UNIVERSITI PUTRA MALAYSIA

CHEMICAL CONSTITUENTS AND BIOLOGICAL ACTIVITIES OF FLAVONOIDS FROM HYDROPONICALLY GROWN PEGAGA (*CENTELLA ASIATICA*, LINN.,URBAN) EXTRACTS

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By

FEZAH BT. OTHMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of Requirements for the Degree of Master of Science

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FEZAH OTHMAN

February 2003

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

Faculty: Science and Environmental Studies

This study was carried out mainly to determine the major flavonoid compounds present in *Centella asiaticae* extracts and their allelopathic, anti-microbial and anti-inflammatory activities. The yield of extracts of *C. asiatica* was also determined and it was found that methanol was the most effective solvent to obtain crude extract from *C. asiatica*. Analysis on the nutrient composition of *C. asiatica* showed that the mean values of the nutrients analysed were about the same among the four groups (leaf, petiole including stolon, root and whole plant). The mineral profiles showed that *C. asiatica* leaf contains significantly ($p<0.05$) higher levels of P, Fe, Na and Mg compared to other parts; Ca and K contents were significantly ($p<0.05$) higher in the root part compared to other parts of *C. asiatica.* The level of vitamin C also was found significantly ($p<0.05$) higher in the leaf part compared to petiole including stolon, root and whole plant of *C. asiatica*.

Determination of total polyphenol content showed that fresh samples of *C. asiatica* exhibited significantly ($p<0.05$) higher content of total polyphenol compared to air-dried samples. Similarly, the content of salicylic acid was found significantly ($p<0.05$) higher in fresh samples (especially from the root part) compared to air-dried samples of *C. asiatica.* In the antioxidant studies using the ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods, it was found that the root extract of *C. asiatica* exhibited significantly ($p<0.05$) higher percentage of total antioxidant activity compared to other *C. asiatica* extracts but lower when compared to the reference antioxidants (α-tocopherol and butylated hydroxytoulene, BHT).

Analysis of flavonoid compounds using HPLC method revealed that catechin, luteolin, quercetin and kaempferol were present as the major flavonoid compounds in the leaf extracts of *C. asiatica*, while catechin, rutin, quercetin and naringin were present as the major flavonoid

extracts. Results from both seed germination and anti-microbial assays indicated that the extracts of *C. asiatica,* especially the methanol extract might contain some bioactive compounds that are able to affect the biological activity of the seeds and microbes used in the experiments. In the anti-inflammatory studies, both *C. asiatica* methanol and water extracts exhibited significant ($p<0.05$) levels of anti-inflammatory activities. Thus, with the presence of flavonoids and other compounds with possible cytotoxicity and antioxidative action in *C. asiatica*, coupled with favourable amounts of minerals and vitamins, it is worth promoting this plant (*C. asiatica*) as a potential anti-inflammatory agent in the future.

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

UNSUR-UNSUR KIMIA DAN AKTIVITI-AKTIVITI BIOLOGI FLAVONOID EKSTRAK PEGAGA (*CENTELLA ASIATICA*, LINN., URBAN) YANG DITANAM SECARA HIDROPONIK

Oleh

FEZAH OTHMAN

Febuari 2003

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

Fakulti: Sains dan Pengajian Alam Sekitar

Kajian ini telah dijalankan terutamanya untuk mengenalpasti sebatian flavonoid utama dan kesan ekstrak-ekstraknya ke atas aktiviti-aktiviti allelopatik, anti-mikrobial dan anti-inflamatori. Hasil-hasil ekstrak *C. asiatica* juga telah dikenalpasti dan didapati metanol merupakan pelarut yang paling berkesan untuk mendapatkan ekstrak kasar dari *C. asiatica*. Analisis terhadap komposisi nutrien *C. asiatica* menunjukkan nilai nutrien purata dalam *C. asiatica* adalah hampir sama di antara empat kumpulan (daun, petiol termasuk stolon, akar dan tumbuhan keseluruhan). Analisis profil mineral bahagian daun menunjukkan kandungan P, Fe, Na dan Mg signifikan ($p<0.05$) paling tinggi berbanding dengan bahagian lain; Ca dan K signifikan ($p<0.05$) paling tinggi di bahagian akar. Paras vitamin C juga telah didapati signifikan ($p<0.05$) paling tinggi pada bahagian daun berbanding dengan bahagian petiol termasuk stolon, akar serta keseluruhan pokok *C. asiatica*.

Penentuan polifenol total menunjukkan sampel segar ada mengandungi polifenol total yang signifikan ($p<0.05$) paling tinggi jika dibanding dengan sampel kering-udara. Kandungan asid salisilik juga didapati signifikan ($p<0.05$) paling tinggi pada sampel segar (terutama pada bahagian akar) berbanding dengan sampel *C. asiatica* yang kering-udara. Dalam kajilidikan antioksidan, menggunakan kaedah ferrik tiosianat (FTC) dan asid tiobarbiturik (TBA), didapati bahagian akar *C. asiatica* menunjukkan aktiviti antioksidan total yang signifikan tertinggi ($p<0.05$) berbanding dengan kawalan dan bahagian lain tetapi lebih rendah daripada kawalan rujukan (tokoferol α dan hidroksitoulena butilat, BHT). Analisis sebatian-sebatian flavonoid dengan menggunakan kaedah HPLC telah menunjukkan katekin, luteolin, kuersetin dan kaempferol adalah komponen flavonoid utama dalam ekstrak daun, sementara katekin, rutin, kuersetin dan naringin adalah komponen flavonoid utama dalam ekstrak akar. Keputusan yang didapati dari asai-asai percambahan biji benih dan anti-mikrobial telah menunjukkan ekstrak *C. asiatica*, terutamanya ekstrak metanol berkemungkinan mengandungi sebatian-sebatian bioaktif yang mampu mempengaruhi aktiviti-aktiviti biologi biji benih dan mikrob yang digunakan di dalam ujikaji-ujikaji.

Dalam kajilidikan aktiviti biologi anti-inflamatori, kedua-dua ekstrak metanol dan air *C. asiatica* menunjukkan paras aktiviti anti-inflamatori yang signifikan ($p<0.05$) berbanding dengan kawalan. Oleh itu, dengan kehadiran flavonoid dan lain-lain sebatian yang bersifat sitotoksik dan antioksidatif pada *C. asiatica*, tumbuhan ini (*C. asiatica*) berpotensi untuk dicadangkan sebagai agen anti-inflamatori pada masa akan datang.

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I certify that an Examination Committee met on 27th February 2003 to conduct the final examination of Fezah Bt. Othman on her Master of Science thesis entitled "Chemical Constituents and Biological Activities of Flavonoids from Hydroponically Grown *Centella asiatica*, L., Urban (Pegaga)" in accordance with Universiti Putra Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) regulation 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that thesis is based on my original work except for equations 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.

FEZAH OTHMAN
Date:: 24 MAR 2003

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5, 12 – HPETE – 5, 12 – hydroperoxyeicosatetraenoic acid

A – absorbance

AAS – atomic absorption spectrophotometer

AMP – adenosine monophosphate

AOA – activity of antioxidant

BAW – n-buthanol: acetic acid: water

BEM – benzene: ethyl acetate: methanol

BHA – butylated hydroxyanisole

BHT – butylated hydroxytoulene

^{13}C NMR – ^{13}C nuclear magnetic resonance

COX – cyclooxygenase

CV – coefficient of variation

Da – dalton

DCPIP – 2, 6 – dichloro-phenol-indophenol

DNA – deoxyribonucleic acid

FTC – ferric thiocyanate

HCl – hydrochloric acid

HPLC – high performance liquid chromatography

LTB_4 – leukotriene B_4

M – Molar

mg – milligram

min – minute

ml – milliliter

mm – millimeter

mM – milliMolar

MPO – myeloperoxidase

NA – nutrient agar

Na_2CO_3 – sodium carbonate

NaOH – sodium hydroxide

NB – nutrient broth

nm – nanometer

NSAIDs – non-steroidal anti-infl

OH – hydroxyl group

PDA – potato dextrose agar

PGE_2 – prostaglandin E_2

PGH_2 – prostaglandin H_2

PVP – polyvinyl pyrrolidone

RNA – ribonucleic acid

ROS – reactive oxygen species

RP-HPLC – reversed-phase high-performance liquid chromatography

t_R – retention time

SEM – standard error of the mean

SD – standard deviation

SOD – superoxide dismutase

TBA – thiobarbituric acid

TBHQ – tert-butylhydroquinone

TECA – titrated extract of *Centella asiatica*

TFA – trifluoroacetic

TLC – thin layer chromatography

TPA – 12- 0- tetradecanoyl-phorbol-acetate

UV – ultra violet

v/v – volume per volume

wt – weight

w/v – weight per volume

CHAPTER 1

INTRODUCTION

Background

Over the years, plants play important roles both as staple foods and sources of natural products for food supplements. The dependence of early human civilisation on herbs and medicinal plants for the purpose of healing the sick was well documented in the history of Egyptians, Chinese and Romans (Esnon, 2000).

Inflammatory-related diseases such as arthritis, asthma and rheumatic fever are always considered serious health problems in many countries. Although many drugs and therapeutic techniques have been developed, treatment of these diseases remains unsatisfactory (Samuelsson, 1993). With the increasing number of global elderly population and rapid changing lifestyle in many countries, the demand for drugs to treat inflammatory-related diseases especially arthritis and asthma is expected to increase.

Many drugs from aspirin to cortisone control inflammation. At present, aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and methotrexate are commonly used for treating arthritis and other inflammatory diseases. Unfortunately, many of these drugs are claimed to have less side effects but usually they also reduce anti-inflammatory activity (Frishman, 2002; Buttgereit *et al.*, 2001; Takeuchi *et al.*, 2001; Fosslien, 1998; Simon, 1997). Therefore, there is an urgent need in finding new anti- inflammatory agents that are more potent but with minimal side effects.

In Malaysia, the indigenous and naturalised plants are recognised by most rural communities as important sources of medicines. It is estimated that there are about 12,000 species of flowering plants and out of these about 1,300 species have been claimed to be medicinal (Teo *et al.*, 1990). One of these plants that naturally have some potential sources of fine phytochemicals and some interesting biological activities is *Centella asiatica. C. asiatica* is usually consumed fresh or as a fresh juice and the Malays sometimes used it as a tonic to treat mental fatigue, anxiety and post partum wounds. A range of biological activities has been found for *C. asiatica* extracts including wound healing activity, anti-inflammatory, anti-microbial and toxicity effects (Teik, 1997). Some of these effects may be attributed to the use of *C. asiatica* in folk medicine.

Today, products of *Centella* such as *Centella* juice, tea and tablets can be easily found at pharmacies and departmental stores. Many researchers and ethno-botanists from developed countries have exploited *C. asiatica* in order to investigate the biological activities of the fine chemicals presents in this plant. As a result, numerous data have been reported on the therapeutic effects of *Centella* especially the triterpenoid compounds from *Centella* (Kartnig, 1988). The triterpenes of *Centella* are extensively studied for their potential as improving factor in wound healing (Shukla *et al*, 1999a; Morriset *et al,* 1987), anti-ageing (Kartnig, 1988) and memory enhancing (Veerendra and Gupta, 2002).

Objectives of The Study

Even though recent studies conducted internationally have confirmed the efficacy of *Centella asiatica* triterpenoids, primarily in the treatment of skin injuries and diseases, data on the content of flavonoids and anti-inflammatory activities of *C. asiatica* are still lacking. In view of this reason, studies were carried out based on the following objectives:

1) To determine and to quantify the major flavonoid compounds present in *C. asiatica* using thin layer chromatography (TLC), spectrophotometry and reversed-phase high-performance liquid chromatography (RP-HPLC) methods. In addition, total antioxidants as well as total polyphenol and salicylic acid content in *C. asiatica* extracts were also determined.

2) To examine the biological activities of *C. asiatica* extracts using seed germination, anti-microbial and anti-inflammatory assays.

CHAPTER 2

LITERATURE REVIEW

Phytochemicals in Functional Medicine

Since the beginning of life, plants have played a major role in influencing human being and similarly, men have influenced the forms and characteristics of plants in helping them to adapt to man's progress. Historically, plants have been beneficial to the medical community and humans have looked to plants as their primary medicinal source (Carper, 1988).

According to Lin (1994), the term phytochemical refers to any compound found in plants and the word is commonly used to define the biologically active molecules in plants that are not classified as vitamins or nutrients by more traditional definitions. While phytochemicals have key functions in plants, they may also play a significant role in human health. Phytochemicals have recently gained much attention from the research community since studies have consistently shown several benefits from consuming these natural phytochemical compounds.

For nearly thirty years, synthetic drug research dominated the pharmaceutical industry until natural drug research began to make a come back around 1990 when it was realised that even with the advanced computerized technology that exists today, scientist cannot abandon nature as a source for drugs

(Pizzorno & Murray, 1987). This is because, plants contain plenty of powerful phytochemical defences, many of which are too complex to be accurately replicated in the laboratory. This chemical complexity is probably due to the building of defence mechanisms to avoid enemies in nature and thus made the plants as a rich source of "medicines" (Das, 1989).

Many important drugs today are plant-derived and the most widely used pharmaceutical in the world is aspirin, which was originally derived from the plant *Filipendula ulmaria* (Middleton & Drzewieki, 1985). Ephedrine, which is derived from the species *Ephedra sinica*, is presently used as a bronchodilator (White *et al.*, 1997). Several drugs come from the opium poppy (*Papaver somniferum*), including morphine, an analgesic and papaverine, an antispasmodic (Calixto *et al.*, 2000). The rosy periwinkle (*Catharanthus roseus*) has given the medical community two very important drugs: vinblastine, a treatment for Hodgkin's disease and vincristine, a treatment for paediatric leukaemia (Noble, 1990). Despite this, less than 1,325 of the world's 265,000 flowering plant species have been thoroughly tested for their medicinal potential (Soepadmo, 1999).

How Are Phytochemicals Produced in Plants?

Plants have developed extensive systems for managing reproduction, response to injury, protection from ultraviolet light, and resistance to disease. Attending to these important functions requires communication between plant cells and between the plant and its environment. Plant use phytochemicals both as a mean of "communication" and as a response to environmental stress.

"Communication" also occurs between phytochemicals and the human organism when we eat plant-based foods (Borris, 1996). The process by which the plant produces the chemicals needed for this communication, i.e., signalling molecules, is referred to as secondary metabolism (Cuppett, 1998). The secondary metabolites produced from this process influence diverse communication activities. This is much agreed by Alaluf *et al.*, (2002) who mentioned that secondary metabolites such as carotenoids, not only provide colour to signal pollinators such as birds and insects but they also protect DNA from damage in dry and/or light intensive environments.

There may be well over 10,000 different phytochemical secondary metabolites that have biological activity in humans. Based on chemical structure and synthesis pathways, these phytochemicals are catergorized into at least 14 general classes: carotenoids, coumarins, flavonoids, glucurates, indoles, isothiocyanates, lignans, monoterpenes, diterpenes and triterpenes, phenolic acids, phtalides, phytates, polyacetylenes, and sulfides. Of these, the flavonoids are the largest class, with over 4,000 different representatives (Harborne and William, 2000).

Flavonoids

The flavonoids are polyphenolic compounds possessing 15 carbon atoms; two benzene rings joined by a linear three-carbon chain. Figure 1 shows chemical structures of some flavonoid compounds.