EXTRACTION OF PHYTOCHEMICALS FROM PERICARPIUM *CITRI RETICULATAE* AND *SPICA PRUNELLA*

by

LOH JIA LIANG

Dissertation submitted in partial fulfillment
of the requirements for the degree
of Bachelor of Health Sciences (Biomedicine)

October 2009

CERTIFICATE

This is to certify that the dissertation entitled

"Extraction of phytochemicals from

Pericarpium *Citri reticulatae* and *Spica prunellae*"

is a bonafide record of research work done by

Mr. Loh Jia Liang

during the period from July 2008 to October 2008 under my supervision.

ACKNOWLEDGEMENTS

First and foremost, I would like to take this opportunity to express my greatest appreciation to my supervisor, Associate Professor Syed Waliullah Shah for his time, invaluable advice, guidance and encouragement in this project. His constructive criticism and reassurance made this project a truly educational experience.

I would also like to extend my sincere thanks and appreciation to all the staff in the Unit Kemudahan Makmal (UKM), especially Madam Rosilawani Ismail, for extending support in terms of chemicals, glassware, equipment, and space required throughout the progress of this project.

My gratitude also goes out to my project partners, Chow Tan Wei and Gan Chin Chear. Their help and companionship in this project has brightened what would have otherwise been a dull and tedious journey.

Special thanks to all my friends, for their ability to put a smile on my face even through the darkest of times.

Last but definitely not least, I would like to express my deepest gratitude to my parents, Loh Chiew Hoong and Khaw Chai Hoon, for their unconditional and consistent love and kindness. I believe, without their love, shelter, care, guidance, and support, I am nothing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
ICR	Institute of Cancer Research
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
TLC	Thin Layer Chromatography
UV-Vis	Ultraviolet Visible Spectroscopy

ABSTRACT

Pericarpium *Citri reticulatae* and *Spica prunella* are two herbs commonly used in Traditional Chinese Medicine concoctions. In this study, a Soxhlet method for the extraction of phytochemicals from Pericarpium *Citri reticulatae* and *Spica prunella* using methanol, n-hexane and distilled water is described. The n-hexane extract yield was very high as compared to the other solvents. Preliminary screening of different classes of phytochemicals in the extracts gave adequate information that was supported by using other sophisticated analytical techniques. It has been observed that the extraction at larger scale be preferred to get sufficient yield and consequently a large number of components into the extracts. The distilled water extract of Pericarpium *Citri reticulatae* contains saponins and possibly flavonoids; the distilled water extract of *Spica prunella* contains saponins; and the methanol extract of *Spica prunella* contains free acids and possibly flavonoids and tannins.

ABSTRAK

Kulit *Citri reticulatae* dan *Spica prunella* merupakan dua jenis herba yang biasa digunakan dalam penyediaan perubatan tradisional Cina. Kajian ini berkisar tentang pengekstrakan bahan fitokimia dari kulit *Citri reticulatae* dan *Spica prunella* berdasarkan suatu kaedah Soxhlet dengan menggunakan metanol, n-heksana, dan air suling. Didapati bahawa hasil ekstrak n-heksana adalah lebih tinggi dibanding dengan pelarut lain. Penyaringan awal kelas fitokimia berbeza yang terdapat dalam ekstrak telah memberikan maklumat cukup yang boleh disahkan oleh teknik analitikal yang lain. Proses pengekstrakan skala besar boleh mengeluarkan hasil yang lebih tinggi dan mempunyai komponen fitokimia yang lebih banyak. Ekstrak air suling kulit *Citri reticulatae* mengandungi saponin dan mungkin juga flavonoid; ekstrak air suling *Spica prunella* mengandungi saponin; dan.ekstrak methanol *Spica prunella* mengandungi asid bebas dan mungkin flavonoid dan tanin juga.

Chapter 1

INTRODUCTION

1.1 Herbal Medicines

A drug is a chemically identified substance, either derived from plants or animal, or produced by synthesis. Plants have been selected and used empirically as drugs for centuries, initially as traditional preparations and then as pure active principles (Kamil, 1993), with this knowledge and accumulated practice passing from generation to generation (Couzinier & Mamatas, 1986).

In recent years, there has been significant interest in alternative healing globally, with particular interest in herbal medicine. Medicinal plants and plant-derived medicines are an integral part of traditional cultures all over the world. A large proportion of the population of developing countries uses traditional medicine alone, or in combination with Western drugs to treat a wide variety of illnesses (Taylor *et al.*, 2000). In most healing cultures, herbal remedies are usually favoured for chronic or self-terminating conditions, while acute or serious illnesses are treated by Western medicine (Wyk & Wink, 2004). The popularity of traditional medicines in developing countries is usually as a result of the high cost of Western pharmaceuticals and healthcare, or because the traditional medicines are more acceptable from a cultural and spiritual perspective. Natural products and their derivatives (including antibiotics) represent more than 50% of all drugs in clinical use in the world.

Effective collaborations between traditional and Western medical practitioners are rare, mainly due to the perception that the use of traditional and herbal medicines has no

scientific basis (Taylor *et al.*, 2000). There is a lack of standardization of herbal medicines in respect to raw materials, methods of production, and in quality control of the finished product. However, with the renewed interest of Western countries in herbal medicines and an increasingly urgent need for the development of new effective drugs which are non-toxic and inexpensive, the investigation of traditionally used medicinal plants is once again receiving scientific attention. Most of the time this involves the isolation and identification of the secondary metabolites produced by the plants and used as the active principles in medical preparations. One of the main factors that make investigations on the plants used in traditional medicine a tricky process is the possibility of synergistic effects resulting from the interaction of the natural compounds, which can result in a loss of activity as the product is purified, and the compounds acting synergistically are lost (Couzinier & Mamatas, 1986).

1.2 Traditional Chinese Medicine

Traditional Chinese medicine (TCM) is an ancient system of medicine, believed to be more than 5000 years old. It is based on two separate theories about the natural laws that govern good health and longevity, namely *ying* and *yang*, and the five elements (*wu xing*). Amongst the earliest records of ancient Chinese herbalism is a text by the Chinese Emperor and Scholar Shen Nong of the Sung Dynasty entitled *Shen Nong Ben Cao Jing* or *The Great Native Herbal* (ca. 2800 BC). This was later translated by Tao Hung Jing and became well known as *Comment on the Divine Husbandman's Classic of the Materia Medica.*

Ying and *yang* denotes opposites that complement each other, such as cold and hot. The five elements in TCM are earth, metal, water, wood, and fire, each of which are linked to the main organ systems of the body (spleen, lungs, kidneys, liver, and heart respectively), the climates (damp, dry, cold, windy, hot), and so on. Medicine is used to restore or maintain balance between these elements and to grant vital energy (*qi*), which has both *ying* and *yang* aspects. Treatment is therefore based not only on symptoms but also on patterns of imbalances, often detected by taking the pulse or observing the patient's tongue.

Chinese herbs are usually given in fixed mixtures or formulas of up to 20 herbs, carefully prepared according to traditional recipes contained in ancient compendia. There are hundreds of these formulas that are commonly used in hospitals and pharmacies alongside Western medicine (Wyk & Wink, 2004).

1.3 Phytochemicals

Phytochemicals are natural non-nutritive, bioactive compounds found in plants. They are a special category of drugs derived from plant sources and are standardized by quantification and elucidation of certain compounds in the plant materials, producing the replicable final product. Phytomedicines include crude vegetable drugs (herbs) and the galenical preparations (extracts, fluid extracts, tinctures etc.) derived from them (Tyler, 1993).

Phytomedicines are commonly used to stimulate the immune system in an attempt to prevent disease, as well as to induce specific cures. They often contain a mixture of substances that have additive or even synergistic effects, so that the health benefits are difficult to test and verify (Wyk & Wink, 2004). One of the criticisms traditionally leveled against natural medicines is the lack of standard levels of biological materials from the

natural plants (Israelsen, 1993). The use of phytomedicines is becoming more scientifically based, with increasing emphasis placed on proven product safety and efficacy (Taylor *et al.*, 2000).The use of plant-based medications has become extremely popular in the United States and Europe, with the botanical industry in the US earning $1.5 billion per annum and the European market nearly three times as much (Ernst, 1998).

1.4 Pericarpium *Citri reticulatae*

The *Citrus reticulatae* Blanco plant is an evergreen tree about three meters high with short straight spines. Its stem is erect, cylindrical, solid, woody, and branched. The tree produces flowers that are white and fragrant in spring. The fruit is hesperidium, compressed-spherical in shape, orange or reddish in colour, and flattened at two ends. The rind of the fruit is officinal; its odour is aromatic and its taste is pungent and bitter (WHO, 1997).

Pericarpium *Citri reticulatae* is the dried peel of the ripe fruit of *Citrus reticulate* Blanco and many other species of the citrus genus of the family Rutaceae (Hou & Jin, 2005). Citrus plants are rich in naturally occurring flavonoids, which are primarily found in the peel (Sheu *et al.*, 2007).

1.5 *Spica prunella*

Prunellae vulgaris Labiatae is a low sprawling perennial herb, 45 cm high, and faintly pubescent. Its flowers are 1.3- 2.0 cm long and are violet-purple in colour, in whorls of 6 crowded inerect, terminal spikes 2.5- 5.0 cm long and bearing a pair of sessile leaves at the base. The floral leaves are bract-like, hairy, purple-marginated, broadly ovate, acute, and overlapping (WHO, 1997).

Spica prunella is the entire plant, including the dried spike of *Prunella vulgaris* of the family Labiatae (Hou & Jin, 2005). It is more commonly known as the selfheal, heal-all, or sicklewort in Western countries. In Chinese medicine, the flower spikes are used, and are known as *Xia Ku Cao*, literally meaning "Summer Dry Herb" (Wyk & Wink, 2004).

1.6 Cultivation and Utilization of Pericarpium *Citri reticulatae*

Citrus species originated from southern and southeastern Asia and have a long and complicated history in cultivation (Wyk & Wink, 2004). It is believed to have originated from the region within Northeast India, South China, Indonesia, and Peninsular Malaysia (Shokrollah *et al.*, 2009). The peel is collected from the ripe fruit, dried, cut into shreds, and used unprepared (Hou & Jin, 2005). Several varieties of *Citrus medica*, the Citron, are used in medicine: the rind and juice are anti-scorbutic (Wheelwright, 1974). Its essential oils are also fragrant and used commercially.

Citri reticulatae is mainly grown in Fujian, Guangdong, and Sichuan provinces of China (Hou & Jin, 2005). In Malaysia, citrus is grown in commercial orchards, backyard orchards and small holdings in various parts of the country. Citrus collections have been

established for conservation purposes, which have notable genetic diversity, particularly of the pummelo and some of the related genera and appear to be fairly well-maintained (Shokrollah *et al.*, 2009). Some are also observed in areas such as the Taman Negara National Park in Pahang and the Danum Valley in Sabah.

Pericarpium *Citri reticulatae*, the rind of citrus has significant value in the preparation of traditional Chinese herbal medicines and foods in China. It has been used in the treatment of indigestion, cough, and detoxification in China for thousands of years (Yi *et al.*, 2007). Mature and immature peels of *Citrus* plants are used in Citrus herbal medicine preparations and are traditionally used to promote the flow of liver 'qi' and alleviate cardiovascular or hernia-like pain and pain in the chest, breast, and hypochondriac region. Apart from resolving and reducing 'qi' accumulations such as food stagnation with pain and distention symptoms, they are also used to dry dampness and transform phlegm, as described in traditional Chinese medical literature (Sheu *et al.*, 2007). Also, it is commonly used in pill and as a decoction with ginger and other carminatives (Hou & Jin, 2005). Now, Pericarpium *Citri reticulatae* is acknowledged in the People's Republic of China pharmacopoeia.

1.7 Cultivation and Utilization of *Spica prunella*

Xia Ku Cao, the spica of *Prunella vulgaris*, is found around the world in China, Europe, Britain and North America. It is grown mainly in the Anhui, Henan, Jiangsu, and Zhejiang provinces of China though they are mostly wild-harvested (Hou & Jin, 2005; Wyk & Wink, 2004). The *prunellae* spikes are gathered in the summer and dried in the sun before use (Youping, 2003).

Prunella vulgaris is a traditional wound-healing plant in Europe and is still used in China as a spasmolytic and sedative for liver and gall problems (Wyk & Wink, 2004). It is used to treat fevers and also as an antirheumatic, alterative, and tonic remedy (Smith & Stuart, 1973). In traditional indications and combinations, *Spica prunella* is used with a combination of other herbs to treat the flaring up of liver fire and the accumulation of phlegm-fire (Geng *et al.*, 1997). It is used together with other plants by Iroquois for steam baths to treat sore legs or stiff knees (Lewis & Elvin-Lewis, 2003). Herbalists have long utilized it to treat sores and lumps in the mouth and especially the throat as well as for treating blood pressure and painful eyes (Youping, 2003; Wheewright, 1974). Prunella spike is also used for hypertension, inflammation for the lymph nodes, lymphoid tuberculosis, mastitis, scrofula, and as an antimicrobial agent (Dong *et al.*, 1998). However, this herb is used with caution in cases where the patient has a weak stomach and spleen (Youping, 2003).

In the present work, Soxhlet extraction of *Spica prunella* and Pericarpium *Citri reticulatae* will be carried out using methanol, n-hexane and hot-water. The phytochemicals extracted into these solvents will be screened by using different tests reported in literature. The elucidation and further characterization will be carried out by other group researchers.

Figure 1.1. *Percarpium Citri reticulatae* (Tangerine peel)

Figure 1.2. *Spica prunella* (Selfheal, Sicklewort, Xia Ku Cao)

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Effects of Pericarpium *Citri reticulatae*

Pericarprium Citri reticulatae is considered by Chinese doctors as a panacea for all sorts of ills; it is a stomachic, stimulative, antispasmodic, antiphlogistic, and dissipates phlegm (Hou & Jin, 2005). It is mainly used to enhance appetite by increasing the secretion of gastric juices, treat dyspeptic complaints and minor sleeplessness (Wyk & Wink, 2004). It is also 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. Tangerine peel is a carminative, *Qi*-regulating stomachic, and mild expectorant for many gastrointestinal and respiratory tract disorders (Hou & Jin, 2005).

The therapeutic dose is safe. No undesirable side effects or toxicity have been reported at the therapeutic dose in classical Chinese material medica. In toxicological tests, no acute toxicity was observed in animals when the decoction was given orally or intravenously (Zhu, 1998).

2.1.1 Antioxidant Activity

The antioxidant activities of flavonoid extract of Pericarpium *Citri reticulatae* (FEPCR), Hesperidine, Nobiletin and Tangeretin were evaluated by various antioxidant assays, including 1,1-Diphenyl-2-picrylhydrazyl (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 (Yi *et*

al., 2008). Methanol extract of Pericarpium *Citri reticulatae* also showed significant antioxidant activity (Su *et al.*, 2008).

2.1.2 Antimicrobial Activity

Antimicrobial assays for flavonoid extract of Pericarpium *Citri reticulatae* (FEPCR) was determined using the agar dilution method with some modifications on six strains of microorganisms including *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Salmonella typhi* and *Enterobacter cloacae*. FEPCR and Hesperidin displayed a broad antimicrobial spectrum and exerted antimicrobial effects in antimicrobial tests but Tangeretin and Nobiletin exhibited low antimicrobial activities. The major antimicrobial component in FEPCR was Hesperidin (Yi *et al.*, 2008).

2.1.3 Antifungal Activity

The effect of essential oils obtained from Pericarpium *Citri reticulatae* by cold press was tested on the growth of moulds commonly associated with food spoilage: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Penicillium verrucosum*, using the agar dilution method. The essential oil extracted from Pericarpium *Citri reticulatae* showed the capacity to reduce or inhibit the growth of moulds at the concentrations assayed and was most effective at reducing the growth of *Aspergillus flavus* (Viuda-Martos *et al.*, 2008).

2.1.4 Antiasthmatic Activity

A study conducted by Shi *et al.* (2009) used different fractions of the alkaloid section of Pericarpium *Citri reticulatae* to screen for beta2-adrenergic receptor (beta(2)AR)

agonistic activity in rat beta(2)AR-transfected CHO-CRE-EGFP cells. The alkaloid sections were characterized and evaluated for their spasmolytic and antitussive activities both *in vitro* and *in vivo* in a guinea pig model. It was demonstrated that synephrine was the alkaloid section component that activated beta(2)AR signaling and stachydrine was the antitussive component that could significantly reduce nitric acid-induced coughing. The alkaloid component and synephrine showed significant spasmolytic effects on acetylcholine chloride (ACh)-induced contractions in isolated guinea pig trachea, and they protected against histamine-induced experimental asthma by prolonging the latent period. The combined use of both synephrine and stachydrines had a more potent spasmolytic activity in comparison with the use of either bioactive compound alone. Thus, it was concluded that synephrine and stachydrine were the key components of the Pericarpium *Citri reticulatae* alkaloid section that mediated asthma relief due to their synergism when used in combination.

2.1.5 Anti-pulmonary Fibrosis Activity

The water, ethanol, and flavonoids-enriched extracts of Pericarpium *Citri reticulatae* was tested for its inhibitory activity on the proliferation of human embryonic lung fibroblasts (HELF). They were given through oral administration to bleomycin (BLM)-induced pulmonary fibrosis rats and analyses of the rat body weight, hydroxyproline levels in serum and lung, scores of alveolitis and fibrosis, as well as the expression of transforming growth factor-β1 (TGF-β1) at the protein and the messenger ribonucleic acid (mRNA) levels in lung were performed. The ethanol extract showed the strongest inhibitory activity on HELF proliferation. Further research using BLM-induced rat model revealed that the ethanol

extract at the doses of 100 and 200 mg/ kg per day caused a marked increase of body weight at first 7 days, significantly lowered the hydroxyproline levels in lung, greatly improved the pathologic scores, as well as inhibited the overexpressions of TGF-β1 protein and mRNA. These results suggest that the ethanol extract of Citrus reticulate has anti-pulmonary fibrosis effects and might have a great potential for the treatment of fibrosis of lung (Zhou *et al.*, 2009).

2.1.6 Adipogenesis Suppression Activity

Intracellular triacylglycerol accumulations of 3T3-L1 cells were significantly reduced by Pericarpium *Citri reticulatae* methanol extract. The suppression effect was dose-dependent, and 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 treatment with the extract. These results suggest that dietary CRP suppresses 3T3-L1 differentiation by down-regulation of adipogenic transcription factors (Sheu *et al.*, 2007).

2.2 Medicinal Effects of *Spica prunella*

No undesirable side effects or toxicity were reported at the therapeutic dose in classical Chinese material medica (Hou & Jin, 2005). Experimental or clinical studies that would support the traditional indications of *Spica prunella (prunella vulgaris)* have not been carried out (Wyk & Wink, 2004).

2.2.1 Immunomodulatory Activity

Sun *et al.* (2005) studied the immunosuppressive activity of the ethanol extract of *Spica prunella* (EESP) consisting of a mixture of triterpenoids, flavonoids, tannins, and polysaccharide on the immune responses in mice by measuring mice splenocyte proliferation *in vitro*. They found that the ethanol extract significantly suppressed concanavalin A (con A)- and lipopolysaccharide (LPS)- stimulated phenocyte proliferation *in vitro* in a concentration-dependent manner. Further investigations of the effects of EESP at three dose levels (single dose of 0.25, 0.5, and 1.0 mg) on the humoral and cellular immune responses of mice subcutaneously immunized with ovalbumin (OVA) showed that EESP significantly suppressed Con A-, LPS and OVA-induced splenocyte proliferation in the immunized mice in a dose-dependent manner. Total IgG, IgG1 and IgG2b levels in the immunized mice were significantly reduced by EESP. Moreover, the suppressing effects on the antibody responses to OVA in the immunized mice were dose-dependently enhanced according to the increase of EESP. The results suggest that EESP could suppress the cellular and humoral response in mice.

The aqueous extract of *Spica prunella* was investigated for its immunomodulatory activities concerning its effect on the mitogenic response of murine splenocytes and nitric oxide production by murine peritoneal macrophages *in vitro*. The extract showed equal stimulation to both the B and T lymphocyte proliferation and suppressed Nitric Oxide production in lipopolysaccharide-stimulated macrophages dose dependently without any cytotoxicity (Harput *et al.*, 2006).

An organic fraction of *Spica prunella*, containing 25.7% and 0.32% rosmarinic acid and caffeic acid, respectively, markedly inhibited 5-lipoxygenase, a key enzyme in

leukotriene B4 biosynthesis, in Ca-ionophore-stimulated bovine polymorphonuclear leukocytes *in vitro* (Psotová *et al.*, 2003).

Methanol extract of *Spica prunella* with rosmarinic acid has been reported to inhibit interleukin-2 (IL-2) gene expression by 50% in Jurkat cells stimulated with anti-CD3 and anti-CD4 antibodies. Moreover, it has been shown to have an inhibitory effect on the intracellular Ca^{2+} increase in Jurkat cells after T cell activation. It has been suggested that rosmarinic acid has the potential to specifically inhibit lymphocyte cell-specific kinase (Lck) Src -homology 2 (SH2) domain binding to its cognate ligand, including ZAP-70, Cbl, HS-1, and PLCgamma1, and the Lck-dependent Ca^{2+} signaling pathway of its downstream effector and to modulate IL-2 gene expression after T cell activation (Ahn *et al.*, 2003).

2.2.2 Antihyperglycemic Activity

The effects of aqueous-ethanol extract of *Spica prunella* (AESP) on blood glucose, exogenous insulin sensitivity and plasma insulin levels in streptozotocin (STZ) induced diabetic ICR mice (STZ diabetic mice) were investigated. Significant decreases in blood glucose levels were observed after the administration of AESP and combined administration of AESP and glibenclamide produced a greater reduction effect in blood glucose levels than either glibenclamide or AESP alone. The antihyperglycemic effects of exogenous insulin on STZ diabetic mice were enhanced and prolonged by AESP. Plasma insulin levels were increased with glibenclamide treatment in STZ diabetic mice, whereas such effect was not observed with AESP. These results show that *Spica prunella* enhances the antihyperglycemic effects of exogenous insulin without stimulating insulin secretion, indicating that insulin sensitivity is increased in STZ diabetic mice (Zheng *et al.*, 2007).

2.2.3 Antiviral activity

In a study conducted by Chiu *et al.* (2004), the effects of the polysaccharide fraction prepared from *Spica prunella* on the expressions of HSV-1 and HSV-2 antigens in their host Vero cells were investigated with flow cytometry. The HSV antigen increased time-dependently in the infected cells, and the *Spica prunella* polysaccharide fraction reduced its expression. The *Spica prunella* polysaccharide fraction also reduces the antigen expression of cyclovir-resistant strain of HSV-1. The amount of HSV antigen-positive cells was reduced when incubated together with the polysaccharide fraction, showing that this polysaccharide fraction has a different mode of anti-HSV action from acyclovir. Results from this study show that the polysaccharide fraction of *Spica prunella* is effective against both the HSV-1 and HSV-2 infections.

In a different study conducted by Brindley *et al.* (2009), the water and ethanol extracts of *Spica prunella* were tested for their ability to inhibit equine infectious anemia virus (EIAV) replication. The aqueous extracts contained more anti-viral activity than the ethanol extracts, displaying potent anti-lentiviral activity against virus in cell lines as well as in primary cell cultures with little to no cellular cytotoxicity. Time-of-addition studies demonstrated that the extracts were effective when added during the first four h of the viral life cycle, suggesting that the botanical constituents were targeting the virion itself or early entry events. Further analysis revealed that the extracts did not destroy EIAV virion integrity, but prevented viral particles from binding to the surface of permissive cells. Modest levels of anti-EIAV activity were also detected when the cells were treated with the extracts prior to infection, indicating that anti-EIAV botanical constituents could interact with both viral particles and permissive cells to interfere with infectivity. Size fractionation

of the extract demonstrated that eight of the nine fractions generated from aqueous extracts displayed anti-viral activity. Separation of ethanol soluble and insoluble compounds in the eight active fractions revealed that ethanol-soluble constituents were responsible for the anti-viral activity in one fraction whereas ethanol-insoluble constituents were important for the anti-viral activity in two of the other fractions. In three of the five fractions that lost activity upon sub-fractionation, anti-viral activity was restored upon reconstitution of the fractions, indicating that synergistic anti-viral activity is present in several of the fractions. These findings indicate that multiple *Prunellae* constituents have profound anti-viral activity against EIAV and the ability of the aqueous extracts to prevent entry of viral particles into permissive cells suggests that these extracts may function as promising microbicides against lentiviruses.

2.3 Phytochemical Extractions Using Soxhlet Apparatus

Solid-liquid extraction, also known as leaching or lixiviation, is one of the oldest ways of solid sample pretreatment (Castro & Garcia-Ayuso, 1998). Solid-liquid extraction is often used to extract a solid natural product from a natural source, such as a plant (Pavia *et al.*, 2002). Classical techniques for the solvent extraction of nutraceuticals from plant matrices are based on the choice of solvent coupled with the use of heat and/or agitation (Wang & Weller, 2006). The Soxhlet apparatus has been the leaching technique most commonly used for the implementation of this step, and is the main reference to which performance of other leaching methods are compared.

An overview of Soxhlet extraction of solid materials was given by Castro & Garcia-Ayuso (1998). In a conventional Soxhlet system, plant material is placed inside a thimble

within the sample chamber, and is gradually filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. Solute is left in the flask and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved. The most outstanding advantages of conventional Soxhlet include:

1. The displacement of the transfer equilibrium by repeatedly bringing fresh portions of the solvent into contact with the solid sample
2. The maintenance of relatively high extraction temperatures within the system with heat from the distillation flask
3. No filtrations required after the leaching step (Castro & Garcia-Ayuso, 1998). The Soxhlet method is also straightforward and cheap (Garcia-Ayuso and Castro, 1999).

2.4 Biologically Active Substances in Pericarpium *Citri reticulatae*

Pericarpium *Citri reticulatae* contains 1- 2% essential oil (mainly limonene, linalool, terpineol), bitter flavanone glycosides (naringin and neohesperidin) and bitter triterpenes (limonin) (Hou & Jin, 2005; Wyk & Wink, 2004). Other components in the oil include isopropenyltoluene, delta-elemene, alpha-copaene, alpha-humulene, beta-sesquiphol-landrene, alpha-humulenol acetate, and 1,8 menthadien-10-ol-acetate. Flavonoids (hesperidin, neohesperidin, narigin, tangeretin, auranetin, and nobiletin) and other

components of hesperidin, carotene, cryptosanthin, vitamins B, C, and P, alkaloid synephrine, and N-methyltyramine have been isolated (Zhu, 1998).

Essential oils of Pericarpium *Citri reticulatae* can be obtained using cold-press and has a density of 0.85 g/mL and refraction index of 1.47 at 20°C. Its boiling point was at 49°C (Viuda-Martos *et al.*, 2008).

Hesperidin, the abundant and inexpensive flavonoid found in Pericarpium *Citri reticulatae*, was more efficiently isolated using ultrasonic extraction compared to traditional Soxhlet extraction. Results showed that solvent, frequency and processing temperature were the most important factors for improving the extracting yields of hesperidin. When performed at the same temperature under the same time using three frequencies, methanol as the solvent improved the extraction yield evidently compared with ethanol or isopropanol. Hesperidin was not degraded by extending the ultrasonic treatment times and using a rotary beaker for materials increased the yields of hesperidin (Ma *et al.*, 2008). Zheng *et al.* (2009) simultaneously determined five bioactive flavonoids present in *Percarpium Citri reticulatae*, which were hesperidin, nobiletin, 3,5,6,7,8,30,40-heptamethoxyflavone, tangeretin, and 5-hydroxy-6,7,8,30,40-pentamethoxyflavone, from 32 samples collected from different districts of China using HPLC with dual wavelength detection.

2.5 Biologically Active Substances in *Spica prunella*

Spica prunella contains triterpenoids, flavonoids, sterol glycosides, and coumarins. The triterpene compounds include ursolic acid and betulinic acid. The flavonoids include delphinidine, cyanidin, and rosmarinic acid. The sterol glycosides include beta-sitosterol-

beta-D-glucoside (Ling, 1995). Other ingredients are alkaloids, oleanolic acid, rutin, hyperoside, caffeic acid, tannin, volatile oil, and vitamins A, C, and K (Zhu, 1998). It also contains sulfated polysaccharide compounds (Lewis & Elvin-Lewis, 2003). The diterpenoid lactones of the labdane type such as ballotenol (main compound), ballotinone, 7α-acetoxymarrubiin and preleosibirin (a prefuranoid) along with flavonoid glycosides, phenylpropanoids (chlorogenic acid) and traces of volatile oils are also present (Wky & Wink, 2004).

Psotová *et al.* (2003) found that the organic fraction of *Spica prunella* total extract contained significant levels of rosmarinic and caffeic acid. Rosmarinic acid was the predominant compound (25.7% w/ w) while the amount of caffeic acid was 0.37% of the organic fraction.

Chiu *et al.* (2004) prepared a polysaccharide fraction from *Spica prunella* by pooling the water extracts obtained by repeated extractions on the same crude herb sample and centrifuging to remove any water-insoluble components. The collected supernatant was concentrated by rotary evaporator and precipitated by 40 and 80% ethanol sequentially at 4°C overnight. The precipitate was collected by centrifugation, and the pellet was dissolved in distilled water and lyophilized as a dark brown powder.

The activity-guided fractionation of the extract of the herb of *Prunella vulgaris* (Labiatae) led to the isolation of four triterpenes, which were betulinic acid, ursolic acid, 2-alpha,3-alpha-dihydroxyurs-12-en-28-oic acid, and 2-alpha-hydroxyursolic acid (Ryu *et al.*, 2000).

Gua *et al.* (2007) isolated three new oleanane-skeleton triterpenoid saponins, 3β,4β,16α-17-carboxy-16,24-dihydroxy-28-norolean-12-en-3-yl-4-O-β-D-xylopyranosyl-β-

D-glucopyranosiduronic acid, (3β,4β,16α)-17-carboxy-16,24-dihydroxy-28-norolean-12-en-3-yl-β-D-glucopyranosiduronic acid methyl ester, and (3β,4β)-24-hydroxy-16-oxo-28-norolean-12-en-3-yl-4-O-β-D-xylopyranosyl-β-D-glucopyranosiduronic acid, together with eight other oleanane-type and ursane-type triterpenoids from the spikes of *Prunella vulgaris*. The *Spica prunella* ethanol extract was concentrated and partitioned sequentially with petroleum ether and n-butanol, followed by various separations using column chromatography.

OBJECTIVES

The objectives of this study are:

1) To extract phytochemicals from Pericarpium *Citri reticulatae* and *Spica prunella* using distilled water, methanol, and n-hexane.

2) To perform preliminary phytochemical screening for major constituents present in extracts of Pericarpium *Citri reticulatae* and *Spica prunella.*

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

All chemicals and reagents used in this study were of analytical reagent grade, obtained from Merck (Germany), and R & M Chemicals (United Kingdom).

3.2 Glassware

All glassware was obtained from Pyrex (United Kingdom) and Scott Duran (Germany). They were all soaked overnight in a 1: 2 v/ v mixture of conc. HNO_3 and conc. HCl, washed with plenty of water/ doubly-distilled water and dried in oven before use.

3.3 Sample Collection and Preparation

The Pericarpium *Citri reticulatae* and *Spica prunella* samples were purchased from a Chinese Herbal shop located in Kebun Sultan, Kota Bharu, Kelantan. Pericarpium *Citri reticulatae* and *Spica prunella* are washed in separate batches. They were washed in separate batches with doubly-distilled water to remove any odd materials, such as soil, dust, seeds, etc. Samples are then spread out evenly on several plastic trays that are lined with tissue paper and allowed to dry for 24 h inside a fume hood. The herbs were then transferred to aluminum foil sheets (folded into makeshift trays) for further drying in an oven at 50°C for 24 h.

The over-dried *Spica prunella* samples were ground using a National MX-895M Microcutter blender to get a mixture of fine and coarse particles. While Pericarpium *Citri*

reticulatae samples were manually broken into smaller fragments before grinding. The ground samples were transferred into beakers, covered with aluminum foil, and stored in a dark and cool place at about 20°C.

3.4 Extraction of Pericarpium *Citri reticulatae* and *Spica prunella*

The stored Pericarpium *Citri reticulatae* and *Spica prunella* samples were Soxhlet-extracted with methanol, n-hexane, and distilled water, described as under.

A pre-cleaned and dried 70.0 mL capacity Soxhlet extraction apparatus was assembled as shown in Fig. 3.1, and the herb (ca. 10.0 g) was placed in a cellulose thimble. Samples were extracted for 4 h with methanol at a rate of 6- 7 cycles/ h. The same procedure was repeated for extraction with n-hexane and distilled water.

The methanol, n-hexane, and distilled water extracts were subjected to rotary evaporation and solvents were dried-off under reduced pressure. The extracts were transferred into pre-labelled volumetric flasks, stoppered and stored in a refrigerator at 4°C until use.

3.5 Preparation of Wagner's Reagent

Iodine (1.3 g) and potassium iodide (2.00 g) were dissolved in distilled water and volume was made to 100.0 mL with distilled water in a volumetric flask. The reagent was stored in a dark and cool area until use.

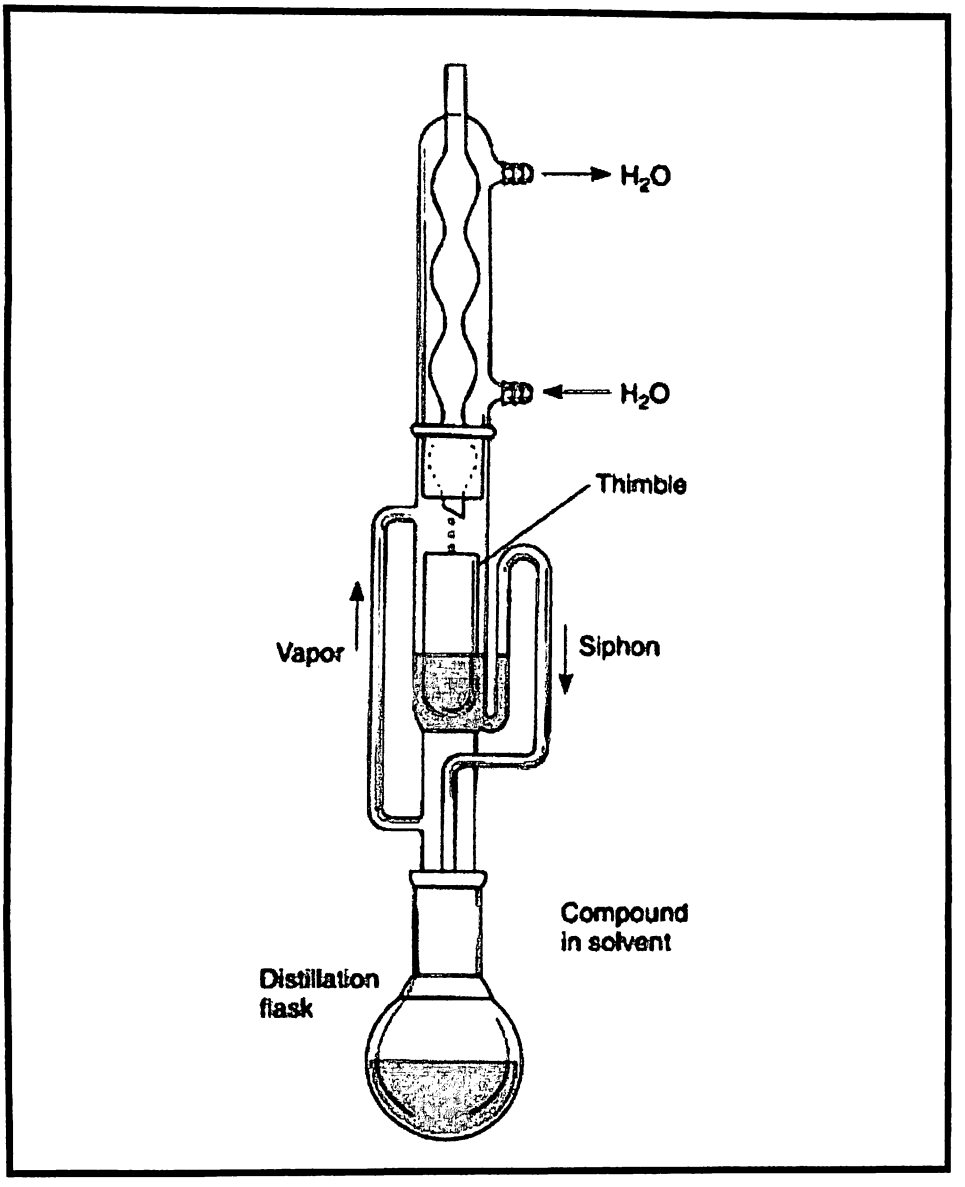

Figure 3.1. A Soxhlet apparatus for solid-liquid extraction (Pavia *et al.*, 2002)

3.6 Phytochemical Screening of Pericarpium *Citri reticulatae* and *Spica prunella* Extracts

Phytochemical screening tests are performed on the extracts obtained from the different extraction methods previously outlined using colour forming and precipitating chemical reagents. The phytochemical tests used in this section are highly sensitive (able to detect phytochemicals even at low concentrations), easy to perform, fast, require minimal equipment, and give clear results.

3.6.1 Preparation of Stock Solutions

The extracts obtained were poured into Petri dishes and evaporated to dryness in a fume hood and then weighed using a Mettler Toledo analytical balance. 0.25 g of each extract (distilled water, methanol, and n-hexane) of Pericarpium *Citri reticulatae* and *Spica prunella* were dissolved in 25.0 mL of its own mother solvents to obtain a stock solution of 1.0% concentration (v/ v). The extracts thus obtained were subjected to preliminary phytochemical screening following the methodology of Kumar *et al.* (2009), Mishra *et al.* (2009), Okunlola *et al.* (2007), and Ahmad & Raji (1993) with modifications.

3.6.2 Test for Saponin

0.5 mL of the plant extract stock solution is used. 2.5 mL of distilled water is added to the extract and the test tube is sealed with parafilm. The test tube is then shaken vigorously for 30 sec and left to stand for 30 min. Froth or honeycomb-like bubble formation that is 3.0 cm or thicker from the surface of the mixture indicates the presence of saponin. If no froth or bubbles are formed in the mixture or the froth and bubbles formed are unstable, 8 drops

of aqueous sodium carbonate (Na_2CO_3) solution is added. The formation of stable froth or bubbles indicates the presence of free acids.

3.6.3 Test for Steroids

1.0 mL of the extract stock solution was dissolved in 10.0 mL of chloroform and an equal volume of concentrated sulfuric acid was added by sides of the test tube. If the upper layer turns red and the sulfuric acid layer turns yellow with green fluorescence, it indicates the presence of steroids.

3.6.4 Test for Amino Acids

1.0 mL of the extract was treated with few drops of Ninhydrin agent. The appearance of purple colour shows the presence of amino acids.

3.6.5 Test for Flavonoids

5 drops of dilute sodium hydroxide was added to 1.0 mL of the extract. An intense yellow colour produced in the plant extract which becomes colourless on addition of a few drops of dilute acid indicates the presence of flavonoids.

3.6.6 Test for Tannins

2.0 mL of iron(III) chloride ($FeCl_3$) was added to 1.0 mL of the extract. Blue-black precipitate indicates the presence of tannins.

3.6.7 Test for Anthraquinones

0.5 mL of the stock solution is shaken with 5.0 mL of chloroform for 5 min. The mixture was filtered, and the filtrate is shaken with an equal volume of 10.0% ammonia solution. A pink, violet or red colour in the ammoniacal layer (lower layer) indicates the presence of free anthraquinones.

3.6.8 Test for Alkaloids

0.5 mL of the stock solution is added to 5.0 mL of 1% aqueous HCl on a steam bath. The mixture was filtered and 1.0 mL of the filtrate is treated with Wagner's Reagent. The formation of precipitates indicates the presence of alkaloids.

CHAPTER 4

RESULTS

4.1 Extraction of Pericarpium *Citri reticulatae* and *Spica prunella*

The extraction of Pericarpium *Citri reticulatae* and *Spica prunella* using distilled water, methanol, and n-hexane respectively seemed to have produced sufficient amounts of extracts.

4.1.1 Visual Inspection of Pericarpium *Citri reticulatae* Distilled Water Extract

After rotary evaporation, the extract obtained from distilled water extraction of Pericarpium *Citri reticulatae* was thick and viscous, and produced a deep orange-brown colouration.

4.1.2 Visual Inspection of Pericarpium *Citri reticulatae* Methanol Extract

After rotary evaporation, the extract obtained from methanol extraction of Pericarpium *Citri reticulatae* was still in a fluid state (excess methanol left over to prevent extracts from sticking to the walls of the rotary evaporation round bottom flask) and had a dull orange colouration. White irregular spherical sediments were found in the extract after storage in the refrigerator.

4.1.3 Visual Inspection of Pericarpium *Citri reticulatae* n-Hexane Extract

After rotary evaporation, the extract obtained from n-hexane extraction of Pericarpium *Citri reticulatae* was in a clear fluid state and imparted a bright orange colouration.

4.1.4 Visual Inspection of *Spica prunella* Distilled Water Extract

After rotary evaporation, the extract obtained from distilled water extraction of *Spica prunella* was thick and viscous, and produced a dark brownish colouration.

4.1.5 Visual Inspection of *Spica prunella* Methanol Extract

After rotary evaporation, the extract obtained from methanol extraction of *Spica prunella* was in a fluid state and imparted a dull green colouration.

4.1.6 Visual Inspection of *Spica prunella* n-Hexane Extract

After rotary evaporation, the extract obtained from n-hexane extraction of *Spica prunella* was in a clear fluid state and imparted a yellowish colouration.

4.2 Dry Weight of Pericarpium *Citri reticulatae* and *Spica prunella* extracts

Table 4.1. The dry weight of Pericarpium *Citri reticulatae* and *Spica prunella* extracts

Solvent	Weight of Extracts (g)	
	Pericarpium *Citri reticulatae*	***Spica prunella***
Distilled Water	4.1947	0.3556
Methanol	7.0806	0.5725
n-Hexane	2.0060	0.3391

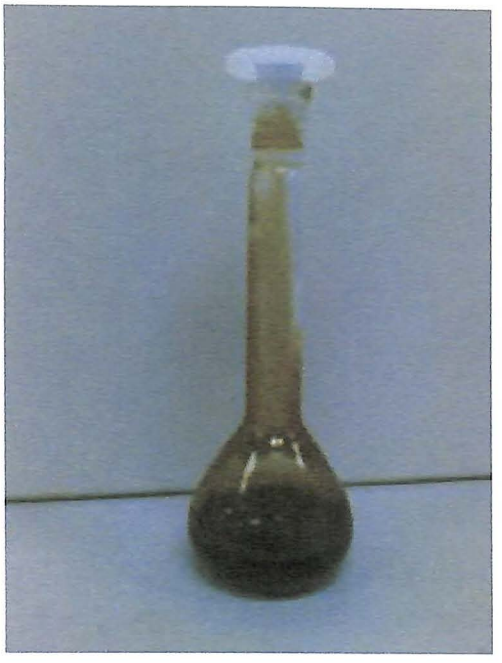

Figure 4.1. Distilled water extract of Pericarpium *Citri reticulatae*

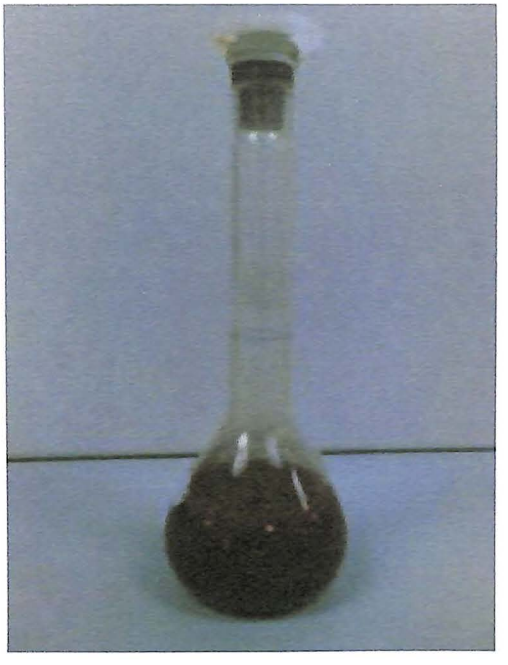

Figure 4.2. Methanol extract of Pericarpium *Citri reticulatae*

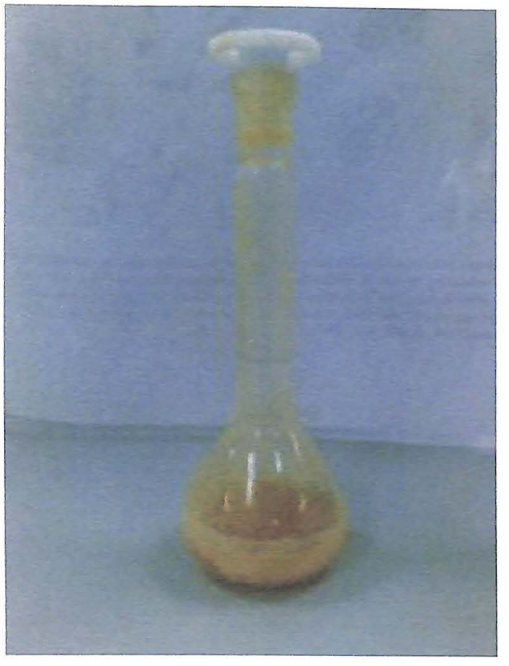

Figure 4.3. n-Hexane extract of Pericarpium *Citri reticulatae*

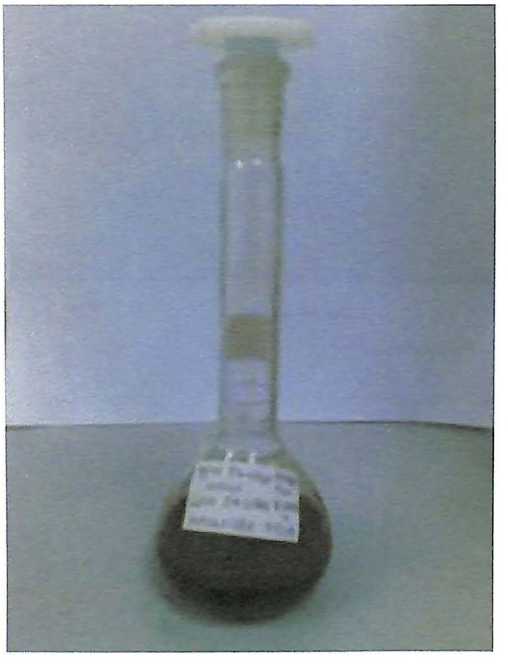

Figure 4.4. Distilled water extract of *Spica prunella*

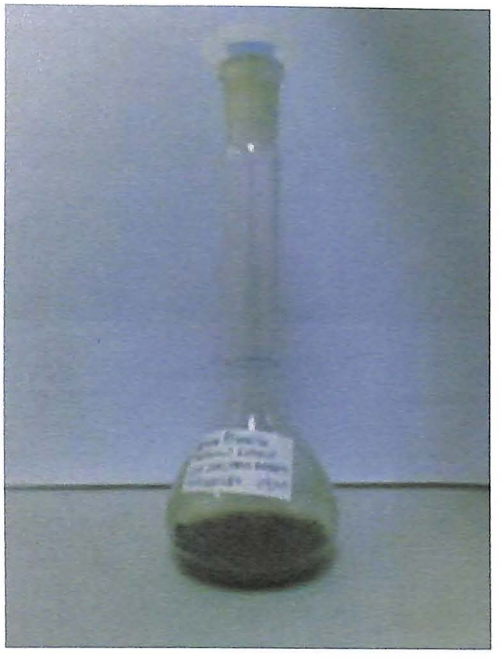

Figure 4.5. Methanol extract of *Spica prunella*

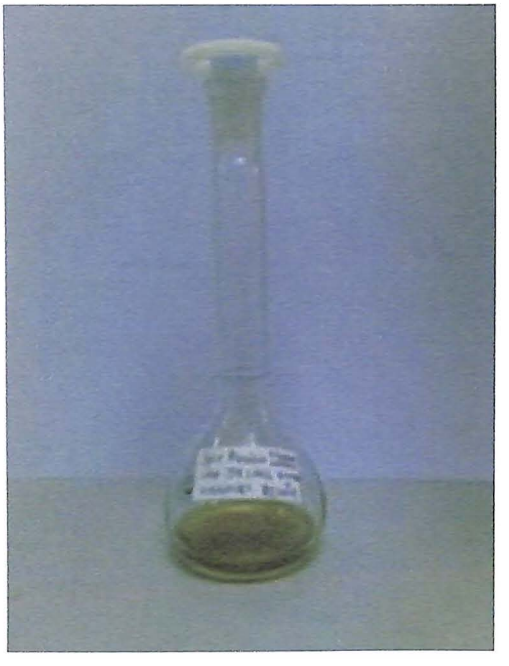

Figure 4.6. n-Hexane extract of *Spica prunella*

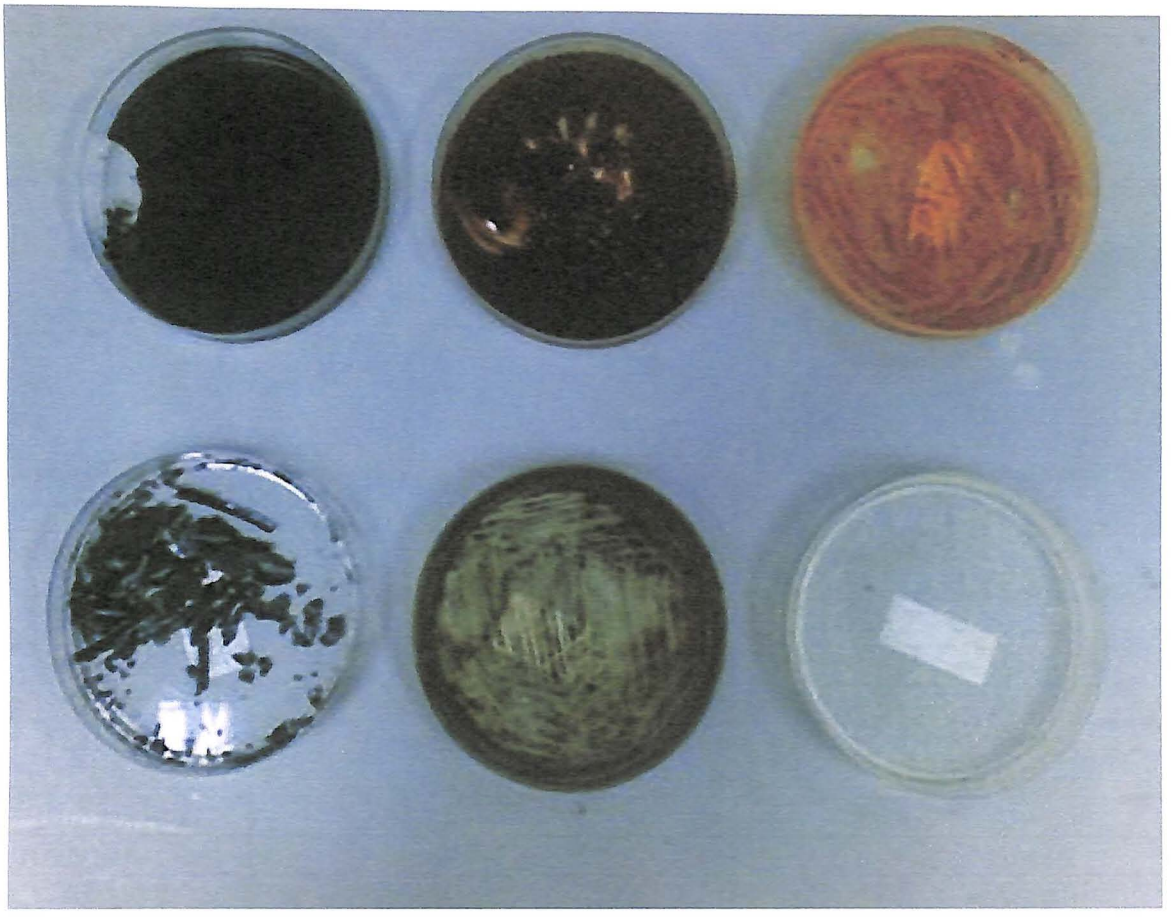

Figure 4.7. Dried distilled water, methanol, and n-hexane extracts of Pericarpium *Citri reticulatae* (top row) and *Spica prunella* (bottom row)

4.3 Phytochemical Screening of Pericarpium *Citri reticulatae* and *Spica prunella* Extracts

The results for the phytochemical screening tests done on the distilled water, methanol, and n-hexane extracts of Pericarpium *Citri reticulatae* and *Spica prunella* are given in Table 4.2 and Table 4.3.

Table 4.2. Phytochemical screening results of Pericarpium *Citri reticulatae* extracts

Phytochemical Test	**Pericarpium *Citri reticulatae***		
	Distilled Water Extract	**Methanol Extract**	**n-Hexane Extract**
Saponins	Present	Absent	Absent
Steroids	Absent	Absent	Absent
Amino Acids	Absent	Absent	Absent
Flavonoids	Ambiguous	Absent	Absent
Tannins	Absent	Absent	Absent
Anthraquinones	Absent	Absent	Absent
Alkaloids	Absent	Absent	Absent

Table 4.3. Phytochemical screening results of *Spica prunella* extracts

Phytochemical Test	***Spica prunella***		
	Distilled Water Extract	**Methanol Extract**	**n-Hexane Extract**
Saponins	Present	Free acids present	Absent
Steroids	Absent	Absent	Absent
Amino Acids	Absent	Absent	Absent
Flavonoids	Absent	Ambiguous	Absent
Tannins	Absent	Ambiguous	Absent
Anthraquinones	Absent	Absent	Absent
Alkaloids	Absent	Absent	Absent

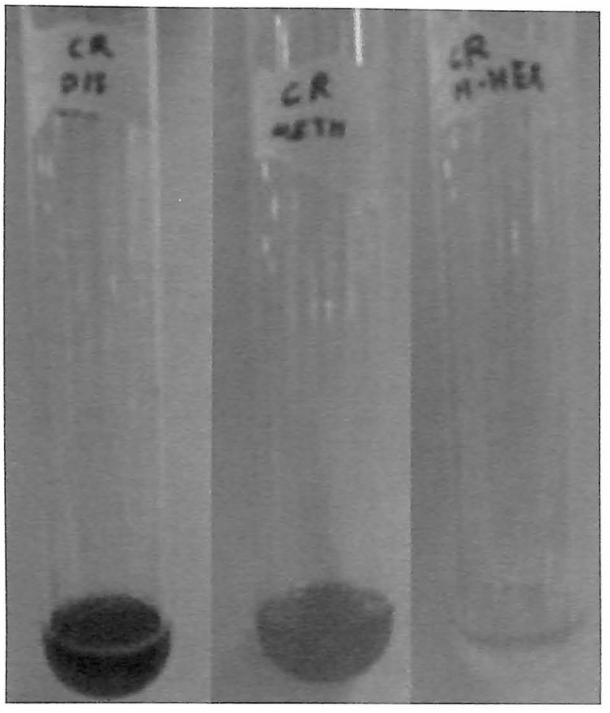

Figure 4.8. Saponin test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts

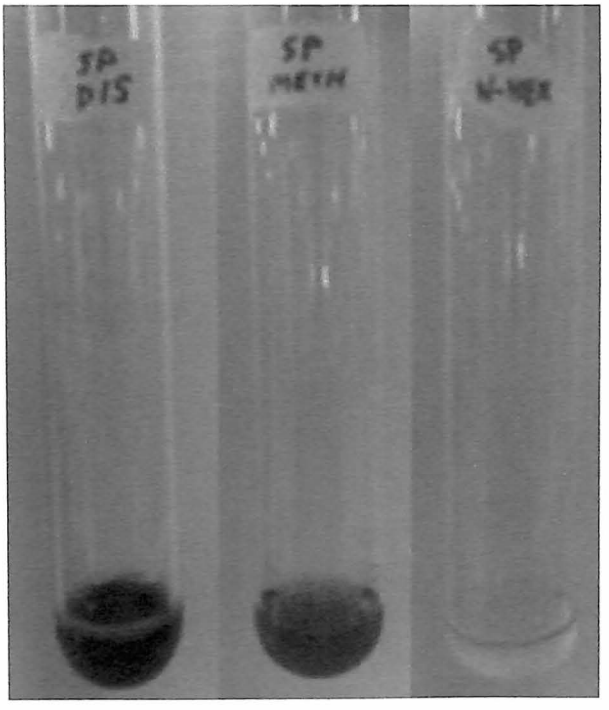

Figure 4.9. Saponin test for (left to right) *Spica prunella* distilled water, methanol, and n-hexane extracts

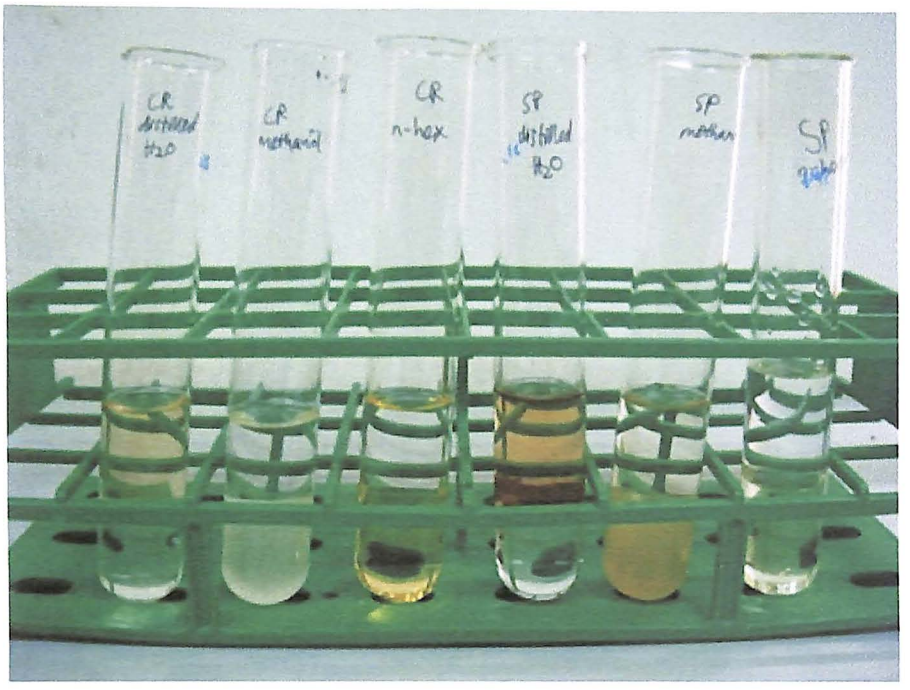

Figure 4.10. Steroid test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* distilled water, methanol, and n-hexane extracts

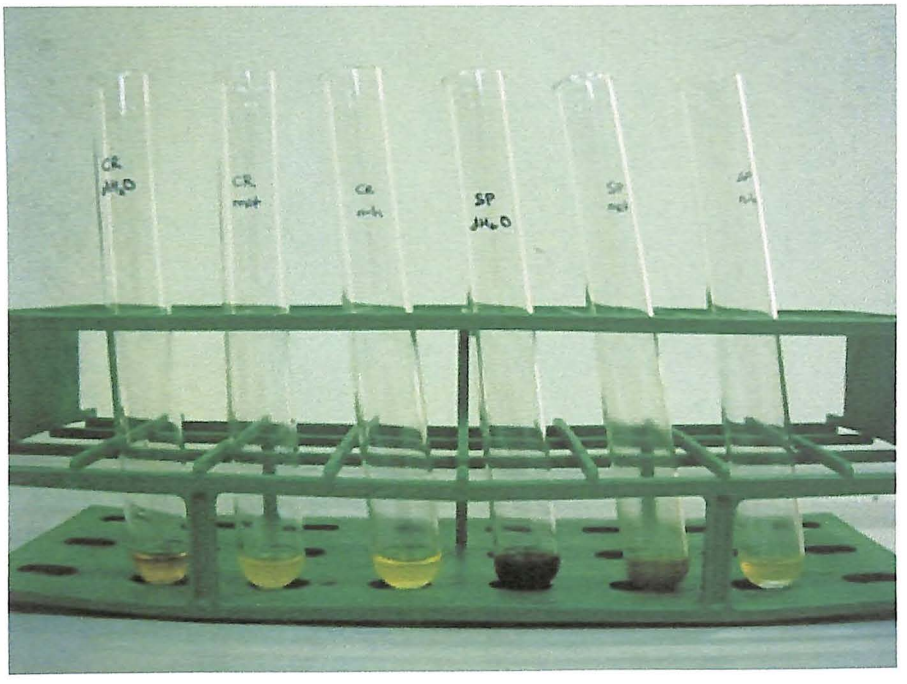

Figure 4.11. Amino acid test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* distilled water, methanol, and n-hexane extracts

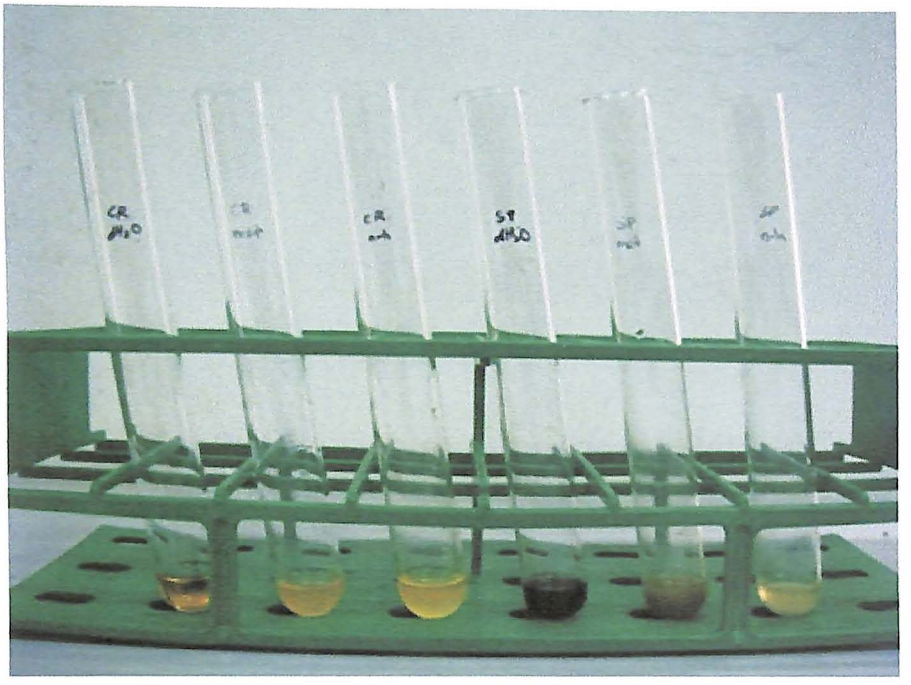

Figure 4.12. Flavonoid test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* distilled water, methanol, and n-hexane extracts

Figure 4.13. Tannin test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* distilled water, methanol, and n-hexane extracts

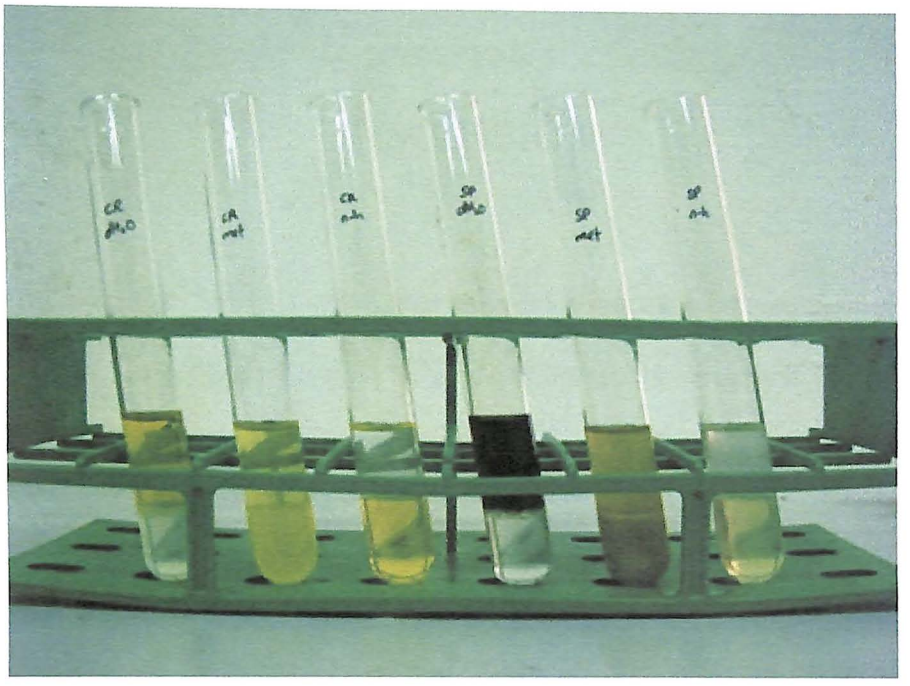

Figure 4.14. Anthraquinone test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* distilled water, methanol, and n-hexane extracts

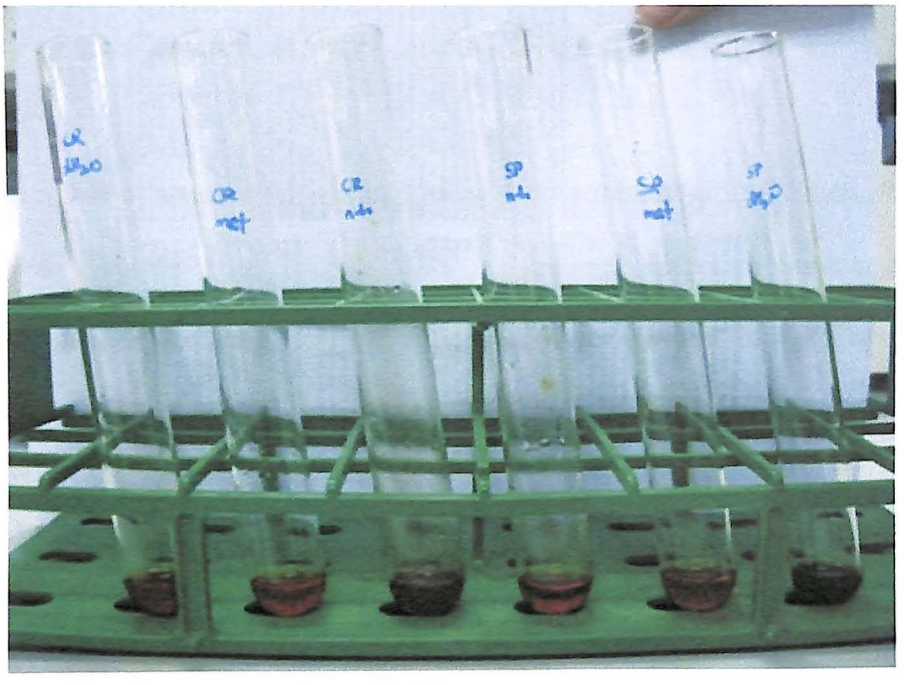

Figure 4.15. Alkaloid test for (left to right) Pericarpium *Citri reticulatae* distilled water, methanol, and n-hexane extracts and *Spica prunella* n-hexane, methanol, and distilled water extracts

CHAPTER 5

DISCUSSION

Extraction is always the first procedure that must be performed on medicinal plant samples before further tests can be performed, be it identification or structural characterization of the active constituents present. It is an important step which forms the basis of further studies involving chromatographic (TLC, HPLC), spectroscopic (UV-Vis, NMR), crystallographic and other hyphenated (GC-MS, LC-MS) techniques.

The extraction techniques commonly used for the isolation of compounds from medicinal plants include liquid-liquid extraction, solid-liquid extraction, and solid phase extraction. With the exception of solid phase extraction, extractions are generally performed using water, methanol, ethanol, n-hexane and other suitable solvents, either individually or as a mixture, in order to extract certain compounds which have greater affinity for them.

The active constituents in Pericarpium *Citri reticulatae* and *Spica prunella* were extracted with distilled water, methanol, and n-hexane. Although Soxhlet extraction is considered a classical and relatively outdated method for extraction, it is still favoured due to its efficiency and low cost (Garcia-Ayuso & Castro, 1999).

The solubility of different natural products vary with different solvents. For example, polar solutes are soluble in polar solvents such as methanol, ethanol, dimethyl formamide (DMF) whereas on-polar solutes dissolve in non-polar solents such as hexane, benzene, cyclohexane, and toluene. Different solvents will yield different extracts and extract compositions (Zarnowski & Suzuki, 2004). Usually, the solubility of natural

products increases with an increase in the polarity levels (Jadhav *et al.*, 2009). According to Kamil (1993), the use of polar solvents in soxhlet extraction for maximum extent of extraction has been conclusively established.

In Jadhav *et al.*'s (2009) study of extraction of vanillin from vanilla pods, they observed that extraction of vanillin was higher in polar solvents such as ethanol, methanol and least in the case of non-polar solvent such as hexane. They proposed ethanol as an optimum solvent for maximum yield of vanillin after the discovery that the extent of extraction of vanillin from cured vanilla beans was the maximum in ethanol. Hexane is the most widely-used solvent in the extraction of edible oils from plant sources. It has a fairly narrow boiling point range of approximately 63– 69°C and is an excellent oil solvent in terms of oil solubility and ease of recovery (Wang & Weller, 2006).

The use of alternative solvents such as isopropanol, ethanol, hydrocarbons, and even water, has increased due to environmental, health, and safety concerns (Wang & Weller, 2006). Mamidipally & Liu (2004) used d-limonene and hexane in the extraction of oil from rice bran and found that d-limonene extracted a significantly higher amount of oil than hexane under any given set of conditions.

Even so, alternative solvents may cost more and often result in less recovery due to decreased molecular affinity between the solvent and solute. Rice bran oil extracted using an aqueous process had a lower content of free fatty acid and lower colour-imparting components than oil extracted using hexane (Hanmoungjai *et al.*, 2000). A co-solvent is sometimes added to increase the polarity of the liquid phase. A mixture of solvents such as isopropanol and hexane has been reported to increase the yield and kinetics of extraction (Li *et al.*, 2004).

On the effect of temperature on extraction, a study dealing with the extraction of vanillin pods using conventional Soxhlet extraction found that an increase in operating temperature from 90°C to 100°C increased the extent of extraction by 30%. The rate of recycle of the condensed solvent, which has a role in determining the extent of extraction, is indirectly controlled by modifying the rate of vapour generation and ensuring sufficient cooling capacity for complete condensation. An increase in temperature is likely to increase the rate of recirculation of the solvent through the sample chamber and hence the extent of extraction increases (Jadhav *et al.*, 2009).

The solvent used is usually recovered by evaporation during Soxhlet extraction. The quality of the final products can be significantly affected by the extraction and evaporation temperatures. Mamidipally & Liu (2004) found that d-limonene extracted rice bran oil was slightly darker compared to hexane extracted oil, probably due to higher extraction and evaporation temperatures used during the d-limonene solvent extraction. The high boiling temperature for solvent recovery can be decreased by using vacuum or membrane separation to recover the solvent (Wang & Weller, 2006).

The quantity of product used has an effect on extraction as well. Jadhav *et al.* (2009) investigated the effect of the quantity of vanilla beans on the extent of vanillin extraction at a constant quantity of ethanol solvent and found that the extent of vanillin extracted per unit of vanilla beans (initially present) depends on the relative proportions of the vanilla beans and solvent. For the case of maximum relative proportion of solvent (1.0 g of vanilla beans to 100 mL of solvent), the rate of extraction almost follows a linear path with time of operation, whereas for minimum relative proportion of the solvent (3.0 g of

vanilla beans to 100 mL of solvent), the rate of extraction decreased with extended extraction time.

Based on the amount of Pericarpium *Citri reticulatae* and *Spica prunella* used for each extraction (ca. 10 g) and the dry weight of the extracts obtained after evaporation of the solvents, the percentage yield of each solvent from the crude herbs can be calculated. In the preparation of distilled water extract from Pericarpium *Citri reticulatae* and *Spica prunella*, a yield of 40.2% and 3.6% was obtained respectively. In the case of the methanol extract, a yield of 70.8% for Pericarpium *Citri reticulatae* and 5.7% for *Spica prunella* was obtained respectively. As for the n-hexane extract from Pericarpium *Citri reticulatae* and *Spica prunella*, a yield of 20.1% and 3.4% was obtained respectively. Based on these calculations it can be said that the yield of extracts from both Pericarpium *Citri reticulatae* and *Spica prunella* increases from n-hexane to distilled water to methanol. Based on their dielectric constants, distilled water is the most polar solvent used (dielectric constant of 80) and n-hexane is the least polar solvent used (dielectric constant of 2.02). Methanol is a polar solvent with a dielectric constant of 33, which lies between distilled water and n-hexane, which might explain why it has the highest percentage of yield for both herbs amongst the three solvents used for extraction.

The phytochemical screening test procedures adopted in this study were chosen based on the availability of chemicals. Although Dragendroff's reagent is more widely accepted and used as the standard reagent when testing for alkaloids, it could not be prepared as bismuth nitrate ($Bi(NO_3)_2$), one of the main components of Dragendroff's reagent, was not available in the laboratory stores.

Phytochemical screening tests reveal the presence or absence of major secondary metabolites such as alkaloids, steroidal compounds, saponins and so on in the extracts of Pericarpium *Citri reticulatae* and *Spica prunella.* Although sufficient extracts seemed to have been obtained during the extraction process for both herbs with the solvents chosen, the results for almost all the phytochemical screening tests performed on the extracts produced negative results. No controls were used for all the phytochemical tests performed because there were no standard solutions available. Thus, the results were interpreted based on the descriptions given in the methodologies used.

The results for the saponin test revealed that only the distilled water extracts of *Percarpium Citri reticulate* and *Spica prunella* contained saponins, while there is a possibility that free acids are present in the methanol extract of *Spica prunella.* The flavonoid test results for the Pericarpium *Citri reticulatae* distilled water extract and *Spica prunella* methanol extract were considered ambiguous because both extracts produced an intense yellow colour with the addition of dilute sodium hydroxide but did not become totally colourless with the addition of a few drops of dilute acid. As for the tannins test, the result for the *Spica prunella* methanol extract was ambiguous because some precipitate was produced upon the addition of iron(III) chloride ($FeCl_3$) but it was not blue-black in colour. With the exception of those previously mentioned, the rest of the results produced from the phytochemical screening tests were negative. If the phytochemical constituents of the extracts obtained are to be interpreted based on these results, the conclusion would be that the distilled water extract of Pericarpium *Citri reticulatae* contains saponins and possibly flavonoids; the distilled water extract of *Spica prunella* contains saponins; and methanol extract of *Spica prunella* contains free acids and possibly flavonoids and tannins.

The results for this study's phytochemical screening test are highly unusual because almost all the phytochemicals screened for are absent in all the extracted material using different solvents. Ma *et al.* (2008) and Zheng *et al.* (2009) found that methanol had the highest extraction yield of flavonoids from *Percarpium Citri reticulatae* but the flavonoid test performed on the Pericarpium *Citri reticulatae* methanol extract was negative. The aqueous extract of *Spica prunella* would be anticipated to contain abundant amounts of carbohydrates, phenolics and other water-soluble constituents (Brindley *et al.*, 2009). Even alkaloids, a phytochemical compound almost always present in herb materials and found to be present in both Pericarpium *Citri reticulatae* and *Spica prunella* by Zhu (1998), was absent in all the different extracts of both herbs. Essential oils should be present in the n-hexane extracts but were not screened for in the phytochemical tests (El-Shazly *et al.*, 2002).

Even though many problems are presented by the diverse methodology utilized by investigators in phytochemical screening, much useful information can be derived from published studies. Positive test results are usually clear cut and on the other hand, must be carefully weighed in terms of being due to real absence of the test material in the sample being evaluated, or to the methodology employed.

The most common problem encountered in the detection of pharmacological activity is that even extracts from single plants are a mixture of several compounds which can be subject to variation in concentration or composition according to ecological changes (Farnsworth, 1993). In the case of higher plant extracts, the majority of false positives can be attributed to the presence of polyphenols, detergents such as saponins and certain pigments or fatty acids (O'Neill & Lewis, 1993). Phenols affect highly purified enzyme-

based targets, while saponins primarily disrupt membranes in cellular targets or dislodge substrates absorbed onto assay wells. Pigments tend to interfere with read-outs in colorimetric or quenched assays. Fatty acids show activity through a variety of mechanisms (O'Neill & Lewis, 1993). The removal of these undesirable compounds from the plant extract is preferable before primary screening, but it is usually easier to run the crude extracts through the primary screening assay, and to introduce measures to discriminate between false and true positive results at a later stage in the process (O'Neill and Lewis, 1993).

The lack of a positive result in a screening assay does not always mean the absence of bioactive constituents (Taylor *et al.*, 2000). The active principle(s) may be present in insufficient quantities in the crude extracts to show any positive results. Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the active principles during the assay. Fractionation of the extracts before screening by partitioning or serial extraction, can in some cases overcome this problem, although this multiplies the number of samples to be tested (Farnsworth, 1993).

The duplication of pharmacological results is important. Failure to do so could result from a variation in the concentration of active compounds due to environmental or genetic factors (Farnsworth, 1993). These include the season, growing environment, area of collection, physiological age of the plant, physiological state (e.g. flowering), and storage conditions. Genetic variation (genotype) usually causes qualitative differences in different samples, while the environmental factors usually affect quantitative results. It has been determined that there was a great deal of regional variability in the flavonoid composition

in Pericarpium *Citri reticulatae* across China. Another cause is the failure to collect the same specimen on separate occasions, which emphasizes the importance of voucher specimens. Initial thin layer chromatogram (TLC) profiles or fingerprints of active extracts as well as the subsequent active fractions, should be performed and filed before the active principle(s) are isolated and identified to enable comparison between samples (Farnsworth, 1993). For traditional Chinese medicinal, the selection of medicinal plants and its quality control is particularly important (Zheng *et al.*, 2009).

The small amount of samples used for the extraction process for each solvent, which is only about 10.0 g due to the limitations in the cellulose thimble capacity and the size of the Soxhlet apparatus, may have also had an effect of the phytochemical content of the extracts. Other studies used higher amounts of herbs samples for extraction. In the study done by Yu *et al.* (2009) on the volatile components of *Citrus Reticulata Blanco*, 20.0 g of herb sample was used in the extraction of volatile oils with 200 mL of distilled water while Reuben *et al.* (2008) used 400 g of pulverized stem bark of *Croton zambesicus* to obtain 52.02 g of methanol extract.

According to Jadhav *et al.*'s (2009) study, the relative proportion of the solvent to the quantity of sample used in the extraction process may have also had an effect on the phytochemical content in the final extract. Yu *et al.*'s (2009) study used a minimum relative proportion of extraction of the solvent (20.0 g of herb samples to 200 mL of solvent) while a maximum relative proportion of the solvent was used in this study (10.0 g of herb samples to 350 mL of solvent). The rate of extraction for the maximum relative proportion of solvent should follow a linear path with time of operation while the rate of extraction for the minimum relative proportion of the solvent should decrease with

extended extraction time. However, it should be noted that the extraction time for Yu *et al.*'s study was for 7 h (high yield) while the extraction time was only 4 h (low yield) for this study.

In this study, it is possible that the phytochemical content in the crude herbs used for extraction had been significantly reduced by the factors previously outlined, resulting in reduced yield in the extracts obtained and subsequently reflected in the majority of negative results seen during phytochemical testing. The screening test could not be repeated as there was insufficient extracts (especially for the n-hexane extract of *Spica prunella*) for the preparation of another set of stock solutions.

CONCLUSION

From this study, it was found that methanol had the highest percentage yield by weight amongst the solvents used for extraction of the phytochemicals present in Pericarpium *Citri reticulatae* and *Spica prunella.*

The information obtained from the preliminary phytochemical screening tests may be supported by other spectroscopic (FTIR, UV-Vis, NMR, MS), chromatographic (TLC, GC, HPLC, GC-MS and HPLC-MS) and related techniques. Although, in the present work, qualitative tests carried out for different classes of compounds were not positive, the results of FTIR, UV-Vis and HPLC revealed the presence of several phytochemicals.

Based on this study, it is concluded that the distilled water extract of Pericarpium *Citri reticulatae* contains saponins and possibly flavonoids; the distilled water extract of *Spica prunella* contains saponins; and methanol extract of *Spica prunella* contains free acids and possibly flavonoids and tannins.

After preliminary phytochemical screening tests have been performed, fractionation and purification procedures can be conducted in order to isolate the active principle. It should also be noted that some compounds are inactive in situ but act synergistically with other constituents of the extract and separation into different fractions during purification will thus result in a decrease or total loss in activity in all the fractions.

REFERENCES

Ahmad, F. and Raji, H. (1993). Naturally produced chemicals and medicinal plants, Dewan Bahasa dan Pustaka Publications, Kuala Lumpur.

Ahn, S. C., Oh, W. K., Kim, B. Y., Kang, D. O., Kim, M. S., Heo, G. Y. and Ahn, J. S. (2003). Inhibitory effects of rosmarinic acid on Lck SH2 domain binding to a synthetic phosphopeptide. *Planta Medica,* **69**, 642-646.

Brindley, M. A., Widrlechner, M. P., McCoy, J. A., Murphy, P., Hauck, C., Rizshsky, L., Nikolau, B. and Maury, W. (2009). Inhibition of lentivirus replication by aqueous extracts of Prunella vulgaris. *Virology Journal,* **6**, 1-13.

Castro, L. d. and Garcia-Ayuso (1998). Soxhlet extraction of solid materials: An outdated technique with a promising innovative future. *Analytica Chimica Acta,* **369**, 1-10.

Chiu, L. C., Zhu, W. and Ooi, V. E. (2004). A polysaccharide fraction from medicinal herb *Prunella vulgaris* downregulates the expression of herpes simplex virus antigen in Vero cells. *Journal of Ethnopharmacology,* **93**, 63-68.

Couzinier, J. P. and Mamatas, S. (1986). Basic and applied research in the pharmaceutical industry into natural substances. In: Advances of the international symposium on medical phytochemistry (Eds, Barton, D. and Ollis, W. D.) John Libbey & Co, London.

Dong, K. S., Wang, X. Q. and Dong, Y. F. (1998). Contemporary clinical chinese materia medica, Zhong Guo Zhong Yi Yao Press, Beijing.

Ernst, E. (1998). Harmless herbs? A review of the recent literature. *American Journal of Medicine,* **140**, 170-178.

El-Shazly, A., Doraia, G. and Wink, M. (2002). Composition and antimicrobial activity of essential oil and hexane-ether extract of Tanacetum santolinoides (DC.) Feinbr. and Fertig. *Zeitschrift für Naturforschung,* **57**, 620-623.

Farnsworth, N. R. (1993). Biological approaches to the screening and evaluation of natural products. In: Biological evaluation of plants with reference to the Malagasy flora (Eds, P. Rasoanaivo and Ratsimamanga-Urverg, S.) International Foundation for Science, Madagascar.

Garcia-Ayuso and Castro, L. D. (1999). A multivariate study of the performance of a microwave-assisted Soxhlet extractor for olive seeds. *Analytica Chimica Acta* **382**, 309-316.

Geng, J. Y., Huang, W. Q., Ren, T. C. and Ma, X. F. (1997). Practical traditional chinese medicine & pharmacology: Medicinal herbs, 2nd Edn. New World Press, Beijing.

Gua, X. J., Li, Y. B., Li, P., Qian, S. H. and Zhang, J. F. (2007). Triterpenoid saponins from the spikes of *Prunella vulgaris*. *Helvetica Chimica Acta,* **90**, 72-78.

Hanmoungjai, P., Pyle, L. and Niranjan, K. (2000). Extraction of rice bran oil using aqueous media. *Journal of Chemical Technology and Biotechnology,* **75**, 348-352.

Harput, U. S., Saracoglu, I. and Ogihara, Y. (2006). Effects of two *Prunella* species on lymphocyte proliferation and nitric oxide production. *Phytotherapy Research,* **20**, 157-159.

Hou, J. P. and Jin, Y. (2005). The Healing Power of Chinese Herbs and Medicinal Recipes, 1st Edn. Haworth Press, Inc, New York.

Israelsen, L. D. (1993). Phytomedicines as a new crop opportunity. In: New Crops (Eds, Janick, J. and Simon, J. E.) Wiley, New York.

Jadhav, D., Rekha, B. N., Gogate, P. R. and Rathod, V. K. (2009). Extraction of vanillin from vanilla pods: A comparison study of conventional Soxhlet and ultrasound assisted extraction. *Journal of Food Engineering,* **93**, 421-426.

Kamil, M. (1993). Biological and pharmacological implications of flavonoids – A review. In: Medicinal plants: New vistas of research, part 2 (Eds, Govil, J. N., Singh, V. K. and Hashmi, S.) Today and Tomorrow's Printers and Publishers, New Delhi.

Kumar, A., Ilavarasan, R., Jayachandran, T., Decaraman, M., Aravindhan, P., Padmanabhan, N. and Krishnan, M. R. V. (2009). Phytochemical investigation on a tropical plant, Syzygium cumini from Kattuppalayam, Erode district, Tamil Nadu, South India. *Pakistan Journal of Nutrition,* **8**, 83-85.

Lewis, W. H. and Elvin-Lewis, M. P. F. (2003). Medical botany: Plants affecting human health, 2nd Edn. John Wiley & Sons, Inc., New Jersey.

Li, H., Pordesimo, L. and Weiss, J. (2004). High intensity ultrasound-assisted extraction of oil from soybeans. *Food Research International,* **37**, 731-738.

Ling, Y. (1995). A new compendium of materia medica, Science Press, Beijing.

Ma, Y., Ye, X., Hao, Y., Xu, G., Xu, G. and Liu, D. (2008). Ultrasound-assisted extraction of hesperidin from Penggan (Citrus reticulata) peel. *Ultrasonics Sonochemistry,* **15**, 227-232.

Mamidipally, P. K. and Liu, S. X. (2004). First approach on rice bran oil extraction using limonene. *European Journal of Lipid Science and Technology,* **106**, 122-125.

Mishra, A. K., Mishra, A., Kehri, H. K., Sharma, B. and Pandey, A. K. (2009). Inhibitory activity of Indian spice plant *Cinnamomum zeylanicum* extracts against *Alternaria solani* and *Curvularia lunata*, the pathogenic dematiaceous moulds. *Annals of Clinical Microbiology and Antimicrobials*, **8**, 9.

O'Neill, M. J. and Lewis, J. A. (1993). The renaissance of plant research in the pharmaceutical industry. In: Human medicinal agents from plants, ACS Symposium Series 534 (Eds, Kinghorn, A. D. and Balandrin, M. F.). American Chemical Society, Washington.

Okunlola, A., Adewoyin, B. A. and Odeku, O. A. (2007). Evaluation of pharmaceutical and microbial qualities of some herbal medicinal products in South Western Nigeria. *Tropical Journal of Pharmaceutical Research*, **6**, 661-670.

Pavia, D. L., Lampman, G. M., Kriz, G. S. and Engel, R. G. (2002). Microscale and macroscale techniques in the organic laboratory, Harcourt College Publishers, Washington.

Psotová, J., Kolá, M., SouSek, J., Ívagera, Z., ViCar, J. and Ulrichová, J. (2003). Biological activities of *Prunella vulgaris* extract. *Phytotherapy Research*, **17**, 1082-1087.

Reuben, K. D., Rahman, F. I. A., Akan, J. C., Usman, H., Sodipo, O. A. and Egwu, G. O. (2008). Phytochemical screening and in vitro antimicrobial investigation of the methanolic extract of *Croton Zambesicus* Muell ARG. stem bark. *European Journal of Scientific Research*, **23**, 134-140.

Ryu, S. Y., Oak, M. H., Yoon, S. K., Cho, D. I., Yoo, G. S., Kim, T. S. and Kim, K. M. (2000). Anti-allergic and anti-inflammatory triterpenes from the herb of *Prunella vulgaris*. *Planta Medica*, **66**, 358-360.

Sheu, F., Chuang, W. I. and Chien, P. J. (2007). *Citri Reticulatae* Pericarpium extract suppresses adipogenesis in 3T3-L1 preadipocytes. *Journal of the Science of Food and Agriculture,* **87**, 2382-2389.

Shi, Q., Liu, Z., Yang, Y., Geng, P., Zhu, Y. Y., Zhang, Q., Bai, F. and Bai, G. (2009). Identification of anti-asthmatic compounds in Pericarpium *Citri reticulatae* and evaluation of their synergistic effects. *Acta pharmacologica Sinica,* **30**, 567-75.

Shokrollah, H., Abdullah, T. L., Sijam, K., Abdullah, S. N. A. and Abdullah, N. A. P. (2009). Differential reaction of Citrus species in Malaysia to Huanglongbing (HLB) disease using grafting method. *American Journal of Agricultural and Biological Sciences,* **4**, 32-38.

Smith, F. P. and Stuart, G. A. (1973) Chinese medicinal herbs, Georgetown Press, San Francisco.

Su, M. S., Shyu, Y. T. and Chien, P. J. (2008). Antioxidant activities of citrus herbal product extracts. *Food Chemistry,* **111**, 892-896.

Sun, H. X., Qin, F. and Pan, Y. J. (2005). *In vitro* and *in vivo* immunosuppressive activity of *Spica Prunellae* ethanol extract on the immune responses in mice. *Journal of Ethnopharmacology,* **101**, 31-36.

Taylor, J. L. S., Rabe, T., McGaw, L. J., Jäger, A. K. and Staden, J. v. (2000). Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation,* **34**, 23-37.

Tyler, V. E. (1993). Phytomedicines in Western Europe: Potential impact on herbal medicines in the United States. In: Human medicinal agents from plants (Eds, Kinghorn, A. D. and Balandrin, M. F.) ACS Symposium Series, Washington.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernandez-Lopez, J. and Perez-Alvarez, J. (2008). Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. *Food Control*, **19**, 1130-1138.

Wang, L. and Weller, C. L. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology*, **17**, 300-312.

Wheelwright, E. G. (1974). Medicinal plants and their history, 1st Edn. Houghton Mifflin Company, New York.

World Health Organization (1997). Medical plants in China, 2nd Edn. World Health Organization, Manila.

Wyk, B. v. and Wink, M. (2004) Medicinal plants of the world, 1st Edn. Briza Publications, Pretoria.

Yi, L. Z., Yuan, D. L., Liang, Y. Z., Xie, P. S. and Zhao, Y. (2007). Quality control and discrimination of Pericarpium *Citri reticulatae* and Pericarpium *Citri reticulatae Viride* based on high-performance liquid chromatographic fingerprints and multivariate statistical analysis. *Analytica Chimica Acta*, **588**, 207-215.

Yi, Z. B., Yu, Y., Liang, Y. Z. and Zeng, B. (2008). *In vitro* antioxidant and antimicrobial activities of the extract of Pericarpium *Citri reticulatae* of a new Citrus cultivar and its main flavonoids. *LWT - Food Science and Technology*, **41**, 597-603.

Yu, L., Li, X., Liu, S., Xu, G. and Liang, Y. (2009). Comparative analysis of volatile constituents in *Citrus reticulata* Blanco using GC–MS and alternative moving window factor analysis. *Journal of Separation Science*, **32**, 3457-3465.

Zarnowski, R. and Suzuki, Y. (2004). Expedient Soxhlet extraction of resorcinolic lipids from wheat grains. *Journal of Food Composition and Analysis,* **17**, 649-664.

Zheng, G., Yang, D., Wang, D., Zhou, F., Yang, X. and Jiang, L. (2009). Simultaneous determination of five bioactive flavonoids in Pericarpium *Citri reticulatae* from China by high-performance liquid chromatography with dual wavelength detection. *Journal of Agricultural and Food Chemistry,* **57**, 6552-6557.

Zheng, J., He, J., Ji, B., Li, Y. and Zhang, X. (2007). Antihyperglycemic activity of *Prunella vulgaris* L. in streptozotocin-induced diabetic mice. *Asia Pacific Journal of Clinical Nutrition,* **16**, 427-431.

Zhou, X. M., Huang, M. M., He, C. C. and Li, J. X. (2009). Inhibitory effects of citrus extracts on the experimental pulmonary fibrosis. *Journal of Ethnopharmacology.* (DOI: 10.1016/j.jep.2009.07.025)

Zhu, Y. (1998). Chinese materia medica - Chemistry, pharmacology and applications, Harwood Academic Publishers, Amsterdam.

Zhu, Y. P. (2003). Xia Ku Cao effective for cold sores. In: Chinese medicine legislation forum (Ed, Youping, Z.) Pan European Federation of TCM Societies, London.