UNIVERSITI PUTRA MALAYSIA

BIOASSAY GUIDED ISOLATION OF ANTIOXIDATIVE COMPOUNDS FROM TWO RUTACEOUS SPECIES MELICOPE GLABRA(BLUME) T.G. HARTLEY AND MICROMELUM MINUTUM (G. FORST) WIGHT AND ARN

NUR KARTINEE KASSIM

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy.

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Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

BIOASSAY GUIDED ISOLATION OF ANTIOXIDATIVE COMPOUNDS FROM TWO RUTACEOUS SPECIES *MELICOPE GLABRA*(BLUME) T.G.HARTLEY AND *MICROMELUM MINUTUM* (G. FORST.) WIGHT AND ARN

By

NUR KARTINEE BINTI KASSIM

December 2013

Chairman : Professor Mawardi Rahmani, PhD
Faculty : Science

Research on the application, characteristics and sources of natural antioxidants especially phenolic had received great interest as synthetic antioxidants were reported to give adverse health effects. *Melicope glabra* **(**Blume) T.G.Hartley and *Micromelum minutum* (G. Forst.) Wight and Arn. (Rutaceae) are edible plants of the Rutaceae family. Both plants are traditionally used in the treatment of various diseases and known to contain a number of rutaceous compounds such as coumarins, lignans and alkaloid. To date, the reports on the bioactive compounds responsible for their medicinal properties are very limited. Thus, the search to identify bioactive compounds particurlarly as antioxidant agent from these unexplored plants are really significant. A bioassay-guided isolation technique by 1, 1-diphenyl-2-dipicrylhydrazyl (DPPH) radical was used to locate and identify the presence of antioxidant components in various extracts of these plants. The three extracts (hexane, ethyl acetate and methanol) of *M. glabra* were screened for antioxidant properties by four different assays; DPPH free radical scavenging, oxidation of *β*-carotene and linoleic acid, oxygen radical antioxidant capacity (ORAC) and total phenolic content (TPC). The results showed that the ethyl acetate and methanol extracts possessed very good antioxidant potential and were selected for activity-guided fractionation. The DPPH IC_{50} values obtained for ethyl acetate and methanol extracts were 24.81 and 13.01µg/mL with the antioxidant activity of 99.5 and 93.0% on the *β*-carotene bleaching assay as compared to α-tocopherol (100%). They also gave high ORAC values (1521 and 2182 µmol TE/g) for the former and latter, respectively. The column chromatograhic separation on active extracts gave five active fractions namely ME 21, ME 24, ME 31, MM 13 and MM 16 with the DPPH IC_{50} values of 17.22, 58.98, 30.21, 17.72 and 49.13 µg/mL respectively. The methanolic extract of *M. minutum* also exhibited good antioxidant activities against radical scavenging, *β*-carotene bleaching and ORAC assays by

exhibiting values of 54.3 µg/mL, 55.19% and 5123 µmol TE/g respectively. The *M. minutum* fraction gave the DPPH IC_{50} of 168.9 µg/ml and ORAC value of 5.75%. Phytochemical investigation on *Melicope glabra* active fractions led to the isolation of ten compounds including one lignan sesamin (**36**), a number of coumarin derivatives (umbelliferone (**37**), scopoletin (**40**), a new pyranocoumarin, glabranin (**41**), scoporone (**42**), 6,7,8-trimethoxycoumarin (**43**) and marmesin (**44))** together with two new glycosides (3-β-D-galactopyranosyl)-O-(2-hydroxy-4-methylenedioxy) cinammate (**38**) and 22-hydroxyfurost-5-ene-(6→O)-α-methylalanyl-3-O-β-glucopyranoside (**39**)). Meanwhile, phytochemical study on *M.minutum* methanol bark extract succesfully yielded one lignan sesamin (**45**) which was previously isolated from the earlier plant, two new coumarins (hydramicromelinin (**46**) and micromelinin (**47**)) along with three glycosides (marmesin glycoside (**48**), maltose (**49**) and sucrose (**50**)). Five of the compounds were identified as new since there has been no previous reports on these compounds. The structure elucidation of the isolates were characterized by different spectroscopic techniques such as UV (ultraviolet), IR (infrared), MS (mass spectra), NMR (nuclear magnetic resonance) and comparison with published data. The isolated compounds, sesamin (**36**), umbelliferone (**37**), scopoletin (**40**), glabranin (**41**), 3-(β-D-galactopyranosyl)-O-(2-hydroxy-4-methylenedioxy) cinammate (**38**) and 22-hydroxyfurost-5-ene-(6→O)-α-methylalanyl-3-O-β-glucopyranoside (**39**) displayed DPPH IC_{50} values of 2508.63, 810.02, 413.19, 240.20, 323.78 and 124.13 µg/mL respectively. In the assesment of antioxidant activities by *β*-carotene bleaching assay on the isolated compounds, sesamin (**36**) displayed the most potent antioxidant with the antioxidant activity of 95.9%. The antioxidant activity observed for other compounds (glabranin (**41**), umbelliferone (**37**) and scopoletin (**40**)) were 74.9, -44.0 and -54.2 % respectively. Umbelliferone (**37**) and scopoletin (**40**) showed slightly prooxidant activities. Two isolated compounds from *M.minutum* namely hydramicromelinin (**46**) and marmesin glycoside (**48**) were also exhibited prooxidant behavior with the antioxidant activity of -116.35 and -34.18%, respectively. The measurement of scavenging activity by ORAC method revealed umbelliferone (**37**) as highly potential antioxidant agent with the ORAC value 24,965 µmol TE/g compared to ascorbic acid (5785 µmol TE/g). Hydramicromelinin (**46**) also showed strong antioxidant activity with the ORAC value of 5539 µmol TE/g. The ORAC values recorded for other compounds; glabranin (**41**), scopoletin (**40**), sesamin (**36**) and marmesin glycoside (**48**) were 2883, 2007, 2319 and 4031µmol TE/g respectively.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN KOMPONEN ANTIOXIDATIF BERPANDUKAN AKTIVITI BIO ASAI DARI DUA RUTACEAE SPESIS *MELICOPE GLABRA*(BLUME) T.G.HARTLEY DAN *MICROMELUM MINUTUM* (G. FORST.) WIGHT DAN ARN

Oleh

NUR KARTINEE KASSIM

Disember 2013

Pengerusi: Professor Mawardi Rahmani, PhD
Fakulti: Sains

Kajian ke atas kegunaan, ciri-ciri dan sumber antioksidan semulajadi terutamanya sebatian fenolik telah mendapat perhatian yang meluas memandangkan antioksidan sintetik dilaporkan memudaratkan kesihatan. *Melicope glabra* (Blume) T.G. Hartley dan *Micromelum minutum* (G. Forst.) Wight dan Arn. (Rutaceae) adalah tumbuhan yang boleh dimakan tergolong dalam keluarga Rutaceae Kedua-dua tumbuhan ini digunakan secara tradisional bagi merawat pelbagai penyakit dan diketahui mengandungi beberapa sebatian rutaceous seperti coumarins, lignan dan alkaloid. Setakat ini, laporan mengenai sebatian bioaktif bertanggungjawab terhadap khasiat perubatan adalah sangat terhad. Oleh itu, penyelidikan bertujuan mengenalpasti sebatian bioaktif terutamaya sebagai ejen antioksidan daripada tumbuhan yang belum diterokai ini adalah sangat berfaedah. Satu teknik pemencilan antioksidan berpandukan aktiviti 1,1-difenil-2-dipikrilhidrazil (DPPH) radikal telah digunakan untuk mencari dan mengenal pasti kehadiran komponen antioksidan dalam pelbagai ekstrak tumbuh-tumbuhan ini. Tiga *M. glabr*a ekstrak (heksana, etil asetat dan metanol) disaring untuk sifat antioksidan menggunakan empat ujian yang berbeza; DPPH memerangkap radikal bebas, pengoksidaan, β-karotena, oksigen kapasiti antioksidan radikal (ORAC) dan jumlah kandungan fenolik (TPC). Keputusan ujian antipengoksidaan menunjukkan ekstrak etil asetat dan metanol mempunyai potensi antipengoksidaan yang kuat dan telah terpilih untuk fraksinasi aktiviti berpandu. Nilai IC_{50} DPPH yang diperolehi oleh etil asetat dan ekstrak metanol adalah masing-masing 24.81 dan 13.01μg/mL dengan aktiviti antioxidan sebanyak 99.5 dan 93.0% ke atas perubahan warna β-karotena berbanding α-tokoferol (100%). Tumbuh-tumbuhan ini turut memberi nilai ORAC yang tinggi iaitu 1521 dan 2182 μmol TE/g. Pemisahan kromatograpi turus ke atas ekstrak-ekstrak aktif ini telah menghasilkan lima fraksi aktif iaitu ME 21, ME 24, ME 31, MM 13 dan 16 MM dengan nilai IC_{50} masing-masing sebanyak 17.22, 58.98, 30.21, 17.72 dan 49.13 μg/mL. Ekstrak metanol *M.minutum* juga

menunjukkan aktiviti antioksidan yang baik terhadap memerangkap radikal, β-karotena pelunturan dan asai ORAC radikal dengan mempamerkan nilai masing-masing iaitu 54.3 μg/mL, 55.19% dan 5123 μmol TE/g. Fraksi dari *M. minutum* memberikan nilai IC_{50} 168.9 μg/ml dan nilai ORAC sebanyak 5.75%. Penyelidikan fitokimia ke atas fraksi-fraksi aktif *M.glabra* membawa kepada pemencilan sepuluh sebatian termasuk satu lignan sesamin (**36**), beberapa terbitan koumarin (umbelliferon (**37**), skopoletin (**40**), satu piranokoumarin baharu, glabranin (**41**), skoparone (**42**), 6,7,8-trimetoksilkoumarin (**43**) dan marmesin (**44**) bersama-sama dengan dua glikosida baru 3-(β-D-galaktopiranosil)-O-(2-hidrosil-4-methilenedioksil) cinammate (**38**) dan 22-hidroksilfurost-5-ena-(6→O)-α-metilalanil-3-O-β-glukopiranosida (**39**). Sementara itu, kajian fitokimia ke atas *M.minutum* ekstrak metanol kulit berjaya menghasilkan satu lignan sesamin (**45**) yang sebelum ini telah dipencilkan daripada tumbuhan yang pertama, dua koumarin baharu (hidramikromelinin (**46**) dan mikromelinin(**47**)) bersama-sama dengan tiga glikosida (glikosida marmesin (**48**), maltosa (**49**) dan sukrosa (**50**)). Lima daripada sebatian ini telah dikenal pasti sebagai baharu kerana tidak ada laporan terdahulu mengenai sebatian ini. Struktur kesemua sebatian dikenalpasti berdasarkan teknik spektroskopi yang berbeza seperti UV (ultralembayung), IR (inframerah), MS (jisim spektrum), NMR (resonans magnetik nuklear) dan juga perbandingan dengan data yang diterbitkan. Beberapa sebatian terpencil, sesamin (**36**), umbelliferon (**37**), skopoletin (**40**), glabranin (**41**), 3-(β-D-galaktopiranosil)-O-(2-hiroksil-4-methilenedioksil) cinammate (**38**) dan 22-hidroksilfurost-5-ena-(6→O)-α-metilalanil-3-O-β-glukopiranosida (**39**) memaparkan nilai IC_{50} DPPH masing-masing iaitu 2508.63, 810.02, 413.19, 240.20, 323.78 dan 124.13 μg/mL mendedahkan sifat antioksidan mereka. Penilaian aktiviti antioksidan oleh cerakinan pelunturan β-karotena pada sebatian-sebatian terpencil, telah menunjukkan sesamin (**36**) sebagai agen antioksidan yang paling kuat dengan nilai aktiviti antioxidan sebanyak 95.9%. Aktiviti antioksidan yang diperhatikan bagi sebatian-sebatian lain (glabranin (**41**), umbelliferon (**37**) dan skopoletin **(40)**) masing-masing adalah 74.9, -44.0 dan -54.2%. Umbelliferon (**37**) dan skopoletin (**40**) menunjukkan sedikit aktiviti prooksidan. Dua sebatian terpencil daripada *M.minutum* iaitu hidramikromelinin (**46)** dan glikosida marmesin (**48**) telah mempamerkan aktiviti prooksidan dengan perencatan peratus masing-masing -116.35% dan -34.18%. Pengukuran aktiviti memerangkap dengan kaedah ORAC mendapati umbelliferon (**37**) sebagai agen antioksidan yang berpotensi tinggi dengan nilai ORAC 24.965 μmolTE/g berbanding asid askorbik (5785 μmolTE/g). Hidramikromelinin (**46**) juga menunjukkan aktiviti antioksidan yang kuat dengan nilai ORAC 5539 μmol TE/g. Nilai-nilai ORAC yang dicatatkan pada sebatian lain; glabranin (**41**), skopoletin **(40)**, sesamin **(36)** dan glikosida marmesin (**48)** masing-masing adalah 2883, 2007, 2319, 4031, 4948 dan 3802 μmol TE/g .

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Approval

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of . The members of the Supervisory Committee were as follows:

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α	alpha
β	beta
δ	chemical shift in ppm
λ_{max}	maximum wavelength in mm
ε	molar absorptivity
^{13}C	carbon -13
AAPH	2,2-Azobis(2-amidino-propane)
APT	Attached Proton Test
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol-d_4
CD_3COCD	deuterated acetone-d_6
COSY	Correlated Spectroscopy
DQF COSY	Double Quantum Filtered COSY
DEPT	Distortionless Enhancement by Polarization Transf
DPPH	1,1'-diphenyl-2-picrylhydrazyl
EtOAC	ethyl acetate
EIMS	Electron Impact Mass Spectrometry
GC-MS	Gas Chromatography-mass spectroscopy
^{1}H	proton
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear Multiple Quantum Coherence
HREIMS	High resolution electron ionization mass spectral

IC_{50}	Inhibition Concentration at 50 percent
t	triplet
s	singlet
m	multiplet
bd	broad doublet
bs	broad singlet
MeOH	methanol
m.p	melting point
MS	Mass Spectrum
m/z	mass per charge
NMR	Nuclear Magnetic Resonance
OD	Optical density
ORAC	Oxygen Radical Capacity
ROS	reaction oxygen species
SD	standard deviation
TLC	Thin Layer Chromatography
IR	Infrared
UV	Ultraviolet

CHAPTER I

INTRODUCTION

The used of plants as medicines in health care have been recognised for thousands of years (Samuelsson, 2004). Among the traditional medicinal systems are Ayurvedic, Unani and Chinese. These systems have contributed to some important drug discoveries and led to the isolation of active compounds. Drug discovery from medicinal plants such as the isolation of morphine from opium had already begun as early as 19th century (Kinghorn, 2001; Samuelsson, 2004). Some of the early drugs for instance cocaine, codeine, digitoxin, and quinine are still in use today (Newman *et al*., 2000; Butler, 2004; Samuelsson, 2004). The strategies for drug discovery research from natural products which include plants, animals or microorganisms have evolved quite significantly over the last few decades. The older strategies focus on the chemistry of the compounds from natural sources, but not on the activity. However, the present stratergies are more focused on the biological activities of the plants and on isolation of target compound(s) rather than trying to isolate all compounds presence in extracts. Thus, the application of appropriate chemical, biological or physical assays are necessary to be incorporated in the extraction and isolation protocol in order to pinpoint the target compound(s) from complex mixtures in natural product extracts. Collection may involve species with known biological activity (e.g., traditionally used herbal remedies) for which active compound(s) have not been isolated and identified.

In a natural products drug discovery program, bioassay plays an important role. A bioassay will be used to guide fractionation of a crude material towards isolation of the pure bioactive compounds. The ability of assay activity-guided fractionation and isolation techniques to give high throughput screening for biological activities of the plants helped the phytochemists to renew its interest in plants as potential sources of new drugs. For these purposes, bioassay tests must be simple, rapid, reliable, reproducible, sensitive, meaningful and, most importantly, predictive. To date, bioassays available are more robust, specific and sensitive to even as low as nanogram amounts of test samples. Most of the modern bioassays are using miroplate readers which require only small amounts of extracts, fractions or compounds. Among the typical assays used in natural product screening are 2,2-diphenyl-1-picryhydrazyl (DPPH) and antibacterial serial dilution assays. Previous studies on ten Chinese medicinal plants extracts with traditional reputations for CNS (Central Nervous System) activities were tested in a series of radio-ligand receptor binding assays, including adrenoceptor (α_1, α_2, β), 5-HT (1,1A, 1C, 2), opiate, benzodiazepine, ion channels (Ca^{++}, K^+), dopamine (1, 2), adenosine 1, muscarinic, Na^+/K^+ ATPase and GABA (A, B) receptors. Bioactivity-guided fractionation resulted in the isolation of individual active compounds including indole alkaloids, proanthocyanins, flavonoids and triterpenes (Phillipson, 1995; Phillipson,1999b).

The continual development of chromatographic and spectroscopic techiques had facilitated the separation, isolation and identification of the biological active compounds. The Phytochemical Society of Europe (PSE) symposium held at Lausanne, Switzerland in 1994 showed that these analytical techniques were becoming more and more sophisticated

(Hostettmann *et al*., 1995). The NMR techniques like COSY, DQF-COSY and TOCSY were available for establishing connectivities between neighbouring protons. HETCOR, HMQC, HSQC revealed the link between ^{1}H and ^{13}C. HMBC is used for long range heteronuclear correlations over 2–3 bonds. The interaction of ^{1}H-^{1}H through space can be evaluated through NOESY, ROESY and TOCSY(HOHAHA). The 1997 PSE symposium at Uppsala, Sweden also highlighted the application of TLC, HPLC hyphenated techniques (e.g. HPLC-PDA, LC-MS, LC-NMR, LC-MS-NMR) for the separation and structure determination of antifungal and antibacterial plant compounds (Bohlin and Bruhn, 1999).

Plants have many phytochemicals with various bioactivities such as antioxidant, anti-inflammatory and anticancer. The study of plants as source of natural antioxidant compounds with free radical scavenging activity have received great interest from many researchers in the last few years. Previous studies have reported that extracts from natural products, such as fruits, vegetables and medicinal herbs, have positive effects against cancer, compared with chemotheraphy or recent hormonal treatments (Wu *et al.*, 2002). Natural antioxidant derived from plant especially phenolics are considerably important as dietary supplement or food preservatives" (Halliwell *et al*, 1995). The natural antioxidant particularly the polyphenol compounds are reported to be found in plant foods (e.g grapes, berries,olives,soy), herbs (e.g oregano) and spices (e.g cinnamon,cumin,turmeric). The important and commomn antioxidants for example ascorbic acid (vitamin C), tocopherol (vitamin E) and tocotrienols and beta carotene (precursor of vitamin A) were derived from plant extracts. They play an important role in oxidative defence mechanisms in biological systems and acting as free radical scavenging agents. Many other plant based dietary polyphenolic constituents are found to be more effective antioxidants *in vitro* than α-tocopherols (vitamins E) or ascorbic acid (vitamin C), and thus might contribute significantly to protective effects *in vivo* (Rice-Evans *et al*., 1997; Jayasri *et al.*, 2009).

In our search for bioactive natural products as antioxidant agent, two genus from Rutacea family namely *Melicope glabra* and *Micromelum minutum* were chosen for investigation. They were among the richest sources of natural products and have been traditionally used in treating various of illnesses such as cough, fever, pain and infected wound. However, to date, not many reports on the bioactive compounds responsible for their medicinal properties. Presence of a number of rutaceous compounds such as coumarins, lignans and alkaloid in the stem and root bark extracts of the rutacaea family may be the answer. It is undisputable that medicinal plants with wide range of biological activities attributed to plant secondary metabolites are an indication that plants can serve as an excellent pool of bioactive compounds with useful therapeutic properties. Prior knowledge about the indigenous use of certain plants of known chemical composition and biological activities of the various plants constituents and an awareness of compounds that have previously been isolated from them, can be used as a directive in the selection process of potential sources (Cordell, 2000). The search to identify new botanical sources for natural antioxidants from these unexplored plants are considered important as minimum studies on the antioxidative properties of both plants have been reported. Natural antioxidants are believed to have minimum health risks to consumers. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) which are widely used to prevent oxidation in food products (Shahidi, 2000) were reported to give adverse effects including

enzymatic and lipid alterations in the *in vivo* test with rodents and monkeys (Branen, 1975). Therefore a part of discussing the characteristic of the isolated compounds, this study also highlighted the antioxidant capacity of the *Melicope glabara* and *Micromelum minutum* extracts as well as the isolated compounds. Phytochemical studies on various *Melicope* species had revealed the occurrence. of alkaloids, flavonoids (Komala *et al.*, 2006), acetophenones (Anderson *et al.*, 2007), coumarins, lignans (Latip *et al.*, 1999), dipeptides and terpenoids (Simonsen *et al.*, 2003). Some of these compounds have been demostrated antibacterial, antifungal, anti-inflammatory and cytotoxic activities (Barrows *et al.*, 2007; Hou *et al.*,1994; Simonsen *et al.*, 2004.)

In our attempt to isolate antioxidant compounds, bioassay guided method was incorporated into the isolation procedures. Only extracts showing significant biological activity in the bioassays, were subjected to the activity-guided fractionation and each fraction then tested for activities. Various chromatographic techniques were applied for the purification of the active fractions in order to isolate the agents which may be responsible for the bioactivities. The structural elucidation of the isolates were determined by various spectroscopic methods (UV, MS, IR and NMR) and were compared to the literature values. The antioxidant activity of the crudes as well as the isolates were evaluated by measuring the free radical scavenging activity by DPPH rapid dot blot staining and spectrophotometric assay, antioxidant activity by coupled oxidation of β-carotene and linoleic assay, β-carotene bleaching on TLC, oxygen radical absorbance capacity (ORAC) assay and total phenolic contents (TPC) of the active crudes were estimated as gallic acid equivalent using a Folin-Ciocalteau assay.

Objectives of Study

The objectives of this study are:

1. To extract and isolate bioactive compounds from *Melicopa glabra* and *Micromelum minutum* by assay guided isolation techniques.
2. To elucidate and identify the structures of the compounds by using modern spectroscopic methods.
3. To investigate the free radical scavenging and antioxidant capacity of the extracts and the isolated compounds.

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