C_3)



Flavonol (C_6-C_3-C_6)



Catechin; a tannin (C_6-C_3-C_6)

Figure 2.3: The structure of some phenolic compounds and their number of basic skeleton (sources: Shahidi and Naczk (2004))

Phenolics found in both edible parts as fruits and vegetables and also in non edible parts. Chalcones and aurones are the yellow pigment of some flowers, whereas anthocyanins are another phenolic pigments that occur as glycoside which responsible for the red and blue colors. Betacyanins are the red pigments for the colour of red beet (*Beta vulgaris*). Benzoic acid (C_6-C_1) and phenolic substances with a phenylpropane structure (C_6-C_3) as eugenol and isoeugenol are common constituents of essential oils (Ribĕreau-gayon, 1972).

Plant phenols are chemically reactive and usually acidic. However, they can be separated from other constituents by their solubility in aqueous sodium carbonate. Total phenolic assay using Folin-Ciocalteu reagent has been widely used to investigate the content of phenolics in the samples studied. Folin-Ciocalteu reagent is nonspecific to phenolic compounds because it can also be reduced by many nonphenolic compounds (e.g.,vitamin C, Cuprum (I)). However, after being adjusted by a sodium carbonate solution to pH ~10, only phenolic compounds react with Folin-Ciocalteu.

In the Folin-Ciocalteu assay, the reducing activity of phenolic was evaluated from its transferring electrons ability. For this reason, Folin-Ciocalteu assay was once grouped into an antioxidant assay category. The blue compounds formed between phenolic and Folin-Ciocalteu reagent was proportional to the concentration of all phenolics in the extracts and was independent with the structure of phenolic compounds. The total phenols assay by Folin-Ciocalteu is convenient, simple, and reproducible. As a result, a