

**SPECTROSCOPIC INVESTIGATION OF
BIOACTIVE CONSTITUENTS IN EXTRACTS OF
CITRI RETICULATAE AND *SPICA PRUNELLA***

by

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Dissertation submitted in partial fulfillment
of the requirement for the degree
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CERTIFICATE

This is to certify that the dissertation entitled
“Spectroscopic Investigation of Bioactive Constituents in
Extracts of *Citri reticulatae* and *Spica prunella*”
is the bonafide record of research work done by

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LIST OF SYMBOLS, ABBREVIATION OR NOMENCLATURE

% T	Percentage of Transmission
AAS	Atomic Absorption Spectrometer
CAM	Complementary and Alternative Medicine
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EC ₅₀	The concentration at which the scavenging/ chelating activity was 50%.
FT-IR	Fourier Transform Infrared
GC-MS	Gas Chromatography Mass Spectrometry
HPLC	High Performance Liquid Chromatography
KBr	Potassium bromide
LC	Liquid Chromatography
NCCAM	National Center for Complementary and Alternative Medicine
NHMRC/MoH	Australian National Health and Medical Research Council (NHMRC) and the New Zealand Ministry of Health (MoH)
NMR	Nuclear Magnetic Resonance
PCR/ CRP	Pericarpium <i>Citri reticulatae</i>
ppm	Parts per million
RDA	Recommended Dietary Allowance
RO	Reverse osmosis
TCHM/ CHM	Traditional Chinese Herbal Medicine
TCM/ CM	Traditional Chinese Medicine
UL	Tolerable Upper Intake Level
UV	Ultraviolet
w/ w	Weight per weight

ABSTRACT

Traditional Chinese Herbal Medicines (TCHMs) are getting more and more popular nowadays in the whole world for improving health condition of human beings as well as preventing and healing diseases. *Pericarpium Citri reticulatae* and *Spica Prunella vulgaris* were chosen for this study as they are widely used in Traditional Chinese Medicine. The objective of this project is to investigate the bioactive constituents of such herbal extracts using spectroscopic method. Prior to the instrumental analysis, preliminary phytochemical screening of both herbs was performed according to the standard protocols reported in literature. The UV-Visible spectral investigations of n-hexane and hot water extracts of *Citri reticulatae* indicated conjugation of C=O, -COOR, C=N, N=N, -COOH, -NO₂ or -CONH₂. Similar trends were observed in the case of methanol, hot water and n-hexane extract of *Spica prunella*. The UV-Visible spectral data was correlated to the FT-IR spectral bands recorded over the 4000- 400 cm⁻¹ range. The FT-IR results indicated presence of the functional groups that can be attributed for polyphenols, alkane groups, condensed system of aromatic rings and ester groups in *Citri reticulatae* and *Spica prunella*. The flame atomic absorption determinations carried out for copper, iron and zinc were $0.034 \pm 0.002 \text{ mg L}^{-1}$, $0.372 \pm 0.006 \text{ mg L}^{-1}$ and $0.081 \pm 0.002 \text{ mg L}^{-1}$ respectively in *Citri reticulatae* and $0.136 \pm 0.003 \text{ mg L}^{-1}$, $3.211 \pm 0.061 \text{ mg L}^{-1}$ and $0.400 \pm 0.006 \text{ mg L}^{-1}$ in *Spica prunella*. The levels of heavy metals such as cadmium were below the detection limits, except for lead which was present in *Spica prunella* at a concentration of $0.317 \pm 0.009 \text{ mg L}^{-1}$. The concentration of all the elements detected was within the safe limits set by WHO.

ABSTRAK

Pada masa sekarang, Herba Tradisional Cina (TCHMs) semakin menjadi popular di seluruh dunia untuk meningkatkan tahap kesihatan manusia, mencegah serta menyembuhkan penyakit. *Citri reticulatae* dan *Spica prunella* telah dipilih dalam kajian ini kerana kedua-duanya merupakan herba yang kerap digunakan dalam Perubatan Tradisional Cina. Objektif bagi projek ini adalah untuk menyiasat komponen bioaktif dalam ekstrak herba dengan menggunakan teknik spektroskopi. Sebelum analisis melalui teknik spektroskopi, kedua-dua herba ini dianalisis dengan menggunakan teknik penyaringan utama mengikuti protokol piawai dalam terjemahan. Analisis spektra UV-Visible dalam ekstrak n-hexane dan air panas *Citri reticulatae* menunjukkan kehadiran konjugasi C=O, -COOR, C=N, N=N, -COOH, -NO₂ or -CONH₂. Keputusan yang sama juga dapat dikesankan dalam ekstrak methanol, air panas dan n-hexane *Spica prunella*. Data dalam analisis spektra UV-Visible dibanding dengan spektra FT-IR yang direkod dalam julat 4000- 400 cm⁻¹. Keputusan FT-IR menunjukkan kehadiran kumpulan berfungsi yang menggambarkan poliphenol, kumpulan alkane, sistem kondensasi bagi gelang aromatik dan kumpulan ester dalam *Citri reticulatae* dan *Spica prunella*. Analisis serapan atom nyalaan telah dijalankan untuk kuprum, besi dan zink dengan kepekatan masing-masing ialah $0.034 \pm 0.002 \text{ mg L}^{-1}$, $0.372 \pm 0.006 \text{ mg L}^{-1}$ dan $0.081 \pm 0.002 \text{ mg L}^{-1}$ dalam *Citri reticulatae* serta $0.136 \pm 0.003 \text{ mg/L}$, $3.211 \pm 0.061 \text{ mg L}^{-1}$ and $0.400 \pm 0.006 \text{ mg L}^{-1}$ dalam *Spica prunella*. Kepekatan logam berat seperti kadmium didapati bawah had pengesanan kecuali plumbum dapat dikesan di dalam *Spica prunella* dengan kepekatan $0.317 \pm 0.009 \text{ mg L}^{-1}$. Kepekatan bagi semua sampel yang telah dianalisa menunjukkan berada di dalam julat keselamatan WHO.

CHAPTER 1

INTRODUCTION

1.1 Overview

With a history of 2000 to 3000 years, Traditional Chinese Medicine (TCM) has formed a unique system to diagnose and cure illness. The TCM approach is fundamentally different from that of Western Medicine. In TCM, the understanding of the human body is based on the holistic understanding of the universe as described in Daoism, and the treatment of illness is based primarily on the diagnosis and differentiation of syndromes (Ody, 2000). As illness is seen as a sign of disharmony within the whole person, so the task of the Traditional Chinese's practitioner is always to restore harmony and balance, thus enabling the body's natural healing mechanisms to work more efficiently.

According to National Center for Complementary and Alternative Medicine (NCCAM, 2009), the theoretical framework of TCM has a number of key components. Yin-yang theory is the concept of two opposing, yet complementary, forces that shape the world and all life. This theory is central to TCM. In the TCM view, a vital energy or life force called *qi* circulates in the body through a system of pathways called meridians. Health is an ongoing process of maintaining balance and harmony in the circulation of *qi*. The TCM approach uses 8 principles to analyze symptoms and categorize conditions: cold/heat, interior/exterior, excess/deficiency, and yin/yang (the chief principles). TCM also uses the theory of 5 elements—fire, earth, metal, water, and wood—to explain how the body works; these elements correspond to particular organs and tissues in the body. These concepts are

documented in the *Huang Di Nei Jing (Inner Canon of the Yellow Emperor)*, the classic Chinese medicine text (NCCAM, 2009).

Among the typical TCM therapies, acupuncture, herbal medicine, and qigong exercises are the mostly described (Yeung, 2007). With acupuncture, treatment is accomplished by stimulating certain areas of the external body. Herbal medicine acts on *zang-fu* organs internally, while qigong tries to restore the orderly information flow inside the network through the regulation of *qi*. Herbs are central to treatment, aided by other therapies such as acupuncture or specialist massager (Ody, 2000). These therapies appear very different in approach yet they all share the same underlying sets of assumptions and insights in the nature of the human body and its place in the universe. Some scientists describe the treatment of diseases through herbal medication, acupuncture, and qigong as an "information therapy".

Due to the expensive practice of the Western Medicine as the use of highly technical diagnoses and procedures, most of the patients search for an alternative. As the therapeutic effects are often complementary to those of Western drugs and minimum side effects of many herbal remedies have recently been demonstrated or verified in numerous modern scientific investigations, there is a recent burst of enthusiasm worldwide on the study of TCM. Besides that, the search for 'miracle cures' for serious disease such as cancer and AIDS, for example, has further fueled the growing interest in Herbal Chinese Medicines (Cai *et al.*, 2002). According to NCCAM (2009), which included questions on the use of various Complementary and Alternative Medicine (CAM) therapies, an estimated 3.1 million U.S. adults had used acupuncture in the previous year. In addition, according to this same survey, approximately 17.0% of adults use natural products, including herbs, making it the most commonly used therapy. In another survey, more than

1/ 3 of the patients at 6 large acupuncture clinics said they also received Chinese herbal treatments at the clinics.

Thus, integrating between Eastern Medicine and Western Medicine is important as Western Medicine is good for acute cases and for patients who need structural repairs, while Herbal Medicine is good for chronic cases as those who require balancing of physical, mental, and spiritual aspects in a long term, painless and cost-effective manner (Hou & Jin, 2005).

Two Chinese herbals are chosen in this project, namely *Citri reticulatae* (Plate 1.1) and *Spica prunella* (Plate 1.2), as they are two of the commonly used Chinese Herbal in the Chinese Society. According to Hou & Jin (2005), *Citri reticulatae* is classified under the herbs that invigorate circulation of vital energy, *qi* and blood. This herb is the dried tangerine peel of the ripe fruit of *Citrus reticulatae* Blanco. It is also known as *Chen-pi* in mandarin. This plant is mainly grown in the Fujian, Guangdong, and Sichuan provinces of China. The peel is collected from the ripe fruit, dried, cut into shreds, and used unprepared (Dong *et al.*, 1998). First taste of it is slightly sweet, then pungent and after taste is bitter, and warm, it acts on the spleen and lung meridians. The herb is considered by Chinese doctors as a panacea for all sorts of illness. It is a stomachic, stimulative, antispasmodic, antiphlogistic and dissipates phlegm. Also, it is used for marasmus in children, dyspnea in the elderly, for fish and crab poisoning, pinworms and mastitis due to stagnation of milk in the breast.

For *Spica prunella*, also known as *Xia ku cao* in mandarin is the entire plant, including dried spike, of *Prunella vulgaris* L. of the family Labiatae. It is grown mainly in the Anhui, Henan, Jiangsu, and Zhejiang provinces of China, where it is collected in the summer and dried in the sun. It is pungent and bitter in taste, and cold, it acts on the liver

and gallbladder meridians. According to Hou & Jin (2005), *Spica prunella* is classified under the group of herbal antipyretics, antimicrobials and detoxicants. Traditionally, this herb was used to treat fevers and also as an antirheumatic, alterative, and tonic remedy (Smith & Stuart, 1973). It is an antipyretic and detoxicant. Its actions resemble those of the chrysanthemum and wild chrysanthemum. However, it is more effective than those for treating eye disorder, such as conjunctivitis (Dong *et al.*, 1998). It is also used for hypertension, lymph node inflammation, lymphoid tuberculosis, mastitis, scrofula, and as an antimicrobial agent (Dong *et al.*, 1998).

1.2 Problem Statement

Traditional Chinese Herbal Medicines (TCHMs) are getting more and more popular nowadays in the whole world for improving health condition of human beings as well as preventing and healing diseases. However TCHM is a multi-component system with components mostly unknown, and only a few compounds are responsible for the pharmaceutical and/ or toxic effects (Liu *et al.*, 2008; Xie, 2006). No single active constituent is responsible for the overall efficacy. The large numbers of other components in the TCHM make the screening and analysis of the bioactive components extremely difficult. So, separation and analysis of the desired chemical components in TCHM are very important subjects for modernization of research on TCM (Liu *et al.*, 2008) and also a key to unlocking the secret of their effectiveness (Cai *et al.*, 2002). The major compound types in Chinese medicinal herbs include alkaloids, saponins, flavonoids, anthraquinones, terpenoids, coumarins, lignans, polysaccharides, polypeptides and proteins. Efficient detection and rapid characterization of these components on a molecular basis play an increasingly important role as analytical support in scientific studies aimed at a better

understanding of the pharmacological basis of CHM. The usual approach to such studies involve the preparation of herbal extracts, testing their pharmacological activity, isolating the individual components of the extracts by using liquid chromatography (LC), and then performing structure elucidation by nuclear magnetic resonance (NMR), mass spectrometry (MS) and other spectroscopic techniques.

In addition, with the large extent of Chinese Medicines being used in foreign countries, the safety assessment in the quality of crude herbs imported from the mainland of China is counted to ensure people's safety during medication and to promote the development of the international trade. Pollutant is a major issue in the world of modernization. Thus, the contamination in the herbal medicines is usually the exogenous harmful substance that can be introduced in the whole process, from the growth of the herbs and plants to the final procedure. The pollutants include residual pesticides, heavy metals, sulphur dioxide or other fumigants when processing or storage, mold pollution by its toxins, organic solvent residues, and the printing ink that can also pollute the medicines when packing. All of them, which have become the potential safety risk in the production of the TCMs, should be strictly controlled and managed (Xue *et al.*, 2008; Fong *et al.*, 2006).

In the present studies, we will employ UV-Visible spectroscopy, FT-IR spectroscopy and AAS to investigate the conjugation, aromatic character of phytochemicals by UV-Visible spectroscopy and confirm it through the functional group analysis by FT-IR spectroscopy. In addition, we will determine the concentration of trace elements present in the herbs studied by AAS.

CHAPTER 2

LITERATURE REVIEWS

2.1 Background Review on *Citri reticulatae* Blanco

According to WHO (1989), *Citri reticulatae* Blanco is an evergreen tree about 3 m high with short straight spines. Stem is usually erect, cylindrical, solid, woody, branched. Leaves are alternate, lanceolate or oval-lanceolate with approximately 5.5- 8 cm long by 2.5- 4 cm wide and attenuated at the 2 extremities, entire or barely crenulate, somewhat coriaceous, articulate. Its petiole flattened on top, winged obscurely. *Citri reticulatae* Blanco is used to flower in spring. The flowers may be small axillary cymes or solitary and axillary, fragrant. The structures of the white flowers are 5 sepals with 5 petals. Stamens are around 18- 24 with polyadelphous appearance while the ovary is located superior and multilocular in appearance. A nectariferous disc is present below the ovary. The fruit produced is a hesperidium, compressed-spherical in shape, orange or reddish in color with flattened at 2 ends. The rind of the fruit is officinal. Its odor is aromatic and its taste is pungent and bitter. Usually, its rind is used for medicinal purposes. It is commonly cultivated especially in China and Vietnam.

2.2 Medicinal Effects of Pericarpium *Citri reticulatae*

Citrus species are cultivated in China for at least 1700 years and Chinese have believed for centuries that food and drugs come from the same source. Those ingredients are commonly used in TCM, and many of them are regulated in China as foodstuff (Lu *et al.*, 2006). For instance, it has been used in the treatment of indigestion, cough and detoxification in China

for thousands of years. According to Yi *et al.* (2008), Pericarpium *Citri Reticulatae* is acknowledged in the People's Republic of China pharmacopoeia. Also, it is used as a condiment.

2.2.1 *In vitro* Antioxidant Activities

According to Su *et al.* (2008), total phenolic content, DPPH free radical-scavenging activity, hydrogen peroxide-scavenging activity, ferrous ion-chelating activity and ferric-reducing antioxidant power (FRAP) of 4 citrus herbal products, *Citri Reticulatae* Pericarpium (CRP), *Citri Reticulatae Viride* Pericarpium (CRVP), *Aurantii Immaturus Fructus* (AIF) and *Aurantii Fructus* (AF) have been compared in their respective methanolic extracts. For each extract, different concentrations ranging from 0.1- 1.0 mg mL⁻¹ were prepared. With regard to EC₅₀ values, CRP showed the second lowest value (EC₅₀ = 0.78 ± 0.04) in DPPH assay and lowest value (EC₅₀ = 0.90 ± 0.02) in hydrogen peroxide-scavenging ability though it contained second highest amount of total phenolic content among the 4 extracts. This indicates that the components with high hydrogen peroxide-scavenging ability were not presented in this extract. In the metal ion-chelating assay, CRP also showed the second lowest value (EC₅₀ = 1.89 ± 0.10). However, in the ferric-reducing antioxidant power (FRAP) assay, CRP showed second highest value (EC₅₀ = 0.25 ± 0.02). Study showed that differences in DPPH free radical-scavenging activity, hydrogen peroxide-scavenging activity, and ferrous ion-chelating activity of all citrus herbal product extracts were significant. AF had the highest antioxidant activity. However, in this study, citrus herbal product extracts did not have good reducing power.

This antioxidant capacity was further studied by Yi *et al.* (2008), a flavonoid extract of Pericarpium *Citri Reticulatae* (FEPCR), the dried rind of the ripe citrus, was obtained

with 80.0% aqueous ethanol. Total flavonoid content of FEPCR was determined by a colorimetric method. Total phenol content was estimated as gallic acid equivalents. The major constituents of FEPCR, including hesperidin, nobiletin and tangeretin, were determined by HPLC analysis. The antioxidant activities of FEPCR, hesperidin, nobiletin and tangeretin were evaluated by various antioxidant assays, including DPPH scavenging, hydroxyl radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and reducing power. All samples showed antioxidant activities to some degree in all the tested methods.

2.2.2 Antimicrobial Activities

The antimicrobial assay was measured Yi *et al.* (2008) as well. They reported that the flavonoid extract of *Pericarpium Citri Reticulatae* (FEPCR) was obtained with 80.0% aqueous ethanol and its major constituents were determined by HPLC analysis. 6 strains of microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Salmonella typhi* and *Enterobacter cloacae* were used in the tests. FEPCR and hesperidin displayed a broad antimicrobial spectrum and exerted antimicrobial effects in antimicrobial tests. But tangeretin and nobiletin exhibited low antimicrobial activities.

2.2.3 Anticancer Properties

Tangeretin, a flavonoid present in citrus fruits, affects certain activities of mammary cancer cells in a manner similar to that of tamoxifen, at least *in vitro*. Like tamoxifen, this flavonoid inhibits growth, activates cell-cell adhesion, and blocks invasion by human MCF-7/6 mammary adenocarcinoma cells. The effects on cell-cell adhesion and invasion of

both tamoxifen and tangeretin are mediated by the E-cadherin/ catenin complex, which serves as an invasion suppressor at the plasma membrane of epithelioid cells. Bracke *et al.* (1999) reported that tamoxifen and the citrus flavonoid tangeretin exhibit similar inhibitory effects on the growth and invasive properties of human mammary cancer cells *in vitro*; furthermore, the 2 agents have displayed additive effects *in vitro*. Tangeretin is 1×10^{-6} M or higher compared to tamoxifen in the activity of inhibiting the cytolytic effect of murine natural killer cells on MCF-7/ 6 cells *in vitro*.

2.2.4 Antiinflammatory Activities

Chen *et al.* (2002) claimed that Pericarpium *Citri reticulatae* is the main material used for extraction of hesperidin for the medicinal industry in China. A related study has been done by Choi *et al.* (2007) on citrus unshiu peel as it is commonly used in the author society. The citrus unshiu peel has been used traditionally as a medicine to improve bronchial and asthmatic conditions or cardiac and blood circulation in Korea, China, and Japan. The effects of citrus unshiu peel water extract (CPWE) on the phorbol myristate acetate (PMA) + calcium ionophore A23187-induced hypoxia-inducible factor-1 α (HIF-1 α) activation and inflammatory cytokine production from the human mast cell line, HMC-1 cells is tested. Human mast cells produce cytokines in respond to PMA and the calcium ionophore A23187. The purpose of Choi's study is to show that mast cells synergistically response to PMA + A23187 for producing cytokines. PMA + A23187 treated-HMC-1 cells are useful *in vitro* model system for studying multifunctional effects of immune and inflammation reactions. CPWE is compared with hesperidin, a common constituent of citrus unshiu. CPWE and hesperidin inhibited the PMA + A23187-induced HIF-1 α expression and the subsequent production of vascular endothelial growth factor (VEGF). In addition, CPWE

suppressed PMA + A23187-induced phosphorylation of the extracellular signal-regulated kinase (ERK). Increased cytokines interleukin (IL)-1b, IL-8, and tumor necrosis factor (TNF)-a level was significantly inhibited by treatment of CPWE or hesperidin. Thus, CPWE and hesperidin are inhibitors of HIF-1a and cytokines on the mast cell-mediated inflammatory responses.

2.2.5 Anticollagen-induced Arthritic Properties

According to Imada *et al.* (2008), aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 have been shown to play crucial roles in cartilage destruction in arthritic diseases, including rheumatoid arthritis and osteoarthritis. In this study, the effects of nobiletin, a citrus polymethoxy flavone, on the expression and production of ADAMTS-4 and -5 *in vitro* and *in vivo* have been examined. Nobiletin interfered with the interleukin (IL)-1b-mediated ADAMTS-4 and -5 mRNA expression in cultured human synovial fibroblasts. Furthermore, intraperitoneal administration of nobiletin also suppressed ADAMTS-4 and -5 mRNA expressions in the joint tissues of collagen-induced arthritic (CIA) mice. Immunohistochemical analysis using an antibody against aggrecan neoepitope (NVTEGE373) revealed that aggrecanase-mediated degradation of aggrecan in cartilage was effectively inhibited by nobiletin. These results provided novel evidence that nobiletin effectively interferes with gene expression of ADAMTS-4 and -5, and thereby prevents cartilage destruction in CIA mice.

2.2.6 Adipogenesis Suppression Properties

Sheu *et al.* (2007) examined how *Citrus* herbal medicines – Pericarpium *Citri Reticulatae* (PCR), Pericarpium *Citri Reticulatae Viride* (PCRV), *Aurantii Immaturus Fructus* (AIF) and *Aurantii Fructus* (AF) – affect the differentiation of 3T3-L1 adipocytes. Methanolic extracts of each sample is tested on 3T3-L1 adipocytes with various concentrations throughout the cell differentiation period of ten days. 8 days after induction for differentiation with 3-isobutyl-1-methylxanthine, dexamethasone and insulin (MDI) medium and simultaneously with the tested PCR, intracellular triacylglycerol accumulations of 3T3-L1 cells were significantly reduced compared with those for PCRV, AIF and AF and those of the vehicle control. This suppression affect was dose-dependent, and decreases in triacylglycerol production were observed for PCR at various concentrations. Additionally, the expression of key transcription factors for the 3T3-L1 adipogenesis gene, including PPAR- γ , C/EBP- α and SREBP-1, was markedly reduced by PCR treatment. These results suggest that dietary PCR suppresses 3T3-L1 differentiation by down-regulation of adipogenic transcription factors. Experimental data may prove useful in further medical examination of the use of PCR for body weight control.

2.2.7 Prevention of UVB-induced Photoinflammation and Photoaging Effects

Exposure to ultraviolet B (UVB) irradiation induces acute skin inflammation such as erythema (sunburn) and edema, while prostaglandin (PGE₂) in the epidermis plays an important role as its prominent mediator. In the study of Tanaka *et al.* (2004), the effect of nobiletin (5,6,7,8,30,40-hexamethoxy flavone) from *Citrus depressa*, on the production of PGE₂ in UVB-irradiated human keratinocytes is investigated. When keratinocytes were irradiated with 60 mJ of UVB cm⁻², the production and gene expression of cyclooxygenase