UNIVERSITI PUTRA MALAYSIA

ANALYSIS OF FLAVONOIDS AND ESSENTIAL OILS FROM CLAUSENA EXCAVATA AND THEIR MEDICINAL PROPERTIES

LIM LAY SEAN

FBSB 2004 9

ANALYSIS OF FLAVONOIDS AND ESSENTIAL OILS FROM *CLAUSENA EXCAVATA* AND THEIR MEDICINAL PROPERTIES

LIM LAY SEAN

MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA

2004

This work is specially dedicated to

My beloved family, friends and teachers

who show me the fund of education

and as my sources of encouragement and support in the completion of this study

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

ANALYSIS OF FLAVONOIDS AND ESSENTIAL OILS FROM *CLAUSENA EXCAVATA* AND THEIR MEDICINAL PROPERTIES

By

LIM LAY SEAN

November 2004

Chairman: **Associate Professor Radzali B. Muse, Ph.D.**

Faculty: **Biotechnology and Biomolecular Sciences**

This study was conducted mainly to determine the major flavonoid compounds and the composition of essential oils predominant in *Clausena excavata*, and also to examine its antioxidant, antibacterial and anticancer activities. The dried yields of *C. excavata*'s leaf crude extracts were also determined and methanol was found to be the best solvent for extracting soluble bioactive compounds from the leaves of *C. excavata*. Total phenolic contents were found abundantly in methanol crude extract of leaves. In the high performance liquid chromatography (HPLC) analysis, rutin and naringin were found predominant in the leaves of *C. excavata*, while in the gas chromatography-mass spectrometry (GC-MS) analysis, the major chemical components identified in the fruit oil were α-ocimene and terpinolene. Terpinolene was also being the main component and remarkably present in the leaf oil. Both of the fruit and leaf oils extracted with dichloromethane in the simultaneous distillation extraction (SDE) method were indicated in very low yield (less than 0.1%). Antioxidant activities of various crude extracts of *Clausena excavata* leaves were dependent on the amount of total phenolics present in the crude extracts. Inhibition of lipid peroxidation and free radical scavenger potential of those crude extracts closely dependent on the particular substitution pattern of free hydroxyl groups on

the flavonoid skeleton. Antioxidant activity of methanol crude leaf's extract was found greater than α-tocopherol in the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods as well. The increase in the concentration of methanol crude leaf extract from 200 to 1000 µg/ml had increased its antioxidant activity. However, methanol crude leaf's extract exhibited weak scavenging activity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. As for the determination of antibacterial activity in both of the disc diffusion and microdilution methods, the growth of all tested gram-positive bacteria, especially *Micrococcus luteus* was found effectively affected by the methanol crude leaf extract. Almost all of the crude extracts were active towards *Micrococcus luteus*. Fruit and leaf oil exhibited their effects on all tested gram-positive bacteria and certain gram-negative bacteria but those tested bacteria strains were just weakly inhibited. Antioxidant activity of flavonoids was associated with anticancer properties. In the MTT assay, however, fruit oil showed better cytotoxic activity than that in methanol crude leaf extract against MCF-7 cells. This implied that *C. excavata* fruit oil might be a good source for the breast cancer treatment due to the presence of possible active anticancer agents.

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

ANALISIS KE ATAS FLAVANOID DAN MINYAK PATI DARIPADA *CLAUSENA EXCAVATA* DAN CIRI-CIRI PERUBATANNYA

Oleh

LIM LAY SEAN

November 2004

Pengerusi: Profesor Madya Radzali B. Muse, Ph.D.

Fakulti: Bioteknologi dan Sains Biomolekul

Kaji selidik ini bertujuan untuk menentukan sebatian flavonoid yang utama dan komposisi minyak pati dalam *Clausena excavata*, juga untuk memeriksa aktiviti anti-oksida, anti-bakteria dan anti-kanser masing-masing. Kadar kering hasil ekstrak mentah daun *C. excavata* juga ditentukan dan methanol telah dikenal pasti sebagai pelarut terbaik untuk mengekstrak sebatian bio-aktif yang larut air daripada daun *C. excavata*. Jumlah kandungan fenolik yang tinggi didapati dalam ekstrak mentah metanol. Dalam analisis HPLC, rutin dan naringin didapati hadir dalam kandungan yang tinggi dalam daun *C. excavata*, sementara dalam GC-MS analisis, komponen kimia utama yang dijumpai dalam minyak pati buah ialah α-ocimene dan terpinolene. Terpinolene juga muncul sebagai komponen utama dalam minyak pati daun dengan kandungan yang cukup menakjubkan. Penghasilan minyak pati daripada buah dan daun yang diekstrak dengan diklorometana adalah sangat sedikit (kurang daripada 0.1%). Aktiviti-aktiviti anti-oksida pelbagai ekstrak mentah daripada daun *C. excavata* adalah bergantung kepada jumlah sebatian fenolik yang hadir di dalam sesuatu esktrak itu. Potensi ekstrak-ekstrak mentah itu sebagai penghalang bagi pengoksidaan lipid dan penderma atom hydrogen kepada radikal bebas adalah sangat bergantung kepada corak penggantian yang khusus kumpulan hidroksil bebas dalam

rangka struktur flavonoid. Dalam kedua-dua ujian FTC dan TBA, aktiviti anti-oksida ekstrak mentah metanol daripada daun didapati lebih berkesan daripada α-tokoferol. Peningkatan kepekatan ekstrak mentah metanol daripada daun dari 200 kepada 1000 μg/ml telah pun meningkatkan aktiviti anti-oksida. Akan tetapi, ekstrak mentah metanol daripada daun kurang berkesan dalam tindak balas terhadap radikal bebas 1,1-diphenyl-2-picrylhydrazyl (DPPH). Dalam penentuan aktiviti anti-bakteria, kaedah penyebaran dengan penggunaan cakera kertas dan kaedah mikro-pencairan medium telah diaplikasikan. Ekstrak mentah metanol daripada daun menunjukkan keberkesanannya untuk merencat pertumbuhan kesemua gram-positif bakteria yang dikaji, terutamanya terhadap *Micrococcus luteus*. Hampir kesemua ekstrak mentah adalah aktif terhadap *Micrococcus luteus*. Minyak pati yang diekstrak daripada kedua-dua buah dan daun menunjukkan aktiviti perencatan ke atas semua gram-positif bakteria dan juga sesetengah gram-negatif bakteria, akan tetapi, kesan perencatan tersebut agak lemah. Aktiviti anti-oksida flavonoid berhubung-kait dengan anti-kanser. Dalam ujian MTT, walau bagaimanapun, minyak pati daripada buah memaparkan kesan sitotoksik yang lebih kuat terhadap sel MCF-7 jika dibandingkan dengan ekstrak mentah metanol daripada daun. Ini menunjukkan bahawa minyak pati daripada buah mempunyai potensi sebagai sumber yang baik untuk rawatan kanser payu dara dengan ada mungkinnya kehadiran ejen anti-kanser.

ACKNOWLEDGEMENTS

My sincere gratitude goes to my supervising committee chairman, Associate Professor Dr. Radzali Muse, from Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM), for his patient supports in guides and constructive advices in completing my work. Besides that, I would like to express my appreciation for the chances given by him for me to attend those valuable seminars and conferences during the study.

Great thanks to committee members Associate Professor Dr. Johari Ramli and Dr. Mohd. Yunus Shukor from Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, UPM, Associate Professor Dr. Mohd. Aspollah Sukari from Department of Chemistry, Faculty of Science, UPM. Their valuable suggestions and advices contributed to the completion of my study.

This multidisciplinary study could not be accomplished without the financial support from Intensification of Research in Priority Area (IRPA) programme, provided by the Ministry of Science Technology and Innovation (MOSTI). Not forget to thank Department of Biochemistry for providing the laboratory facilities and Department of Chemistry for providing the facility to carry out GC-MS analysis. At the same time, I would like to show my appreciation to Dr. Muhajir Hamid for providing the laboratory facilities to conduct cytotoxicity test. I am also being grateful for the helps given by the lab assistants and department stuffs.

Special thanks to my lab mates and friends, who provided supports, assistance and encouragement to me along my lab works and study. I would like to show my appreciation to Mr. Tung Sow Hoong for his technical support through out the study and leading me in the completion of this thesis. Last but not least, my deepest thanks to my family members, especially my parents, Lim Choon Huat and Tan Ah Jah, for their continuous supports and understanding.

I certify that an Examination Committee met on 3rd November 2004 to conduct the final examination of Lim Lay Sean on her Master of Science thesis entitled "Analysis of Flavonoids and Essential Oils from *Clausena excavata* and Their Medicinal Properties" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

MUHAJIR HAMID, Ph.D.
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

MOHD. ARIF SYED, Ph.D.
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

MOHD. PUAD ABDULLAH, Ph.D.
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

SHAIDA FARIZA SULAIMAN, Ph.D.
Associate Professor
School of Biological Sciences
Universiti Sains Malaysia
(Independent Examiner)

_____
ZAKARIAH ABD. RASHID, Ph.D.
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

RADZALI MUSE, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

JOHARI RAMLI, Ph.D.
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

MOHD. ASPOLLAH SUKARI, Ph.D.
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Member)

MOHD. YUNUS SHUKOR, Ph.D.
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, Ph.D.
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

DECLARATION

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

_____

LIM LAY SEAN

Date :

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Abs	- absorbance
AIDS	- acquired immunodeficiency syndrome
AR	- analytical reagent
BHA	- butylated hydroxyanisole
BHT	- butylated hydroxytolune
CO_2	- carbon dioxide
DMSO	- dimethylsulphoxide
DPPH	- 1,1-diphenyl-2-picrylhydrazyl
FBS	- foetal bovine serum
FID	- flame ionized detector
FTC	- ferric thiocyanate
g	- gram
GC	- gas chromatography
HCl	- hydrochloric acid
H_2O	- water
HPLC	- high performance liquid chromatography
IC_{50}	- 50 % inhibitory concentration
i.d.	- internal diameter
kg	- kilogram
LDL	- low density lipoprotein
M	- Molar
MeOH	- methanol
mg	- milligram
MIC	- minimum inhibitory concentration
min	- minute
MS	- mass spectrometry
MTT	- 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NCCLS	- National Committee for Clinical Laboratory Standards
nm	- nanometer
OD	- optical density
ppm	- parts per million
PUFAs	- polyunsaturated fatty acids
ROS	- reactive oxygen species
RP	- reversed phase
RPMI 1640	- Roswell Park Memorial Institute 1640
SDA	- simultaneous distillation adsorption
SDE	- simultaneous distillation extraction
TBA	- thiobarbituric acid
TBQH	- *tert*-butylhydroquinone
TFA	- trifluoroacetic acid
TLC	- thin-layer chromatography
UV	- ultraviolet
µg	- microgram
µl	- microliter
%	- percent
ºC	- degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Introduction

Two hundred years of modern chemistry and biology have described the role of primary metabolites in basic life functions such as cell division and growth, respiration, storage and reproduction. In biology, the concept of secondary metabolite can be attributed to Kossel (1891). He was the first to define these metabolites as opposed to primary metabolites. Thirty years later, an important step forward was made by Czapek (1921) who dedicated an entire volume of his 'plant biochemistry' series to what he named 'end products'. According to him, these products could well derive from nitrogen metabolism by what he called 'secondary modifications' such as deamination. Compared to the main molecules found in plants, these secondary metabolites were soon defined by their low abundance, often less than 1% of the total carbon, or a storage usually occurring in dedicated cells or organs.

In the middle of the 20th century, improvement of analytical techniques such as chromatography allowed the recovery of more and more of these molecules, and this was the basis for the establishment of the phytochemistry discipline. The improvement of biochemical and biotechnological techniques, and also the rise of molecular biology have been clearly demonstrated that secondary metabolites play a major role in human health and adaptation of plants to their environment.

Plant secondary compounds are usually classified according to their biosynthetic pathways. Three large molecule families are generally considered: (i) phenolics (e.g. flavonoids), (ii) terpenes (e. g. monoterpenes) and steroids, and (iii) alkaloids (Harborne, 1999).

Plant phenolics are an important group of secondary metabolites, which have diverse medicinal applications. Flavonoids are a broad class of low molecular weight secondary plant phenolics characterised by the flavan nucleus. Flavonoids are widely distributed in the leaves, seeds, barks and flowers of plants and over 4,000 flavonoids have been identified. Flavonoids have many functions in the biochemistry, physiology and ecology of plants, and they are important in both human and animal nutrition. In plants, flavonoids have functions in protecting against UV light (UV-B screening pigments), in warding off pathogenic microorganisms (phytoalexins) or pests (antifeedants), in the fertility and germination of pollen, in activating bacterial nodulation genes (nitrogen fixation) and in regulating plant growth and enzyme activity. Plant coloration is not only attractive for pollinators and seed distribution, but also provides aesthetically valuable characteristics for humans. The antioxidant activity of flavonoids towards free radicals and reactive oxygen species, and their potential oestrogenic and anticancer activity, such as antiproliferation, promotion of differentiation and apoptosis, draws attention to their health-protecting role in human diet and animal feed (Harborne and Williams, 2000).

Essential oils are odorous products obtained from natural raw materials such as leaves, fruits, flowers, roots and wood of many seasonal or perennial plants. They are generally of complex composition and contain alcohols, aldehydes, ketones,

phenols, esters, ethers, and terpenes in varying proportions. An estimated 3,000 essential oils are known of which approximately 300 are of commercial importance. On account of their aroma and highly volatile nature, essential oils have been traditionally used as basic raw materials in perfumes and flavouring. They are used in the preparation of beverages, medicines, and personal care and household products such as cosmetics, toiletries and cleaning preparations. They are also used in antiseptics, deodorants, disinfectants and in flavouring of foods and beverages. Essential oils such as monoterpenes are commonly accumulated in *Citrus* species of the Rutaceae family and other families such as Labiatae, Pinaceae and Umbelliferae. Monoterpenes have boiling point at 140-180 ºC. They can be separated using steam or hydrodistillation and identified using gas chromatography / mass spectrometry technique.

In the industrialised countries, people are seeking alternative herbal medicine because of the side effect from the strong modern drugs. According to World Health Organisation (WHO), 70 – 90 % of world population especially from developing countries use plant remedies for their health care. It has been indicated that Peninsular Malaysia and the neighbouring islands have more than 6,000 to 7,000 species of higher plants that have therapeutic or medicinal properties. They have been used for many generations in various systems of traditional medicines. Malaysia has over 4,000 Chinese herbal stores which import medicinal plants from Indonesia, China and India, and the locally available species are neglected or under-utilised.

Plants are potential sources of natural antioxidants such as e. g. flavonoids and other polyphenols. Natural antioxidants have importance for nutritional and therapeutic applications. Understanding the nutritional and therapeutic role of natural antioxidants is essential for the development of functional foods, which refers to the improvement of conventional foods with added health benefits. This is becoming very significant at a time when food is playing a major role in disease prevention in a global population that is projected to increase to 9 billion by the year 2050. Disease prevention and management through the diet are potentially the most effective tools to improve health and reduce the increasing health-care costs for the expanding global population (Shetty, 2003).

As dietary compounds, flavonoids are widely known as effective antioxidants that inhibit lipid peroxidation and offer protection against oxidative damage to membrane functions. Currently use of synthetic antioxidants has been suspected to cause or promote negative health effects. Both powerful synthetic antioxidants of butylated hydroxyanisole (BHA) and butylated hydroxytolune (BHT) have been widely used for many years to retard lipid oxidation. Nevertheless, these synthetic antioxidants are suspected to be carcinogenic and have been restricted use in foods (Madavi & Salunkhe, 1995). Hence, there is a need to substitute them with naturally occurring antioxidants.

Antioxidants are important not only for food protection but also as a defence mechanism of living cells against oxidative damage. It is well known that humans, as they grow older, become less active, have an increased probability of illness, and generally experience a loss of optimum function of all physiological systems. Lipid

peroxidation causes serious damage to the human body. Many researchers have shown that lipid peroxidation *in vivo* is the primary cause of many of the cardiovascular diseases such as atherosclerosis, and also in cancer and aging. The endogenous antioxidants distributed in and around living cells, which regulate the various oxidation-reduction reactions, are seen as a potential class of determinants of longevity (Cutler, 1984). In order to replenish the age-induced loss in the capability of endogenous antioxidant defence mechanisms, there is a need to identify new phytochemicals that could be made readily available by the regular intake of conventional foods.

Recent publications indicate that there is much evidence that plant antioxidants play an important role in biological systems in vitro as agents for antioxidative defence. Antioxidant compounds have already been found in numerous plant materials such as oilseeds, cereal crops, vegetables, fruits, leaves and leaf wax, barks and roots, spices, herbs and crude plant drugs (Ramarathnam *et al*, 1995). Like many green leaves, rosemary contains β-carotene, ascorbic acid, tocopherol, and selenium. Many other antioxidants could complement the conventional vitamins. Classically, rosemary is considered a good antioxidant herb. It contains close to two dozen named antioxidants. Antioxidants from rosemary can be competed with those powerful synthetic antioxidants such as BHA and BHT (Duke and Bogenschutz-Godwin, 1999).

Microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over spoilage and poisoning of foods by microorganisms is increasing due to the increase in outbreaks of food