INVESTIGATIONS INTO THE EFFECTS OF TRADITIONAL CHINESE MEDICINAL HERBS USED IN THE TREATMENT OF HUMAN BREAST CANCER

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LIST OF ABBREVIATIONS

ABTS: 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

AM: *Astragalus membranaceus*

BALB: Bagg albino

BRCA1: breast cancer gene 2

BRCA2: breast cancer gene 1

BrdU: 5-bromo-2-deoxyuridine

BW: body weight

CA: *Curcuma aromatica*

ConA: Concanavalin A

DMSO: Dimethyl sulphoside

DOX: Doxorubicin

EDTA: Ethylenediaminetetraacetic acid

EORTC: European Organization of Research and Treatment of Cancer

EGF: epidermal growth factor

FBS: fetal bovine serum

5-FU: 5-fluorouracil

HPLC: high performance liquid chromatography

IC\textsubscript{50}: 50\% inhibition concentration

IL-2: interleukin-2
IL-6: interleukin-6

i.p.: intraperitoneal

LPS: Lipopolysaccharide

6-MP: 6-mercaptopurine

MTP: micro titer plate

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Thiazol blue)

NSAID: non-steroidal anti-inflammatory drug

O.D.: optical density

PBS: Phosphate-buffered saline

QOL: quality-of-life

QLQ: quality-of-life questionnaire

QLQ-C30: quality-of-life core questionnaire

QLQ-BR23: quality-of-life breast cancer supplementary questionnaire

ROS: reactive oxygen species

RS: Raw Score

S: score

s.c.: subcutaneous

SB: Scutellaria barbata

SDS: Dodecyl-sulphate sodium salt

SN: Solanum nigrum
TCM: traditional Chinese medicine

TNF-\(\alpha\): tumor necrosis alpha

WHO IARC: World Health Organization, International Agency for Research on Cancer
LIST OF PUBLICATIONS


LIST OF CONFERENCE PRESENTATIONS


6. Slater A, Tan BKH: Immunomodulating property of *Astragalus membranaceus* and *Curcuma aromatica* in breast cancer patients. 2nd International Conference on Natural Products, Singapore 1-4 July 2002
SUMMARY

The incidence of breast cancer is the highest among Singaporean women, with more than 1000 new cases diagnosed each year. There is an urgent need for new forms of treatment i.e. complementary/alternative medicine (CAM). Traditional Chinese Medicine (TCM) is the most popular type of CAM for the treatment of cancer among the Chinese population in Singapore. In most cases it is used to complement conventional treatment (surgery, radio-, chemo- and/or hormonal therapy).

We sought a scientific evaluation of the medicinal herbs that might have immunomodulatory and/or anti-tumor effects and could palliate the side-effects (nausea, insomnia, pain and fatigue) of chemo- and radiotherapy.

The investigation was carried out in two phases. In the clinical phase of our study, a quality-of-life (QOL) analysis of completed questionnaires from 6 patients was performed using the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QLQ-C30 version 3.0) and the supplementary breast cancer module (QLQ-BR23). We compared the herbal prescription of the patients who had better scores on the symptoms scale (i.e. less fatigue, pain, dyspnoea, insomnia and appetite loss).

In the laboratory phase, we selected the 2 herbs, *Astragalus membranaceus* (AM) and *Curcuma aromatica* (CA) that we found to be the most frequently prescribed for their possible immunomodulatory effect. We tested these herbs for their effects on lymphocyte activation and cytokine (IL-2, IL-6, TNF-α) production.

We also selected *Solanum nigrum* (SN) which was used for its cytotoxic property against breast cancer. We evaluated the tumor cell growth inhibitory effect of the
crude ethanolic extract of SN on MCF-7, T-47D, ZR-75 and MDA-MB -231 human breast cancer cell lines. The lowest ED$_{50}$=7.75 µg/ml was obtained with the estrogen-dependent MCF-7 cell line. We also demonstrated apoptosis as the cause of cell death, both by ELISA and histopathology.

Experiments were also conducted in oophorectomized nude mice models with breast tumor implants. The anti-tumor activity of SN was assumed in these animals by measuring tumor growth and life span prolongation as parameters. We found that SN treatment caused irreversible tumor regression where the tumor size was relatively small (MCF-7 and MDA-MB-231) or when it was given simultaneously with the tumor implant. SN 300 mg/kg BW was effective against small volume tumors but not efficient against larger, multiple tumors. SN 500 mg/kg BW could be used as prevention (producing a prophylactic rate of 75%) but was not an efficient dose of invasive tumors. Mice bearing human breast tumors treated with SN maintained their original body weight without any change in behavior.

This study suggests there is potential usefulness in using herbal TCM to treat breast cancer patients.
CHAPTER ONE: GENERAL INTRODUCTION
1.1 Traditional Chinese Medicine (TCM)

Complementary and alternative medicine (CAM) is a booming healthcare industry in Europe and U.S.A. and is used by 25%-69% of the general population of developed countries (Richardson et al., 2000). CAM has been defined as “diagnosis, treatment and/or prevention which complements mainstream medicine by contributing to a common whole, by satisfying a demand not met by orthodoxy or by diversifying the conceptual frameworks of medicine” (Ernst et al., 1995). It has also been described as “medical and healthcare practices outside the realm of conventional medicine, which are yet to be validated using scientific methods” (National Center for Complementary and Alternative Medicine, Strategic Plan, 2000).

In South-East Asia, the most frequently used CAM interventions are Traditional Chinese Medicine (TCM) and Ayurvedic Medicine systems (Dobs, 2001). TCM includes different treatment modalities such as herbal medicine, acupuncture, moxibustion, oriental massage, qi-gong and diet. The practice of herbal TCM (“phytotherapy”) has been defined as “the use of products derived from plants to treat diseases or eliminate pain” (The Cochrane Collaboration, 1993).

In Singapore, acupuncture and herbal TCM are the most popular forms of alternative medicine due to the majority of the population being ethnic Chinese (personal communication with Dr. Teo Eng Kiat; 2001), principal of the Singapore College of TCM and Chairman, TCM Practitioners Board Examination Committee, Singapore. Singaporeans of Chinese descent, who comprise 76.8% of the total population (Singapore Department of Statistics, 2000), have used TCM for many decades, and tend to turn to this form of treatment for a variety of diseases, from simple colds to advanced cancer (Ho et al., 1980).
According to Dr. Teo, as many as 40% of the Singapore population seek TCM treatment as the primary form of treatment. Statistics compiled in 1994 indicate that an estimated 12% of the daily outpatient attendance of about 10,000 people was seen by TCM practitioners (Hsu et al., 1995).

With the recent legislation requiring all TCM practitioners in Singapore to upgrade their skills and knowledge to obtain certification to practice by the local health regulatory authority, the percentage of TCM users is likely to increase in the coming years (Health Science Authorities, Singapore, 2002).

One important reason for the choice of TCM is economic - it is considered a more affordable form of health care, compared to the more sophisticated form of Western medicine. Some of the clinics in Singapore, like the Chung Hwa Free Clinic, offer free consultation and treatment to cancer sufferers. Some of the patients who seek TCM treatment do so because they believe it has less side effects and toxicity, and can improve their immune system. Other patients have incurable end-stage disease (e.g. chronic renal failure, liver cirrhosis, cancer), where conventional treatment has failed to cure, and TCM is tried as a last choice treatment to relieve their symptoms and prolong life (Ernst et al., 1998; Richardson et al., 2000).

Some cancer patients in Singapore do take TCM at some stage of their illness. Most of these patients do not disclose this to their physicians as they fear losing their doctor’s trust or being discriminated against. Thus primary care physicians usually underestimate their patients’ use of complementary medicine. In USA, 72% of the patients fail to report to their physicians the use of CAM. In Israel, one study showed that only 18.7% of CAM-using patients reported having ever used CAM (Eisenberg et al., 1993; Kitai et al., 1998; Giveon et al., 2003). A similar problem was reported in
Canada, where only 46.4% of breast cancer patients reported their use of CAM to their physician (Boon et al., 2000).

The current approach of physicians to complementary medicine in primary care is essentially “don’t ask, don’t tell” (Perlman et al., 1999).

There is a generally low acceptance of herbal TCM by oncologists and other conventional medical practitioners here and elsewhere. Their main concerns are that herbal medicine might be contaminated with high levels of toxic heavy metals, such as mercury or lead (Wong et al., 1985) and could cause direct adverse effects such as allergic reactions, nausea, vomiting and sedation (Ernst et al., 1996; Shaw et al., 1997). There is also concern that TCM treatment could indirectly interfere with the effects of radio- or chemotherapy, delaying the recovery of patients and putting them at unnecessary risk (Brown, 1986; Kaegi, 1998). There is an urgent need that the health care practitioners understand the CAM products their patients are using and are familiar with the information about the safety and efficacy of these herbal products (Smith et al., 1999).

Western medicine is based on an understanding of anatomical structures or organs and the cause of disease is viewed as the pathological function of these organs – a diseased structure causes dysfunction of the body. In contrast, TCM is based on the “system theory” and the human body is viewed as a part of a complex structure of organs – liver, heart, spleen, lung and kidney (these do not correspond to the exact anatomical structures known in Western medicine). This system undergoes spatial, functional and temporal changes and allows open exchange of substance and energy with the environment and interaction/ interrelation between the organs/ compartments in the body. The main substance of energy transfer is qi which can’t be seen but can be detected by modern technology (Seto et al., 1992). The imbalance in the fluctuation of
internal and external energy and a weakened self-regulatory power can cause disease. This is a chain reaction of chemical, physiological and biological changes. In TCM, the etiology of a disease is attributed to the malfunction of the system which causes a change in the organs – the emphasis is based on the function/ dysfunction of an organ which triggers pathological structural changes in the organ. The main principle of TCM treatment is to cure this deviation by eliminating the pathological factors and by strengthening the self healing (Cheng, 2005).

Appropriate application of dosages of Chinese herbs based on differentiation of symptoms and signs is important in order to achieve better therapeutic results with less adverse effects. The diagnosis is not disease- but patient-specific. The TCM physicians place great emphasis on the severity of disease, the form/ formula/ dosage of the herbs, gender and age of the patients. Different patients may have different reactions to the same herbs and the same formulae with different dosage/ amounts/ proportions of Chinese herbs may have different functions and indications, thus making the treatment individually tailored (Deng, 2002). This also makes TCM-related clinical trial designs very challenging.

1.2 Herbs used in TCM for cancer treatment

In TCM, cancer is viewed as the result of imbalance of the whole body-mind network by taking into account the human body as whole. The tumor is due to stagnation or accumulation of pathological elements (\textit{qi}, blood, phlegm or body fluids). This functional disorder leads to structural changes (tumor growth) when the body loses its capacity for recovery. The aim of herbal treatment is to strengthen and restore the \textit{yin} and \textit{yang} balance, the \textit{Five Organs System}, the \textit{external} and \textit{internal factors} in order to cure the cancer. In conventional medicine, the therapeutic aim is to destroy the cancer
cells and prevent their spread by applying chemo-, radio, hormonal and surgical treatments.

There is emerging evidence that TCM can play an important role in the supportive treatment of cancer patients. Though the way cancer and its treatment is viewed in Western (disease-specific) and Chinese medicine (patient-specific) is very different, the holistic approach of TCM could possibly be integrated into conventional Western medicine to palliate the side-effects of the latter (Wong et al., 2001).

Plants have been used as medicinal agents for centuries, and they continue to play an important role in primary health care. More than 50% of all drugs in clinical use are derivatives of natural products and 12 of the world’s 25 best-selling pharmaceuticals originate from plants (Baker et al., 1995).

Natural product extracts like *Aloe vera* (to treat burns and skin abrasions), *Echinacea* (to boost the immune system and fight common colds), *Allium sativum* (to lower high cholesterol levels), *Ginger* (for immunostimulation and inhibition of cancer metastasis), *Ginkgo biloba* (antioxidant, for improving microcirculation in the brain, reducing tendency for blood clot formation), *Glucosamine* (to rebuild and maintain worn cartilage), *Artemisia annua* (for treating fever and drug resistant malaria), *Green tea* (antioxidant), *Lingzhi* (to stimulate the immune system, anticancer properties), *Royal jelly* (high vitamin, amino acid and mineral content), *Vitex Agnus castus* (to balance estrogen-progesterone level), *Lavandula augustifolia* (anti-inflammatory), *Pinus sylvestris* (adrenal cortex stimulant) and *Tanacetum annum* (anti-histamine) are popular health supplements (Dewick, 1999; Effert, 2005; Schnaubelt, 2005).

The most well-known anti-cancer drugs derived from natural products are the *Vinca alkaloids* (Vinscristine, Vinblastin, Vindesin), *Epipodophyllotoxins* (Etoposide),
**Taxanes** (Paclitaxel, Docetaxel), **Camptothecins** (Topotecan, Irinotecan), and the antibiotics (Doxorubicin, Mitomycin, Bleomycin) [Rang et al., 2003].

According to TCM, herbal treatment can eliminate the disease indirectly by boosting the host immune system (immunostimulation) and directly by eliminating the disease (cytotoxicity). The herbs prescribed for tumors are usually (a) tonifying herbs which act on multiple organ systems and stimulate the immune system and (b) tumor-dispersing herbs which eliminate the pathological external factors (i.e. stress, unhealthy diet and lifestyle).

The guiding principles in TCM treatment of tumors are: “supplementing qi and nourishing blood” (in biomedical terms: to stimulate the bone marrow and increase the white blood cell count); “dispersing the lucid and sending down the turbid” (to protect renal function, decrease serum urea nitrogen and creatinine level); relieving abdominal distention (due to accumulation of gas from long-term bed rest), “regulating the flow of qi”, (removing blood stasis to alleviate the pain and reduce fever due to infections)[Zhou et al., 2001].

The specific aim of herbal TCM is to slow disease progression by modulating the neuro-endocrine and immune systems, to inhibit the tumor growth and to improve the quality of life of the patients. Different herbs with different disease fighting pattern are used simultaneously, assuming that groups of chemicals in these herbs produce a cluster of actions on various cells, cellular organs and multiple receptors, thus achieving a more efficient cure. In TCM, these herbs are used for their organ system properties (the way they can rectify the functional and structural deviation in the body) and not for their chemical properties. In Western medicine usually a single chemical compound is used to act on a single cell type and receptor, however (especially with chemotherapeutic drugs) the physicians tend to use multi-drug treatments to palliate the side-effects of the
anticancer drugs. TCM takes into consideration the property of a whole herb thus using multiple chemical compounds which work synergistically on multiple systems, receptors and enzymes (Swee, 2005). For example, a mixture of TCM herbs for breast cancer patients would contain herbs which aim not only to shrink the tumor itself but also palliate insomnia and anxiety, enhance appetite and boost the immune system, thus having an integrated effect on the different symptoms of a breast cancer patient.

Chinese herbs have been reported to have the effect of blocking precancerous lesions by removing carcinogenic factors and by chemoprophylaxis, as well as slow the development of tumors by inhibiting cancer cell infiltration into the surrounding tissue and thus reduce metastasis (Wu et al., 2001; Niu et al., 2003).

Treatment with CAM interventions could also reduce the oxidative stress in cancer cells (Nelson et al., 2001).

At the cellular level, the tumor-reversing effects of Chinese herbs could occur either by inducing early stage apoptosis of tumor cells or by repressing proliferation and causing arrest of cancer cells at the G₀/G₁ stage (Yano et al., 1994).

The use of TCM is very much embedded in Chinese culture both in Singapore and Hong Kong. As many breast cancer patients believe that diet is responsible for their cancer, food, diet and herbs should also play an important role in the management of their illness (Simpson, 2003).

In Singapore, herbal TCM is the most popular type of alternative medicine. Most of the patients seek TCM treatment because they believe it has less side-effects and toxicity, and can enhance their immune system.

In the breast cancer patients we studied, herbal TCM was used to complement conventional treatment (surgery, radio-, chemo- and hormonal therapy). We sought a
scientific evaluation of the immunomodulatory effects of some Chinese herbs used widely in breast cancer patients at two TCM Clinics that we surveyed.

While the patients in this study had the same diagnosis (breast cancer), their diagnosis according to TCM principles could be very different and variable at each visit (stagnation of qi, blood, phlegm, body fluids; dampness type; heat type etc).

Each TCM practitioner has his/her preference for the type of herb(s) to prescribe, whether raw herbs or processed mixtures of herbs. It is also very challenging for the practitioner to standardize the prescriptions since it is given individually for every patient depending on his/her medical condition.

The most commonly prescribed TCM herbs for our six breast cancer patients were: herbs to tonify the qi - Codonopsis pilosula, Astragalus membranaceus, Atractylodes macrocephala, Ziziphus jujuba (red date), Lycium barbarum (wolfberry fruit); herb to invigorate the blood - Curcuma aromatica; herb to relieve food stagnation – Crataegus pinnatifida; herbs to drain dampness - Poria cocos, Coix lacryma Jobi (Job’s tears seed); herbs to clear heat and relieve toxicity- Scutellaria barbata, Solanum nigrum (Reid, 1993; Keys, 1997).

Herbs that tonify qi decrease lethargy, fatigue, lack of appetite, abdominal pain, diarrhea, dyspnoea, edema and stimulate the body and the sympathetic nervous system. Herbs that invigorate the blood alleviate pain, while those which relieve food stagnation stimulate digestion and increase gastrointestinal secretion. Herbs which drain dampness are diuretics and have tranquilizing effect, while those which clear heat and relieve toxicity have anti-inflammatory, anti-infectious and diuretic effects. The combination of these herbs might explain their efficacy in improving the quality-of-life (QOL) in cancer patients (Read, 1977).
The effects of the prescription herbs initially were evaluated through a QOL analysis using the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QLQ-C30 version 3.0) and the supplementary breast cancer module (QLQ-BR23). We compared the herbal prescriptions of the patients who had better scores on the symptoms scale (i.e. less fatigue, pain, dyspnoea, insomnia and appetite loss) and selected the 6 most frequently used herbs: *Astragalus membranaceus* (AM), *Curcuma aromatica* (CA), *Solanum nigrum* (SN), *Scutellaria barbata* (SB) for studies on their immunomodulatory and cytotoxic effect. *Crataegus pinnatifida* (CP) and *Ziziphus jujube* (ZJ) were well documented in the literature for their appetite enhancer and anxiolytic effect respectively, thus in our research project we did not conduct biomedical experiments on these 2 herbs.
Picture 1. Dried root of *Astragalus membranaceus*
*Astragalus membranaceus* L. (AM) (Huang Qi, Mongolian Milk Vetch,) is native to China, Korea and Japan. It contains glycosides (astragalosides I, II, III, IV, V, VI, VII) [Hirotani et al., 1994], isoflavonoids (Wu et al., 2005; Zhang et al., 2005), polysaccharides (astroglucan A, B and C), more than forty types of saponins (Yang et al., 2005), vitamin A, betaine, beta-sitosterol (Kim et al.2003), hexuroic acid, rumatakenin and sugars (He et al., 1990). In TCM, AM has been used to correct the deficiency of *qi*, repair and tonify emptiness. It is used as a diuretic, a cardiotonic and to relief fatigue hypotension (Wu et al., 2000; Fetrow et al., 2001).

In Western medicine, it has been reported that the saponins and polysaccharide compounds found in the *Astragalus* root support and stimulate the cellular immune system, strengthen the natural defence mechanism by enhancing antibody production through increased T helper cell activity (Yoshida et al., 1997; Shan et al., 1999; Lee et al., 2005). It was reported that Astragaloside VII is a potent IL-2 inducer thus being a strong immunostimulator (Yesilada et al., 2005). It was shown that the extract of AM stem and leaves could promote lymphocyte proliferation, elevate T cell count and enhance IL-2-induced LAK activity thus having antineoplastic effect (Wang et al., 1992; Jiao et al., 1999). It was also reported that AM is effective in treating Coxsackie B virus induced myocardial muscle injury (Yang et al., 1990; Yuan et al., 1990). Used in combination with other herbs, it could reduce serum virus count in some AIDS patients (Chang et al., 1988; Yao et al., 1992). The saponins have been found to have anti-diabetic (Li et al., 2004), neuro-protective (Luo et al., 2004), cardio-protective (Zhang et al., 2005), anti-inflammatory (Zhang et al., 2003), diuretic and antihypertensive properties as well (Yang et al., 2005).
Picture 2. Dried roots of *Curcuma aromatica*
Curcuma aromatica L. (CA) (Yu Qin, Wild Turmeric) is a member of the Zingiberaceae family, like ginger and cardamon. It grows in tropical countries like Samoa, Tonga, Jamaica, Peru, Brazil, Jordan, India, Indonesia and South-China (Govindarajan, 1980). It contains, among others, curcumin, curcumenol, tumerone, atlantone, diaryl heptanoids, zingiberone, sugars, resins, proteins, different vitamins and minerals (Kojima et al., 1998; Navarro et al., 2002; Xia et al., 2005; Yang et al., 2005).

It is known to be an energy regulator and is used in energy stagnant illnesses. CA is a mass-reducing herb and has been used to remove the stagnation of qi, reduce inflammation, chest pain and colic (Bensky et al., 1986; Reid, 1993; Dewick, 1997; Dharmananda 1997; Fetrow et al., 2001; Park et al., 2004; Jayaprakasha et al., 2005).

In Western medicine, it has been reported to enhance fibrinolysis (Srivastava et al., 1989), to treat atherosclerosis (Ashraf et al., 2005), pain (Navarro et al., 2002), cholelithiasis (Niederau et al., 1999), irritable bowel syndrome (Brinkhaus et al., 2005), bacterial and fungal infections (Grosvernor et al., 1995). It has been shown to be useful in the prevention and treatment of breast cancer (Inano et al., 2000), colorectal cancer (Goel et al., 2001), skin (Huang et al., 1997) and liver cancer (Cheng et al., 2001).

Curcumin, a potent bioactive compound in CA, was shown to have anti-HIV activity (Mazumder et al., 1995) to inhibit carcinogenesis by being a strong antioxidant (Selvam et al., 1995; Abas et al., 2006) and to induce apoptosis in cancer cells without cytotoxic effect on healthy cells (Duvoix et al., 2005). Curcuminoids are effective by inhibiting leukotriene biosynthesis, reducing prostaglandin formation thus having a potential anti-inflammatory and anticoagulant effect (Selvam et al., 2005).
Picture 3. Reconstruction of *Solanum nigrum* (authenticated Dr. Ruth Kiew and stored as a voucher specimen BT4, Herbarium, Singapore Botanical gardens by)
**Solanum nigrum L** (SN) (Black nightshade) is a common herb that grows wildly and can be found worldwide. SN has been used in many different countries (China, India, Israel, Turkey, Madagascar, Lesotho, Tanzania, Mexico, Brazil, Hawaii, Cook Islands) as traditional folk medicine and for its various biological activities (Dafni et al., 1994; Ankli et al., 2002). It belongs to the *Solanaceae* family and contains, depending on the parts of the plant (stem, leaves, fruit), steroidal glycosides (α and β2-solamargine, solamargine, degalactotigonin), steroidal alkaloids (solasodine, solanidine), flavonols, sitosterol, stigmasterol (Dewick, 1997; Keys, 1997; Hu et al., 1999; Fetrow et al., 2001; Zhao, 2004). There is a lot of debate over whether or not the leaves or fruit of SN are poisonous and carcinogenic. However, in some countries it is consumed as a vegetable (Purchase et al., 1975; Blankmeyer et al., 1998, Sammon, 1992). It was reported that the unripe fruit contains the highest concentration of solasodine which is a cytotoxic substance for cancer cells. It is active against anaphylactic and hypoglycaemic shocks, and shocks resulting from burns (Eltayeb et al., 1997; Latoxan, 2005). It was shown that SN and some of its compounds has antitumor (Chiang et al., 1991; Yen et al., 2001; Son et al., 2003; Cai et al., 2004), antifungal (Locher et al., 1995), cholesterol-lowering (Lee et al., 2005), antipyretic and diuretic (Bensky et al., 1986) effects. It is also used to treat inflammation, edema, mastitis (Sultana et al., 1995), liver complaints (Goodman et al., 1988), neurological conditions (Perez et al., 1998) and for cytoprotection in drug induced kidney damage (Kumar et al., 2001). The steroidal glycosides from *Solanaceae* species can be used to treat herpes simplex and skin cancer (Cham et al., 1987; Bensky et al., 1986; Ikeda et al., 2000). It has also been reported that solamargine together with cisplatin was effective in treating lung and liver cancer (Kuo et al., 2001).
Scutellaria barbata (SB) (Skullcap) contains flavonoids (scutallarein, scutellarin), sesquiterpenes, wogonin, lignin, resin etc. It is known to have anti-inflammatory, anticonvulsant, sedative and cardioprotective action (Fetrow et al., 2001). It was reported that SB induces apoptosis in human leukemia cells (Cha et al., 2004) and has anti-cancer/ cytotoxic activity in lung and ovarian cancer (Yin et al., 2004; Powell et al., 2003).

Ziziphus jujuba (ZJ) (Spina Date Seed) is rich in phytoestrogens, which are known to have anxiolytic activity and increase appetite (Lund et al., 2001). It is used in TCM treatment of insomnia, neurasthenia and palpitation (Zhao, 2004).

Crataegus pinnatifida (CP) (Hawtorn) has high content of bioflavonoids and was reported to have antioxidant and cardioprotective properties (Fetrow et al., 2001). It was published that the corosolic acid isolated from the fruit of CP is a protein kinase C inhibitor and has cytotoxic effect on leukemia cells (Ahn et al., 1998). In TCM it is used for dyspepsia, diarrhea, abdominal pain, hypertension and hyperlipidemia (Zhao, 2004).

Herbal TCM (due to the many different compounds used together) usually exhibit synergistic antitumor activity. One of the mechanisms of TCM in cancer treatment is immune system modulation by stimulatory, suppressive or regulatory activity or a combination of these effects (Patwardhan et al., 2005). Some of the most common TCM- stimulated cytokines are Tumor Necrosis Factor- \( \alpha \) (TNF-\( \alpha \)), Interleukin-2 (IL-2), and Interleukin-6 (IL-6). TNF-\( \alpha \) (cachectin) is a potent mediator of immune and inflammatory responses. It is produced by different activated cells including macrophages, granulocytes, T and B lymphocytes, natural killer (NK) cells, fibroblasts, and certain tumor cells. TNF can co-stimulate the proliferation of activated T (T\(_{\text{helper}}\)) and B lymphocytes. TNF-\( \alpha \) is selectively cytotoxic for some transformed cell lines and
can exert cytotoxic effects against certain solid tumors in lower dose. In vivo, TNF-\(\alpha\) serves as a primary mediator in protective immune responses against microbial and viral pathogens. However, TNF-\(\alpha\) in higher dose has also been implicated as an inflammatory mediator in a number of pathologic responses including septic shock, cachexia and autoimmune diseases (Levinson et al., 1996; Parslow et al., 2003). IL-6 is secreted by T-helper cells and macrophages. It is a multifunctional cytokine which regulates immune responses, acute-phase reactions and hematopoiesis. It potentiates the effect of IL-1 and TNF-\(\alpha\) to further promote T-cell activation. IL-6 also enhances B lymphocyte proliferation and differentiation and promotes multi-potent hematopoietic cells. It is also an endogenous pyrogen.

IL-2 (T-cell growth factor, TCGF) is a lymphokine which is produced by antigen-activated T-helper cells. It is involved in activating all types of acquired immune response: T\(_{\text{cytotoxic}}\), T\(_{\text{helper}}\), B-cell growth and differentiation; lymphokine-activated killer (LAK) cells and NK cell generation and proliferation. It plays an important immunoregulatory role and it is frequently used in cancer therapy. IL-2 also induces other lymphokines such as interferon and B-cell growth factor (Gupta et al., 1996; Weir et al., 1996a; Weir et al., 1996b; Kresina, 1998).

Another effect of TCM in cancer treatment is cytotoxicity, inducing cell death in solid tumors. It was shown that apoptosis (programmed cell death) is the most common type of cell death, though it has been reported that cancer cells can undergo autoschizis, a novel type of necrosis (Jamison et al., 2002). It has been reported that most cytotoxic drugs induce necrosis (of cancer cells in-vitro) at high doses and apoptosis at low doses (Schwartzman et al., 1993).
Apoptosis is a biochemical and physiological response characterized by distinct morphological changes, including pre-lytic DNA fragmentation, cell shrinkage and appearance of apoptotic bodies. The underlying mechanism of apoptosis induction involves different signal transduction pathways controlled by pro- and anti-apoptotic factors (death signals, genetic regulation by transcription factor manipulation, activation of effector enzymes) [Renehan et al., 2005].

The most common apoptosis initiators are: (a) stress (ultraviolet and $\gamma$-irradiation, hypoxia, growth factor deprivation), cytotoxic T-cells releasing granzyme-B – these trigger changes in the nucleus (p53, cyclin D, cyclin E regulation) and/or mitochondria (cytochrome c release, (b) cytotoxic drugs and cytokines which bind to the “death receptors” (Fas, Apo1, TNFR1, TNRF2). These activate either the effector caspases-3, -6,-7 or through caspase-9 / poly (ADP)-ribose polymerase (PARP) pathway to cause DNA fragmentation in the nucleus (Hengartner, 2000; Desagher et al., 2000; Kaufmann et al., 2001; Reed, 2001; Renehan et al., 2005).

The protein bcl-2 has been shown to play an important role in the apoptotic pathway both as pro- and anti-apoptotic regulator (Song et al., 1999). Overproduction of bcl-2 can result in blockage of apoptosis. The anti-apoptotic property of bcl-2 is possibly modulated by the protein Bax, and the Bcl-2: Bax ratio could determine whether a cell would undergo apoptosis. Among other chemicals, steroid sex hormones (estrogen) and certain natural compounds (flavonoids, terpenoids, alkaloids, polysaccharides) may regulate Bax and Bcl-2 levels. Breast epithelium undergoes cyclic apoptosis and fluctuation in Bcl-2 protein level during the menstrual cycle and pregnancy. Changes in Bcl-2 levels have also been associated with breast cancer (Oltvai et al., 1993; Gee et al., 1994; Reed, 1994; Sato et al., 1994; Sabourin et al., 1994; Wang et al., 1995).
1.3 Breast cancer: significance in modern day society

Breast cancer is the most common cancer in Singaporean women and the 3 major ethnic groups (Chinese, Malay, Indian) are equally affected. The incidence rate is about 5% and more than 1000 new cases are diagnosed yearly (Singapore Cancer Society, 2002), while 300 deaths from this cancer occur per year (WHO IARC, 2005). According to the World Health Organization (WHO), more than 1.2 million people will be diagnosed with breast cancer in 2005 worldwide. While breast cancer is less common at a young age (in their thirties), younger women tend to have more aggressive breast cancers than older women. The survival rates are lower among younger women (Imaginis, 2005).


The risk factors associated with breast cancer are: female, age above 40, family history (having a first degree relative with breast cancer), late first pregnancy, early onset of
menses, late menopause, diet (high intake of saturated animal fats, decreased fruit and vegetable intake), alcohol consumption, weight gain, lack of physical exercise and intake of hormones (oral contraceptive pills, hormone replacement therapy) [Ng et al., 1997].

In Singapore, the median age at diagnosis of breast cancer is 50 years, approximately 10 years younger than the Caucasian population (Seow et al., 1996). This could be due to the different type of BRCA1 mutations observed in patients of Asian ethnicity, especially in those younger than age 36 years (Ho et al., 2000). Breast carcinoma presenting at a younger age is usually the invasive type thus requiring very complex treatment.

The conventional breast cancer treatment involves multimodal approaches, such as surgery, radio-, chemo- and/or hormonal therapy. In most of the breast cancer cases we observed in Singapore, herbal TCM was used to complement conventional treatment.

It has been established earlier that most of the breast cancer cases are caused by hormonal (estrogen, progesterone) imbalance, estrogen being a risk factor in the development of this disease. Preventive and therapeutic treatments often target estrogen. The most common drugs used are (a) Selective Estrogen Receptor Modulators (SERMs): Tamoxifen, Toremifene,Raloxifene; Soy, Flaxseed in CAM (b) Aromatase Inhibitors: Anastrozole, Exemestane, Letrozole; Genistein in CAM (c) Hormonal agents: Fulvestrant, Goserelin acetate, Megestrol.

A wide range of conventional chemotherapy is also commonly used: Capecitabe, Cyclophosphamide, Docetaxel, Doxorubicin, Epirubicin, Fluorouracil (5-FU), Paclitaxel, Vinorelbine (Jellin, 2005).

Against HER2+ breast cancer the current treatment is Transtuzumab, which is a recombinant antibody against the HER2 gene (Piccart-Gebhart et al., 2005).
There are also promising results with the angiogenesis inhibiting anti-Vascular Endothelial Growth Factor (anti-VEGF) drug, Bevacizumab.

It has been reported that certain Chinese medicinal plants have estrogenic activities thus might have estrogen agonist/antagonist effect. Some of these herbs (*Epimedium brevicornum, Antrodia camphorata*) were tested *in-vitro* for MCF-7 human cancer cell growth inhibition, others are already used for the management of menopausal symptoms (Yap *et al.*, 2005; Yang *et al.*, 2005; Zhang *et al.*, 2005).

There is very little available scientific evidence to validate the efficacy of TCM. Singapore, with its dual health-care system, is in a very unique position for clinical and laboratory studies to be done to evaluate the effectiveness of the combined use of Western and traditional medicine in the treatment of disease states like breast cancer.

### 1.4 Rationale and purpose of this study

Breast carcinoma is a common cancer in women and is a leading cause of cancer-related deaths in Singapore and worldwide (Polyak, 2001). Early detection and intervention have shown promising results. However, the incidence of this disease is constantly growing, causing a greater impact on health care services to demand new cures for this disease.

It is known that CAM therapies provide psychological benefits (optimism, hope for disease control, cure and longer survival) for cancer patients (Di Gianni *et al.*, 2002). The objective of our study was to obtain data that may show whether TCM use could have beneficial effect on breast cancer patients through improving their quality of life (by palliating the side effects of conventional therapy), stimulating their immune system and inhibiting tumor growth.
The conventional treatment for breast cancer includes surgery, radio-, chemo- and/or hormonal therapy. The side-effects of these medical interventions and the stress of living with breast cancer can negatively affect the patients’ quality of life. QOL research has become an important part of the assessment of cancer treatment in Western society. However only two studies (one in Shanghai and the other in Hong Kong) have been conducted on Chinese breast cancer patients (Yu et al., 2000; Fung et al., 2001; Cui et al., 2004).

The purpose of our study was to gain scientific evidence that TCM, an ancient type of medicine based on empirics and widely used for centuries, could be potentially useful in the modern biomedical setting.

The results obtained from our study may contribute towards improved treatment of patients with breast cancer.
CHAPTER TWO: MATERIALS AND METHODS
2.1 Materials

2.1.1. Cell lines and cell cultures

All cell lines are adherent type and were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA).

MCF-7

Picture 5.MCF-7 cells

MCF-7 was derived from pleural effusion of a metastasised adenocarcinoma (epithelial) of the human breast. It is estrogen receptor positive and its growth can be inhibited by TNF-α. The MCF-7 line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic
estrogen receptors and the capability of forming domes. Doubling time is 29 hours. If exposed to estrogen, it can develop into tumors in nude mice (ATCC, 2005).

**MDA-MB-231**

**Picture 6**. MDA-MB-231 cells

![MDA-MB-231 cells](image)

MDA-MB-231 was derived from metastasised adenocarcinoma (epithelial) of the human breast. It expresses epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α). Doubling time is around 19 hours. In nude mice, MDA-MB-231 forms poorly differentiated (grade III) adenocarcinoma (ATCC, 2005).

**T-47-D**

T-47-D was derived from a patient with infiltrating ductal carcinoma of the breast. It expresses androgen, progesterone, glucocorticoid, prolactin, calcitonin and estrogen receptors. Doubling time is 32 hours (ATCC, 2005).
ZR-75-1
T-47-D was derived from a patient with infiltrating ductal carcinoma of the breast. It is estrogen receptor positive. Doubling time is 80 hours. It forms tumors in nude mice (ATCC, 2005).

2.1.2. Animals
Inbred male and female BALB/c mice, 5 to 6 weeks old, were obtained from the Animal Holding Unit, National University of Singapore.
Inbred female oophorectomized BALB/c nude mice, 8 to 12 weeks old, were obtained from the Animal Research Centre (Canning Vale, Australia). The autosomal recessive nude gene in homozygous (nu/nu) mice causes the lack of fur and an abnormal thymus. The deficiency in T cell function allows athymic mice to accept and grow xenografts as well as allografts of normal and malignant tissues.
All animal experiments were conducted in accordance with the Animal Welfare Act and the Animal Research Extension Act, in facilities approved by the Institutional Animal Care and Use Committee.

2.1.3. Chemicals and reagents

*BDH Laboratory Supplies (UK)*
Dimethyl Sulphoxide (DMSO)

*Hyclone (UK)*
Inactivated fetal bovine serum (FBS)

*Merck KgaA (Darmstadt, Germany)*
Methanol, Ethanol, Hexane, HCl, H$_2$SO$_4$, Dodecyl-sulphate sodium salt (SDS), Ethyl acetate, n-butanol

**Innovative Research of America (Sarasota, Florida, USA).**

60 days release 17-β-estradiol (0.72 mg) pellets for nude mice

**Sigma-Aldrich (St Louis, MO, USA)**

All other chemicals and reagents used in the present study

### 2.1.4. Kits

**BD Biosciences Pharmingen (San Diego, CA, USA)**

Mouse IL-2 BD OptEIA$^\text{TM}$ ELISA Set

Mouse IL-6 BD OptEIA$^\text{TM}$ ELISA Set

Mouse TNF-α (Mono/Mono) BD OptEIA$^\text{TM}$ ELISA Set

**Roche Diagnostics GmbH, Roche Applied Sciences (Penzberg, Germany)**

Cell Death Detection ELISA$^\text{PLUS}$

Cellular DNA Fragmentation ELISA

### 2.1.5. Facilities/ Equipment

-86°C Freezer (Forma Scientific)

-20°C & 4°C ACMA Refrigerator

Balance (Precisa 40SM-200A, Switzerland)

Biological Safety Cabinet (NUAIRE, Plymouth, USA)

CO$_2$ Incubator (SANYO Electronic Co. Ltd, Japan)

ELX 800 Microplate Reader (Bio-Tek Instruments Inc., USA)

Hemocytometer (Fortuna, Germany)
HPLC with Phenomenex Synergi 4μ Hydro-RP80A column (Japan)
Kubota Centrifuge KR400 (Kubota Seisakusho Co. Ltd., Japan)
Nikon Light Microscope (Nikon Co. Ltd, Japan)
Beckman pH Meter (Fullerton, CA, USA)
Tecan Sunrise Microplate Reader (Grödig, Austria)
UV-1601 Spectrophotometer (Shimadzu, Japan)

2.2. Methods

2.2.1. Quality-of-life (QOL) study

2.2.1.1. QOL study design

Our study started in February 2001 at two TCM clinics in Singapore. One clinic is the Chung Hwa Free Clinic at Toa Payoh, which is financed by individual donors and Buddhist charity organizations. Most of the patients here are from low-income households. The other clinic is Teo Acupuncture Hall, a private clinic at Rochor Centre, which provides TCM treatment for mainly higher income patients.

We chose breast cancer patients for the study since the incidence of this disease is increasing worldwide, creating a need for a more satisfactory treatment. Breast cancer patients are also ideal candidates for observing the side-effects of chemo- and radiotherapy since they have little complications from the tumor itself (compared to gastric or pancreatic cancer), thus any change in quality-of-life is most likely to be due to the applied treatment itself.

Thirteen female Chinese patients, age 31 to 53 years, who previously had been diagnosed with breast cancer by Western-trained physicians, were selected as the first subjects for our study between February 2001 and July 2001. After a month, seven
patients dropped out (mainly because of not returning the questionnaires) and we continued to follow up the remaining six patients. Two had been treated with radiotherapy, chemotherapy and the hormone receptor modulator, Tamoxifen; one with radiotherapy and Tamoxifen; two with Tamoxifen only; one with herbal TCM only. It is noted that five of the six patients recruited had some form of surgery as part of their prior conventional medical treatment (Table 1).

Table 1. Conventional treatment of selected patients

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Modality of treatment at the time of study initiation</th>
<th>Number with prior surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>R, C, T</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>R, T</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>T</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>TCM only</td>
<td>1</td>
</tr>
</tbody>
</table>

R: radio-therapy C: chemotherapy T: Tamoxifen TCM: Traditional Chinese Medicine

Our inclusion criteria were that the patients (a) must have primary, not metastatic, breast cancer diagnosed by a biomedical practitioner, (b) should not have any other treated or untreated underlying medical condition, (c) should have had previous or current conventional treatment for breast cancer, (d) should comply with TCM and (e) should be able to come back for follow-up. After initial evaluation of the patients’ medical history, with regard to the modality of breast cancer treatment including previous surgery and the type of herbs used (if any), we explained the nature of our non-invasive, purely observational study and obtained the patients’ consent to
participate in the study by showing them the questionnaire in their preferred language (English or Chinese).

These patients were followed up for their herbal TCM treatment by the same Chinese physician, Dr. Teo. They responded weekly, bi-weekly, monthly or bi-monthly to the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QOL-C30 version 3.0) and to the supplementary breast cancer module (QOL-BR23) questionnaire (Aaronson et al., 1993) [see Picture 7a, 7b, 8a, 8b and Table 2 and Table 3 for their composition].
32

Picture 7a.EORTC QOL-C30 (version 3) questionnaire

<table>
<thead>
<tr>
<th>Question</th>
<th>Not at all</th>
<th>A little</th>
<th>Quite a bit</th>
<th>Very much</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you have any trouble doing strenuous activities, like carrying a heavy shopping bag or a suitcase?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>2. Do you have any trouble taking a long walk?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>3. Do you have any trouble taking a short walk outside of the house?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4. Do you need to stay in bed or a chair during the day?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5. Do you need help with eating, dressing, washing yourself or using the toilet?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td><strong>During the past week:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Were you limited in doing either your work or other daily activities?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7. Were you limited in pursuing your hobbies or other leisure time activities?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>8. Were you short of breath?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>9. Have you had pain?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>10. Did you need to rest?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>11. Have you had trouble sleeping?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>12. Have you felt weak?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>13. Have you lacked appetite?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>14. Have you felt nauseated?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>15. Have you vomited?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>16. Have you been constipated?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Please go on to the next page
### Picture 7b. EORTC QOL-C30 (version 3) questionnaire

#### During the past week:

<table>
<thead>
<tr>
<th>Question</th>
<th>Not at all</th>
<th>A little</th>
<th>Quite a bit</th>
<th>Very much</th>
</tr>
</thead>
<tbody>
<tr>
<td>17. Have you had diarrhea?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>18. Were you tired?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>19. Did pain interfere with your daily activities?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>20. Have you had difficulty in concentrating on things, like reading a newspaper or watching television?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>21. Did you feel tense?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>22. Did you worry?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>23. Did you feel irritable?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>24. Did you feel depressed?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>25. Have you had difficulty remembering things?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>26. Has your physical condition or medical treatment interfered with your family life?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>27. Has your physical condition or medical treatment interfered with your social activities?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>28. Has your physical condition or medical treatment caused you financial difficulties?</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

#### For the following questions please circle the number between 1 and 7 that best applies to you

29. How would you rate your overall health during the past week?
- 1 = Very poor
- 2 = Poor
- 3 = Fair
- 4 = Good
- 5 = Very good
- 6 = Excellent

30. How would you rate your overall quality of life during the past week?
- 1 = Very poor
- 2 = Poor
- 3 = Fair
- 4 = Good
- 5 = Very good
- 6 = Excellent

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The EORTC QOL-BR23 questionnaire is designed to assess the quality of life of patients with breast cancer. The questionnaire consists of several questions that ask patients to rate the extent to which they have experienced certain symptoms or problems during the past week and during the past four weeks.

### During the past week:

1. Did you have a dry mouth?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

2. Did food and drink taste different than usual?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

3. Were your eyes painful, irritated or watery?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

4. Have you lost any hair?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

5. Answer this question only if you had any hair loss: Were you upset by the loss of hair?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

6. Did you feel ill or unwell?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

7. Did you have hot flushes?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

8. Did you have headaches?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

9. Have you felt physically less attractive as a result of your disease or treatment?
   - Not at all: 1
   - A little: 2
   - Quite a bit: 3
   - Very much: 4

10. Have you been feeling less feminine as a result of your disease or treatment?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

11. Did you find it difficult to look at yourself naked?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

12. Have you been dissatisfied with your body?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

13. Were you worried about your health in the future?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

### During the past four weeks:

14. To what extent were you interested in sex?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

15. To what extent were you sexually active?
    - (with or without intercourse)
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

16. Answer this question only if you have been sexually active. To what extent was sex enjoyable for you?
    - Not at all: 1
    - A little: 2
    - Quite a bit: 3
    - Very much: 4

Please go on to the next page.
**Picture 8b. EORTC QOL-BR23 questionnaire**

<table>
<thead>
<tr>
<th>Question</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>17. Did you have any pain in your arm or shoulder?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. Did you have a swollen arm or hand?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. Was it difficult to raise your arm or to move it sideways?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20. Have you had any pain in the area of your affected breast?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21. Was the area of your affected breast swollen?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22. Was the area of your affected breast oversensitive?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23. Have you had skin problems on or in the area of your affected breast (e.g. itchy, dry, flaky)?</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Composition of EORTC QLQ-C30 (version 3.0)

<table>
<thead>
<tr>
<th>Scale</th>
<th>QLQ-C30 item numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLOBAL HEALTH STATUS</td>
<td></td>
</tr>
<tr>
<td>Global health status/QoL</td>
<td>QL2</td>
</tr>
<tr>
<td></td>
<td>29, 30</td>
</tr>
<tr>
<td>FUNCTIONAL SCALES</td>
<td></td>
</tr>
<tr>
<td>Physical functioning</td>
<td>PF2</td>
</tr>
<tr>
<td></td>
<td>1 to 5</td>
</tr>
<tr>
<td>Role functioning</td>
<td>RF2</td>
</tr>
<tr>
<td></td>
<td>6, 7</td>
</tr>
<tr>
<td>Emotional functioning</td>
<td>EF</td>
</tr>
<tr>
<td></td>
<td>21 to 24</td>
</tr>
<tr>
<td>Cognitive functioning</td>
<td>CF</td>
</tr>
<tr>
<td></td>
<td>20, 25</td>
</tr>
<tr>
<td>Social functioning</td>
<td>SF</td>
</tr>
<tr>
<td></td>
<td>26, 27</td>
</tr>
<tr>
<td>SYMPTOM SCALES</td>
<td></td>
</tr>
<tr>
<td>Fatigue</td>
<td>FA</td>
</tr>
<tr>
<td></td>
<td>10, 12, 18</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td>NV</td>
</tr>
<tr>
<td></td>
<td>14, 15</td>
</tr>
<tr>
<td>Pain</td>
<td>PA</td>
</tr>
<tr>
<td></td>
<td>9, 19</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>DY</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Insomnia</td>
<td>SL</td>
</tr>
<tr>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Appetite loss</td>
<td>AP</td>
</tr>
<tr>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Constipation</td>
<td>CO</td>
</tr>
<tr>
<td></td>
<td>16</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>DI</td>
</tr>
<tr>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Financial difficulties</td>
<td>FI</td>
</tr>
<tr>
<td></td>
<td>28</td>
</tr>
</tbody>
</table>
The QLQ-C30 questionnaire is designed to measure the physical, psychological and social functions of the cancer patients (Kaasa et al., 1995). It is composed of both multi-item and single-item scales. These include five functional scales (physical, role, emotional, social and cognitive function), three symptom scales (fatigue, pain and nausea), a global health status scale and six single items (dyspnoea, insomnia, appetite loss, diarrhea, constipation and financial difficulties). The physical and role function scales have a choice of “yes/no” for response; the two global quality-of-life questions have a 7-point linear analogue scale for answers (1 being “very poor”, 7 being “excellent”). All other items have a 1 (“not at all”) to 4 (“very much”) point score on the answer scale.
Table 3. Composition of EORTC breast cancer module QLQ-BR23

<table>
<thead>
<tr>
<th>Scale</th>
<th>QLQ-BR23 item numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FUNCTIONAL SCALES</strong></td>
<td></td>
</tr>
<tr>
<td>Body image</td>
<td>BRBI</td>
</tr>
<tr>
<td>Sexual functioning</td>
<td>BRSEF</td>
</tr>
<tr>
<td>Sexual enjoyment</td>
<td>BRSEE</td>
</tr>
<tr>
<td>Future perspective</td>
<td>BRFU</td>
</tr>
<tr>
<td><strong>SYMPTOM SCALES</strong></td>
<td></td>
</tr>
<tr>
<td>Systemic therapy side</td>
<td>BRST</td>
</tr>
<tr>
<td>effects</td>
<td></td>
</tr>
<tr>
<td>Breast symptoms</td>
<td>BRBS</td>
</tr>
<tr>
<td>Arm symptoms</td>
<td>BRAS</td>
</tr>
<tr>
<td>Upset by hair loss</td>
<td>BRHL</td>
</tr>
</tbody>
</table>
The breast cancer BR-23 module is meant for use among patients varying in disease stage and treatment modality (i.e. surgery, chemotherapy, radiotherapy and hormonal treatment) (Sprangers et al., 1996). The module comprises 23 questions assessing disease symptoms, side effects of treatment (surgery, chemotherapy, radiotherapy and hormonal treatment), body image, sexual functioning and future perspective. Validation studies on the module have been completed in The Netherlands, Spain and the United States. It had been field-tested in a large cross-cultural study involving 12 countries (EORTC Protocol 15931) (Fayers et al., 1998). All of the scales and single-item measures range in scores from 0 to 100. A high score for an item on the functional scale represents a high / healthy level of functioning. Similarly, a high score for the global health status represents a high quality-of-life, but a high score for an item on the symptom scale represents a high level of problems.

Patients on chemo- and radiotherapy answered the questionnaire more frequently because it was assumed that their state of well-being would be more likely to fluctuate during follow-up. The questionnaire was self-administered in English or in validated Chinese (Mandarin and “Taiwanese Chinese”) translation, depending on the patients’ language preference.

After completing the first QLQ-30 and BR-23 questionnaires, five patients had their first herbal TCM treatment, while one patient (who previously had mastectomy) was already using TCM at the time of induction into the study. They subsequently made several visits to the clinic, the frequency varying between 3 times per week to once in 2 months. Their herbal prescriptions were altered according to their condition at each visit, and comprised different air-dried plants which were soaked in water for half-an-hour and then boiled for two hours. The supernatant decoction was then consumed
twice a day, in the morning and in the evening, before or after meals. The patients were requested by the TCM practitioner to avoid taking any other type of medicine within 1 hour of taking the decoction to minimize the possibility of direct drug interactions with TCM.

Scores were obtained from the completed questionnaires and transformed for interpretation and comparison in two ways: (a) baseline (pre-treatment) scores were compared with post-TCM treatment scores and (b) baseline scores were compared with scores of breast cancer patients published in the manual of EORTC QLQ-30 Reference Values (Fayers et al., 1999). Our patients were matched by age, gender and initial conditions with those from the reference population (Canada, Norway, Sweden, Denmark). The Reference Values (general population data based on large random samples from the general population in the above-mentioned countries) are based on pre-treatment data and give baseline quality-of-life scores for patients according to specific cancer sites (breast, prostate, lung, brain etc.) and stage of disease (local/loco-regional/advanced).

All of our patients were diagnosed with early or advanced but not yet metastasised breast cancer and were treated by Western-trained physicians. Comparing our patients’ baseline mean scores to the published EORTC reference values, we observed a moderate difference in physical and emotional functioning, pain and appetite loss, and a little change on the role functioning and dyspnoea scale. This might indicate that our six patients in Singapore were different from the reference population in their psychological and physiological responses to their cancer, and might have a higher threshold of pain perception.

The patients used herbal TCM as an alternative, complementing the conventional therapy. The prescription was adjusted at each visit according to the actual condition
at the time of the medical consultation by adding or removing herbs and increasing or
decreasing the dosage and frequency of the herbal medication. The better quality of
life could be attributed partially to the patients’ favorable prognosis and the use of
moderately toxic conventional therapies (Burstein et al., 1999). The higher scores on
the functional scale and lower scores on the symptoms scale could also indicate that
herbal TCM can ease some of the symptoms in cancer patients.

SN 300 mg/kg BW small volume tumors not efficient larger, multiple tumors SN 500
mg/kg BW could be use as prevention (prophylactic rate 75%) not efficient dose of
tumors

2.2.1.2. Scoring procedures for the EORTC QLQ-30 and BR-23
questionnaires

As a first step, we estimated the average of the items on the functional or symptom
scale on the QLQ-30 and BR-23 questionnaires. This average is the Raw Score (RS) =
(I₁ + I₂ +…+ Iₙ) / n (n: number of items). As a next step we applied a linear
transformation to 0-100 to obtain the Score (S) which was calculated for the
respective scales as it is shown below (range is the difference between the maximum
and the minimum possible value of RS):

Functional scales:   S = 1-(RS-1)/range x 100
Symptom scales / items and Global health status/ QOL:   S = (RS-1)/range x 100

2.2.2. Extraction of herbs

The dried forms of Astragalus membranaceus (AM) root, Curcuma aromatica (CA)
tuber, Scutellaria barbata (SB) leaves and Solanum nigrum (SN) leaves were
purchased from Teo Acupuncture & Medical Hall, where the breast cancer patients
obtained their prescription. The herbs were harvested in Southern China (Personal communications with Teo Eng Kiat, 2001).

2.2.2.1. Preparation of water and ethanolic extract of *Astragalus membranaceus, Curcuma aromatica, Scutellaria barbata and Solanum nigrum*

The dried herbs were crushed into small pieces with an electronic micronizer. As a first step in the fractionation process, we chose the ethanolic extract of these herbs. 170 g of AM, 180 g of CA, 120g of SB and 130 g of SN were extracted with 80% ethanol at room temperature until exhaustion. The preparation was filtered with Whatman filter paper GF/A, concentrated under reduced pressure and freeze dried. The yield was 19%, 13.3%, 9.2% and 6.5% respectively.

We also prepared boiled water extracts of 200 g each of AM, CA, SB and SN by covering the herbs with sufficient water, bringing to boiling point. After cooling, we filtered the extract and proceeded to freeze dry. The yield was less compared to the ethanolic extracts, 12.2%, 7.8%, 4.7% and 2.9% respectively.

2.2.2.2. Fractionation of the water boiled extract of *Astragalus membranaceus*

As a next step we also fractionated the water-boiled extract of AM. The water extract of AM was partitioned to obtain ethyl acetate (0.2% yield), butanol (3.3% yield) and the remaining water fraction (15.9% yield).
2.2.2.3. Characterization of the ethanolic and water extract of *Solanum nigrum*

Following the *in-vitro* cytotoxic experiments with the ethanolic extract of *Solanum nigrum*, we were interested to further investigate and compare the ethanolic and water extract of this herb. The reason being was to see whether there is any major difference between the ethanolic extract used in our laboratory testing and the water extract the patients were taking.

We ran both extracts on reversed-phase high performance liquid chromatography (HPLC). It was a linear gradient elution at a flow rate of 0.8ml/min on Phenomenex Synergi 4µ Hydro-RP80A column (250 x 4.6mm, 4 micron) with acetonitrile and de-ionized water as mobile phase. Detection was done with a Shimadzu Diode Array detector. During the linear gradient elution program, the percentage of acetonitrile in mobile phase changed as follows: 0.1 to 5 mins: 0%; 5 to 30 mins from 0% to 100%; 45 to 50 mins: from 100% to 0%; 50 to 60mins: 0%.

The peaks obtained were detected at four different wavelengths: UV 240, 254, 260 and 280nm.

2.2.3. Breast cancer cell culture

The human breast cancer cells were cultured for 4-8 passages: MCF-7, T-47D and ZR-75 cells were cultured in RPMI-1640, while MDA-MB-231 cells were cultured in DMEM medium at 37°C in 5% CO₂ humidified incubator. The medium contained 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100µg/ml). Before the cellular DNA fragmentation experiment, the cells were labeled with BrdU overnight.
2.2.4. Cell viability assays by MTT test and trypan blue exclusion

Evaluation of cell activation in the immunomodulatory and cytotoxicity studies was carried out using the MTT tetrazolium assay. Briefly, after 48-72 h incubation of tissue culture plates at 37°C in 5% humidified CO₂ incubator, 10 μl MTT (stock solution 5 mg/ml PBS) was added to each well. The plates were again incubated for 4 h after which 100 μl/well 10% SDS in 0.01N HCl was added to dissolve the formazan crystals. After overnight incubation, the plates were read in a microplate reader (ELX 800, Bio-Tek Instruments, USA) at 570 nm. Wells with medium, MTT and SDS, but without cells, were used as blanks (Gerlier et al., 1986; Swamy et al., 2000; Liu, 2002).

2.2.5. Lymphocyte-activation assay

Fresh splenocytes were prepared aseptically into single cell suspension (Nakamura et al., 1986; Wu et al., 1990). These were suspended at 1.6 x 10⁶ cells/ml in medium containing RPMI 1640 supplemented with 10% heat-inactivated FBS. A total of 90 μl of the cells were added to each well of a flat-bottom 96-well plate using a multi-channel pipette. Studies were carried out to evaluate the effects of 10.0, 1.0 and 0.1μg/ml AM and CA extracts on the proliferation of mouse lymphocytes under various conditions, in the absence or presence of 10 μl of (a) suboptimal doses of ConA (0.3 μg/ml) or LPS (1.0 μg/ml) and (b) optimal doses of ConA (3.0 μg/ml) or LPS (6.0 μg/ml).

The spleen-cell suspensions were incubated for 48-72 h at 37°C in 5% humidified CO₂ incubator with 10 μl serially diluted extracts, starting with highest concentration of 10.0 μg/ml for each extract in quadruplicate wells. Experiments were repeated in four replicates for extracts showing stimulation of the splenocytes. Evaluation of cell activation was carried out using the MTT tetrazolium assay.
2.2.6. Assay of cytokines IL-2, IL-6 and TNF-α

6-8 weeks old BALB/c female mice were fed orally for 14 days with AM (50, 100, 200 mg/BW kg). Blood samples were collected by cardiac puncture and the serum was analyzed for IL-2, IL-6 and TNF-α.

To measure cytokine production we used an assay that employs the quantitative 'sandwich' enzyme immunoassay technique. The sample was blood serum, obtained by cardiac puncture from BALB/c 8 weeks old female mice.

Nunc Maxisorb 96 MTP was coated with 100 μl/well diluted capture antibody and incubated overnight at 4°C. After washing 3 times, the wells were blocked with 200 μl/well assay diluent. Following an hour incubation at room temperature (RT), the plates were washed 3 times. 100 μl/well standard solution, serial dilution and serum sample was added to the plates. After 2 hours of incubation at RT, the plates were washed 5 times.

For the TNF-α and IL-6 assays 100 μl/well detection antibody plus Avidin-HRP was added. After 1 hour of incubation at RT, the wells were washed 7 times and 100 μl substrate solution was added to each well. Following 30 minute incubation at RT in the dark, 50 μl/well stop solution was added.

For the IL-2 assay 100 μl/well detection antibody was added first, then after 5 washes 100 μl/well Avidin-HRP was added.

As a last step for all 3 assays, the MTP was read @ 450 nm within 30 minutes. Color development was in proportion to the amount of cytokine bound in the initial step. By comparing the optical density of the samples to the standard/serial dilution curve, the concentration of the cytokine in the unknown samples was then determined.
2.2.7. **In-vitro cytotoxicity assay**

Sub-cultured, trypsinized human breast cancer cells MCF-7, T-47D, ZR-75 and MDA-MB-231 were centrifuged @ 900 rpm for 5 minutes. After discarding the supernatant, the cells were re-suspended in medium. The cell suspension was diluted to $5 \times 10^5$ cell/ml concentration using trypan blue to determine total cell counts and viable cell number (non-viable cells dye blue). 95 μl of cell suspension was added to each well. After 2 hour incubation, 5 μl of serially diluted AM, CA, SB and SN extracts were added to the respective wells. 5 μl 6-MP was the positive control and 10% DMSO in sterile water was the negative control. After a 48 hour incubation at 37°C in 5% humidified CO$_2$ incubator, MTT-tetrazolium assay (see 2.2.4.) was applied and the absorbance was read @ 570 nm using microplate reader. The inhibition rate and ED$_{50}$ were calculated as follows:

\[
\text{inhibition rate}\% = \frac{(T \text{ cells with extract} - \text{cells with medium})}{(T \text{ cells with solvent} - T \text{ cells with medium})} \times 100\%
\]

T: optical density of cells under different treatments (with or without extract)

ED$_{50}$ is the calculated effective dose which inhibits growth by 50%

### 2.2.8. **In-vitro analysis to determine the type of cell death**

#### 2.2.8.1. DNA fragmentation ELISA assay (Fig.1)

After the promising *in-vitro* cytotoxic experiments we were focusing on SN as a potential cytotoxic agent which could be used in breast cancer treatment.

Exponentially growing human breast cancer cells MCF-7 were diluted with culture medium to obtain a cell concentration of $1 \times 10^5$ cells/ml. 100 μl of diluted cells ($10^4$ cells) were added to each microplate well. The cells were incubated with SN extract
for 4, 8, 12 and 24 hours at 37° C and 5% CO₂. Negative control was 10% DMSO in sterile water, positive control was DNA-histone-complex, background control was the incubation buffer. After the respective incubation periods, the MTPs were centrifuged (200 x g for 10 minutes). The supernatant (S1: contained necrotic DNA that leaked through the membrane during the incubation) was used to analyze necrosis, the pellet was lysed and centrifuged again. The supernatant (S2: the cytoplasmic fraction) was used to detect apoptosis. 20 µl of S1 and S2 were transferred into the streptavidin-coated MTP and continued with ELISA: 80 µl of the Immunoreagent was added to each well and incubated on a microplate shaker (300 rpm for 2 hours at 15–25°C). After rinsing MTP 3 times, 100 µl ABTS solution/well was added. After incubation on a plate shaker (250 rpm 10-20 min) the samples were measured @ 405 nm against ABTS solution as a blank (reference wavelength @ 490 nm).

Figure 1. Cell death detection ELISA<sup>PLUS</sup> (Roche, 2005a)

A: sample preparation
B: ELISA
The values were averaged and the background value (Incubation buffer + ABTS solution) was substracted. The specific enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated from these values using the following formula:

\[
\text{enrichment factor} = \frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without SN treatment)}}
\]

\[\text{mU} = \text{absorbance} \times 10^{-3}\]

2.2.8.2. Cellular DNA fragmentation assay (Fig.2)

Sub-cultured MDA-MB-231 breast cancer cells (2-4 x 10^5) in tissue culture flask were pre-labeled with the non-radioactive thymidine analogue BrdU overnight at 37°C in 5% humidified CO2 incubator. The labeled cells were further incubated in the presence of SN extract at different concentrations (10-400 μg/ml) for 2, 4 and 6 hours. The negative control was 10% DMSO in sterile water, the positive control was camptothecin (0.1 μg/ml).

At the end of each incubation period, the cells were centrifuged @ 900 rpm for 5 minutes. The supernatant (S1) was analyzed for necrosis and the cellular lysate was further centrifuged. The supernatant of this cellular lysate (S2) was used for apoptosis detection.
The wells of a MTP were coated with anti-DNA antibody, S1 and S2 samples were added into duplicate wells and incubated for 90 minutes. After washing the MTP, the samples (some of them containing immunocomplexed BrdU-labeled DNA-fragments) were denaturized by microwave irradiation (500W for 5 minutes). Anti-BrdU antibody peroxidase conjugate (Anti-BrdU-POD) was added to form an immunocomplex with the BrdU-labeled DNA. Following 90 minutes of incubation, peroxidase substrate (TMB) was added to the wells and after 10-30 minutes of incubation the absorbance was measured @ 405nm wavelength using an ELISA plate reader.

2.2.9. Animal handling

2.2.9.1. Housing and maintenance

The animals (Swiss albino, BALB/c, BALB/c nude mice) were kept in filter-covered plastic cages, housed in a temperature-controlled (28°C) room with a diurnal 12 hours light cycle, and provided with tap water and standard rodent diet ad libitum. The nude mice were kept in a pathogen-free environment under similar maintenance
2.2.9.2. Mice gavage

BALB/c mice were restrained by grasping the nape, and a gavage tube was then inserted into the oral cavity of the mouse, moved downwards into the esophagus and 1 ml herbal extract was administered.

2.2.9.3. Blood collection by intracardiac puncture

BALB/c mice were euthanized by cervical dislocation and a hypodermic needle was inserted on the left lateral side of the sternum. Blood was aspirated and centrifuged to separate plasma and serum.

2.2.9.4. Intraperitoneal (i.p.) injection

After restraining the mouse, the abdominal area was wiped with alcohol swab and a hypodermic needle was inserted into the lower left/ right quadrant of abdomen. To confirm proper placement of the needle, the syringe was aspirated and the herbal extract (average 0.2 ml) was then administered in a steady, fluid motion.

2.2.9.5. Acute toxicity test

For the investigation of the acute toxicity of SN and to estimate the LD$_{50}$, we used both normal (SN water extract) and nude (SN ethanolic extract) BALB/c mice. We tested ethanolic extract in nude mice since the in-vitro experiments demonstrated stronger cytotoxic effect than the water extract and our future in-vivo experiments involved nude mice.

The doses were 10, 100, 1000, 2000, 5000 mg/BW intraperitonially (i.p.) in normal and 1500, 1750, 1875, 2000, 2250, 2500 mg/BW i.p. in nude mice. Both experiments included control groups, each treatment group had 3 animals. Acute poisonous effect,
body weight and behavior change was observed daily for a period of 2 weeks. The tests were carried out according to the protocols established by Lorke, 1983.

2.2.10. Breast tumor model in BALB/c nude mice

2.2.10.1. Tumor xenografts

In the first phase, 50 mice received subcutaneous intrascapular sustained release 17-\(\beta\)-estradiol (0.72 mg, released over 60 days) pellet implant (Hawkins et al., 2000). 24 mice were inoculated with \(10^7\) MCF-7 cancer cells into the left and/or right flank within 4 weeks of estradiol pellet implant.

To determinate the size of the subcutaneous tumors, we used the ellipsoid volume formula (Osborne et al., 1985; Ware et al., 1985; Riondel et al., 1986) which was reported to be the most accurate estimation of actual tumor volume (Tomayko et al., 1989) and was applied by other laboratories in several breast cancer xenograft models (Gottardis et al., 1988; Mimnaugh et al., 1991; Cameron et al., 1997). The tumors were measured every four days with calipers in three dimensions (length (A), width (B), depth (C)) in order to calculate tumor volume, \(V = A \times B \times C \times \pi / 6 \text{ mm}^3\).

2.2.10.2. In-vivo treatment protocols

Animals with tumors larger than \(8 \times \pi / 6 \text{ mm}^3\) (length, width and dimension measuring at least 2mm respectively), were selected for treatment with SN extract administered via i.p. injection: 300 mg/kg BW in 0.2 ml vehicle for three consecutive days per week. The negative control was vehicle (10% DMSO in saline) alone, also for three consecutive days each week. The positive control was 5 mg/BW kg Doxorubicin in sterile saline, given once weekly.

In the second phase of the study, 25 mice were implanted with \(10^7\) MDA-MB-231 cancer cell implant into the left and/or right flank. 10 mice started to be treated at the
same time of the tumor implant (prophylactic study), 15 mice were selected for treatment only after the tumor volume, V, was larger than $V = A \times B \times C \times \pi / 6 \text{mm}^3$ (therapeutic study). The SN extract was administered via i.p. injection: 500 mg/kg BW in 0.2 ml vehicle for three consecutive days per week. The negative control was vehicle alone, also injected i.p. for three consecutive days each week. The positive control was Doxorubicin 5 mg/kg BW in sterile saline, given i.p. once weekly. Despite that the patients were consuming the TCM drugs *per os*, in our cytotoxic laboratory experiments all nude mice were given i.p. injection as a more accurate and reliable administration route and easily adjustable dose of the SN extract. This is in line with the common cytotoxic/chemotherapeutic drug administration in cancer treatment.

2.2.11. Histopathological analysis of tumor tissue section

The uterine specimens and solid breast tumors were fixed in 10% formalin and embedded in paraffin. The sections/slides were stained with haematoxylin and eosin and viewed under light microscope at 40x, 100x or 200x magnification.

2.2.12. Statistical analysis

The significance of the differences between control and treated values was analyzed using Students’ t-test. Differences with $p$ values < 0.05 were considered to be statistically significant.
CHAPTER THREE: RESULTS AND DISCUSSION
3.1 Quality-of-life (QOL) study in breast cancer patients

3.1.1. Aims and experimental approach

The objective of the first part of our study was to conduct a QOL analysis among breast cancer patients who are using conventional therapy and herbal Traditional Chinese Medicine (TCM), in order to identify the medicinal herbs that might have immunomodulator and/or anti-tumor effects and could palliate the side effects of chemo- and radio-therapy.

A total of 13 female Chinese patients, age 31 to 53 years, were recruited to respond to the EORTC questionnaires QLQ-C30 (version 3.0) and to the supplementary breast cancer module (QLQ-BR23).

The questionnaire was administered in English or in validated Chinese translation. During the study, 7 patients dropped out, thus we had only 6 cases to follow up at the start (baseline) and 11-13 weeks after the herbal TCM treatment. The scores from the questionnaires were transformed, interpreted and compared against breast cancer patients published in the EORTC QLQ-30 Reference Values manual.
3.1.2 Results

Figure 3. QLQ-30 scores for 2 breast cancer patients receiving radio-, chemo-, hormonal and herbal TCM therapy
For two patients receiving chemo-, radio-, hormonal and herbal TCM therapy (Fig. 3), the scores for global health status were 70.3% pre-treatment (70.3% post-treatment). The mean scores for each of the functional scales pre- and (post) treatment were: physical 85.0% (90.0%), role 75.0% (66.7%), emotional 79.2% (83.3%), cognitive 83.3% (83.3%), and social 66.7% (83.3%).

The mean scores for each of the symptom scales were: fatigue 33.3% (27.8%), nausea and vomiting 25.0% (16.7%) and pain 8.3% (8.3%), dyspnoea 33.3% (16.7%), insomnia 66.7% (16.7%), appetite loss 0% (0%), constipation 0% (16.7%), diarrhea 16.7% (0%), and financial difficulties 16.7% (33.3%).

On comparing the differences (Δ) between pre- and post-TCM treatment scores, we found moderate improvements in social functioning (Δ=16.7%), dyspnoea (Δ=16.7%), diarrhea (Δ=16.7%) and clinically very significant decrease of insomnia (Δ=50.0%), and little improvement in nausea and vomiting (Δ=8.3) and fatigue (Δ=5.5%). The scores were a little worse for role functioning (Δ=8.3%) and moderately worse for constipation (Δ=16.7%) and financial difficulties (Δ=16.7%).
Figure 4. QLQ-30 scores for 1 breast cancer patient receiving radio-, hormonal and herbal TCM therapy
For one patient receiving radio-, hormonal and herbal TCM therapy (Fig. 4), the mean scores for global health status were 33.3% (33.3%). The mean scores for the functional scales were: physical 60.0% (66.7%), role 66.7% (50.0%), emotional 50.0% (50.0%), cognitive 50.0% (50.0%) and social 33.3% (66.7%). The mean scores for the symptom scales were: fatigue 55.7% (44.3%), nausea and vomiting 0% (0%) and pain 33.3% (33.3%), dyspnoea 0% (0%), insomnia 0% (33.3%), appetite loss 33.3% (33.3%), constipation 0% (0%), diarrhea 0% (0%), and financial difficulties 33.3% (33.3%).

On comparing the differences between the pre- and post-TCM treatment scores, we found little improvement in physical functioning ($\Delta = 6.7\%$), moderately less fatigue ($\Delta = 11.3\%$) and very significant improvement in social functioning ($\Delta = 33.3\%$). The scores were moderately worse for role functioning ($\Delta = 16.7\%$) and there was a very significant ($\Delta = 33.3\%$) increase in insomnia.
Figure 5. QLQ-30 scores for 2 breast cancer patients receiving hormonal and herbal TCM therapy
For two patients receiving hormonal and herbal TCM therapy (Fig. 5), the mean scores for global health status were 54.2% (62.5%). The mean scores for the functional scales were: physical 90.0% (93.3%), role 100.0% (91.7%), emotional 91.7% (83.3%), cognitive 91.7% (83.3%), and social 75.0% (75.0%).

The mean scores for the symptom scales were: fatigue 22.2% (11.2%), nausea and vomiting 0% (0%) and pain 16.7% (16.7%), dyspnoea 0% (16.7%), insomnia 33.3% (33.3%), appetite loss 0% (0%), constipation 50.0% (33.3%), diarrhea 0% (0%), and financial difficulties 0% (0%).

On comparing pre- and post-TCM treatment scores, we found insignificant change for global health status ($\Delta=8.3\%$), moderate improvement for fatigue ($\Delta=11.0\%$) and constipation ($\Delta=16.7\%$). The scores were little worse on role, emotional and cognitive function scales and the patients had much more dyspnoea.
Figure 6. QLQ-30 scores for breast cancer patient receiving only herbal TCM therapy

Baseline vs. after 11 weeks of TCM treatment:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001

Criteria:
- quality of life
- physical functioning
- role functioning
- emotional functioning
- cognitive functioning
- social functioning
- fatigue
- nausea and vomiting
- pain
- dyspnoea
- insomnia
- appetite loss
- constipation
- diarrhea
- financial difficulties
For 1 patient receiving only herbal TCM therapy (Fig. 6), we used one survey point instead of a mean of data. The scores for global health status were 100.0% (post-TCM treatment 100.0%). The scores for the functional scales were: physical 93.3% (100.0%), role 100.0% (100.0%), emotional 100.0% (100.0%), cognitive 100.0% (100.0%), and social 100.0% (100.0%).

The scores for the symptom scales were: fatigue 33.3% (11.0%), nausea and vomiting 0% (0%) and pain 16.7% (0%), dyspnoea 0% (0%), insomnia 33.3% (0%), appetite loss 0% (0%), constipation 0% (0%), diarrhea 0% (0%), and financial difficulties 0% (0%).

We found little difference between pre- and post-TCM treatment scores on the physical functioning scale (Δ=6.7%), moderately less pain (Δ=16.7%) and clinically very significant improvement for fatigue (Δ=22.3%) and insomnia (Δ=33.3%).
Figure 7. QLQ-30 reference scores compared to the baseline scores of 6 breast cancer patient in Singapore receiving herbal TCM therapy
We also calculated each patient’s baseline score on each scale of the QLQ-30 questionnaire, then took the means of the scores of the six patients as our referral values, and compared these values with the reference values given in the QLQ-C30 Reference Value Manual (Fayers et al., 1999).

The mean score of our six patients for global health status was 63.7% (reference value 66.3%).

The mean scores for the functional scales were: physical 83.9% (73.6%), role 86.1% (76.6%), emotional 81.9% (67.3%), cognitive 83.3% (83.1%), and social 69.4% (77.3%).

The mean scores for the symptom scales were: fatigue 33.3% (31.4%), nausea and vomiting 8.3% (8.8%), pain 16.7% (29.1%), dyspnoea 11.1% (20.1%), insomnia 38.9% (31.1%), appetite loss 5.6% (19.9%), constipation 16.7% (13.9%), diarrhea 5.6% (7.0%), and financial difficulties 11.1% (13.4%).

On comparing the six breast cancer patients’ baseline scores from Singapore with the scores from the QLQ-C30 Reference Value Manual of breast cancer patients (Fig. 7) [Fayers et al., 1999], we found moderately lower scoring on the symptom scales of pain and appetite loss and little less dyspnoea among our patients. On the functional scales, the scores for our patients were little better for role function and moderately higher for physical and emotional functions; there was no difference between patient populations on the other scales.
Figure 8. BR-23 scores of breast cancer patients at baseline and after 11-13 weeks of TCM treatment
To interpret the scores from the QLQ-BR23 questionnaire, we compared the mean QLQ-BR23 scores of our patients before and after herbal TCM treatment (Fig. 8). The scoring for the following items was as follows: body image pre-TCM treatment 84.7% (post-TCM treatment 83.3%), sexual functioning 30.6% (43.3%), sexual enjoyment 66.7% (44.4%), future perspective 27.8% (55.6%). The scores for the following items on the symptom scales were systemic therapy side-effects 23.9% (18.0%), breast symptoms 12.5% (18.5%), arm symptoms 12.9% (11.1%) and “upset by hair loss” 33.3% (16.7%).

As reference values for the BR-23 module are not available, we compared our findings as changes in scores over the 11-13 week period of follow up, using the baseline measurement as reference value. We concluded from the findings that after herbal TCM treatment, our patients had very much more positive “future perspective”. They also had a little better sexual functioning and the systemic therapy side effects decreased a little. On the other hand, their sexual enjoyment decreased markedly and they had a little increase in breast symptoms.
Figure 9. Single item score distribution of 6 breast cancer patients at baseline and after 11-13 weeks of TCM.
On the single item score distribution (Fig. 9), we can see that the proportion of patients complaining of having “quite a bit” or “very much” symptoms decreased after herbal TCM treatment: at baseline, 50% of the patients were complaining of “quite a bit” of insomnia, which was reduced to 0% after treatment; 20% of the patients reported “very much” to having constipation, this changed to 50% of patients complaining of “a little bit” of constipation. But the proportion of patients who had “a little bit” of dyspnoea increased from 33% to 50%. After the treatment, no patients complained of having insomnia, appetite loss or diarrhea.

As reference values for the BR-23 module are not available, we compared our findings as changes in scores over the 11-13 week period of follow up, using the baseline measurement as reference value. We concluded from the findings that after herbal TCM treatment, our patients had very much more positive “future perspective”. They also had a little better sexual functioning and a little less systemic therapy side effects. On the other hand, their sexual enjoyment decreased markedly and they had a little increase in breast symptoms.

3.1.3. Discussion

The responses of the 6 breast cancer patients to herbal TCM were generally favorable. The two patients who were using radio, chemo, hormonal (Tamoxifen) and herbal TCM therapy had much less dyspnoea, insomnia and diarrhea. The patient who was receiving radio-, hormonal therapy and herbal TCM had moderately less fatigue, while the two patients who were on herbal TCM treatment at the same time with Tamoxifen had much less constipation. When herbal TCM was the only treatment, the
patient had a very significant decrease in fatigue, pain and insomnia without
decreasing other functions or increasing disease symptoms. These might indicate that
herbal TCM had reduced the side effects of radio-, chemo- and hormonal therapy and
could be especially useful in palliative cancer treatment.

Our study has several limitations. Interpreting quality of life data can be approached
in different ways. According to Fayers (2001), one method is to use the normative
approach and compare the scores against published data. In our study, we used the
QLQ-C30 Reference Value Manual scores (derived from population in Canada,
Denmark, Norway and Sweden) as our population-based reference values because
there are no reference values for Asian populations as yet.

One of the points we need to discuss is how we can compare our findings with
reference values from a different population having national and cultural differences
i.e. Singapore’s Chinese population vs Scandinavian population. A Singapore
population-based set of national reference values is long overdue. Such values will be
of great use to future Asian QOL studies investigating the efficacy of TCM.

The second issue is how to decide whether a finding is significant. Clinical
significance is subjective and statistical significance is not an appropriate method to
define it, because “for any given change or treatment effect, the P values are affected
by the sample size” (Fayers, 2001). Osoba et al., (1998) found that when the scale
scores changed by 5 to 10 points (on the 0-100 scale), patients described their
condition as a “little” better or worse. A change of 10 to 20 points was described as a
“moderate” change, while one greater than 20 was “very much” better or worse. In
our study, we found that the 1 patient who was receiving only herbal TCM therapy
had a “little” (6.7 points) better post-TCM treatment score on physical functioning,
moderately less pain (16.7 points) and “very much” less fatigue (22.3 points) and insomnia (33.3 points). Other score differences of less than 5 points were thus considered to be clinically insignificant.

The third issue is the possible influence of repeated measurements from the learning effect through using the same questionnaire in the same patient over relatively short time intervals (1-2 weeks). This influence could perhaps be minimized by administering only 2 questionnaires per patient (baseline and post-treatment).

The fourth issue is the sample size and selection of the patients. We recruited 13 patients for this study, but over time 7 dropped out – questionnaires were not returned perhaps due to the lack of incentives.

Last but not least is the issue of the extent of missing values, which were the highest (51%) on the sexual functioning scale, thus affecting the validity of the scores for that scale. Our impression was that in line with Asian standard pattern of behavior - the patients were not willing to answer questions related to their sexual life.

It needs to be pointed out that the findings from these case studies are subjective; evidence from laboratory tests and other investigations (i.e. computer tomography scans, X-ray, ultrasound) would be helpful to document the clinical improvement of the patients and so provide further support for the role of herbal TCM in the care of cancer patients.
3.2. Extraction and characteristics of *Solanum nigrum*

3.2.1. Aims and experimental approach

It has been shown that SN crude extract and some of its individual compounds have growth inhibiting effects on different cancer cell lines: colon, nasopharynx, uterine and cervix, glioma, melanoma (Chiang *et al.*, 1991), human hepatoma (Kuo *et al.*, 2001) breast cancer (Son *et al.*, 2003), basal cell and squamous cell carcinoma (Cham *et al.*, 1987). Cai *et al.*, in 2004 reported that phenolic compounds found in SN and other traditional Chinese medicinal herbs were the dominant antioxidant components to which the anti-cancer property of these herbs are attributed to.

It has also been reported that the bioactive compounds are most likely to be the steroidal glycosides β2-solamargine, solamargine and galactotigonin (Hu *et al.*, 1999). As the breast cancer patients used the boiled water extract of SN but (as a standard, first line laboratory procedure) we used ethanolic extract of SN in our experiments, we were interested to know whether there was a major difference between the water and ethanolic extracts of SN.

Our aim was to characterize the SN.

We ran both SN ethanolic and SN water extract on UV spectrophotometer and HPLC.
3.2.2. Results

The UV spectrophotometer scan suggested that SN water extract has a high peak at a wavelength of 200-400nm (A). The SN ethanolic extract showed 2 peaks, one is similar to the water extract, the second one at 650-700nm (B).
The HPLC chromatogram showed three similar peaks (A, B, C) in both extracts, most of the compounds being highly polar.
3.2.3. Discussion

From the above experiments we can conclude that most of the SN compounds are highly polar. Both the water and ethanolic extract had a high peak at 200-500 nm where most lipids, steroids (solamargine, solanidine), vitamins and flavoproteins show absorbance. The peak at 650-700 nm in the SN ethanolic extract was perhaps due to the porphyrins, chlorophylls and derivates (Teledyne, 2003).

These experiments revealed that there was no major difference between the boiled water and ethanolic extracts of SN. The components present in both extracts perhaps were heat resistant steroidal glycosides.

3.3. Results of *in-vitro* immunomodulatory studies

3.3.1. Aims and experimental approach

Following the EORTC QOL-C30 and QLQ-BR23 analysis, we evaluated the herbal prescription of the patients and selected the 2 most frequently used medicinal herbs, *Astragalus membranaceus* (AM) and *Curcuma aromatica* (CA) for their possible immunomodulatory property.

The immunopotentiating effect of the roots of AM has been associated with its polysaccharide fractions (Shao *et al.*, 2004)

Lymphocyte activation assay was carried out to evaluate the effects of the ethanolic extracts of AM and CA on the proliferation of mouse lymphocytes under various conditions: in the presence of optimal/suboptimal doses or absence of the mitogens, concanavalin A (ConA) and lipopolysaccharide (LPS). The MTT tetrazolium assay was carried out to evaluate lymphocyte activation by measuring lymphocyte activation.
We also compared whether the AM or CA ethanolic extract has stronger immunomodulatory property. After obtaining the results, we carried out lymphocyte proliferation assays comparing ethanolic, aqueous and fractionated aqueous AM extracts. This was

Furthermore, it has been reported in the literature that AM has cytokine production modulatory effect (Mao et al., 2004), thus we were interested in the levels of IL-2, IL-6 and TNF-α cytokines in the mouse serum following AM treatment.

### 3.3.2. Results

#### 3.3.2.1. Effect of ethanolic extract of *Astragalus membranaceus* and *Curcuma aromatica*

Table 4. Effect of different concentrations of 80% ethanol extract of *Astragalus membranaceus* and *Curcuma aromatica* on BALB/c mice lymphocyte proliferation.

<table>
<thead>
<tr>
<th>µg/ml</th>
<th>AM</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10.0</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

No mitogen: + stimulation, - inhibition, empty square: no statistically significant change (p value < 0.05).

Results are from six individual experiments.
As shown in Table 4, in the absence of mitogens, both 0.1 μg/ml and 1.0 μg/ml of AM with 10.0 μg/ml of CA stimulated splenocyte proliferation. Neither extract caused significant splenocyte proliferation in the presence of B cell mitogen, LPS.

In the presence of suboptimal dose of ConA, AM at 0.1 μg/ml and 10.0 μg/ml significantly potentiated splenocyte activity. AM depressed splenocyte activity in the presence of optimal dose of ConA.

In the presence of optimal dose of ConA, CA at 0.1 μg/ml, 1.0 μg/ml and 10.0 μg/ml significantly potentiated splenocyte activity.

3.3.2.2. Effect of the fractions of *Astragalus membranaceus* water extract

Lymphocyte activation assay was carried out according to (Nakamura et al., 1986; Wu et al., 1990) to evaluate the effects of 10 μg/ml, 1 μg/ml and 0.1μg/ml ethanolic, aqueous and fractionated aqueous AM extract on the proliferation of mouse lymphocytes under various conditions:

[a] in the presence of suboptimal doses of ConA (0.3 μg/ml), LPS (1 μg/ml)

[b] In the presence of optimal doses of ConA (3 μg/ml), LPS (6 μg/ml)

[c] without mitogens.

**C0.3:** conA suboptimal, **C3:** optimal, **LPS1:** LPS suboptimal, **LPS6** optimal dose
**AM0.1, AM 1, AM10:** *Astragalus membranaceus* 0.1, 1, 10 mg/ml concentration

The results are from 2 different experiments, each concentration combination in 6 wells per experiment.
Figure 12. Immunomodulatory effect of the ethanolic extract of AM on mouse lymphocytes

* statistically significant (p value < 0.05) ** statistically significant (p value < 0.005)

Figure 13. Immunomodulatory effect of the aqueous extract of AM on mouse splenocytes
Figure 14. Immunomodulatory effect of butanolic fraction of aqueous extract of AM on mouse splenocytes

Figure 15. Immunomodulatory effect of ethyl acetate fraction of aqueous extract of AM on mouse splenocytes
The ethanolic AM extract has shown to be a stronger immunomodulator than the boiled aqueous AM extract or any of its fractions (butanol, ethyl acetate or remaining water fraction). All boiled water fractions showed a similar trend in the lymphocyte activation assay: in the presence of suboptimal dose of ConA the AM extracts stimulated the lymphocyte activity, while in the presence of optimal dose of mitogen ConA the AM extracts in higher concentration suppressed lymphocyte proliferation. However, these changes were not statistically significant. None of the AM extracts stimulated the LPS induced splenocytes proliferation.
3.3.3. Discussion

The results indicate that both AM and CA have immuno-stimulatory effect. AM acts best in the presence of suboptimal dose of ConA, while CA caused significant splenocyte proliferation in the presence of optimal dose of ConA. The responder cells are probably T-cells, as there was increased splenocyte proliferation with the T-cell mitogen, ConA. The suppression of splenocytes in the presence of optimal doses of ConA with 10.0 and 1.0 μg/ml dose of AM might be due to the toxic effect of this extract. It appears that AM has a stronger immunomodulating property as it potentiated splenocyte activity at a lower dose than CA.

The ethanolic extract of AM displayed stronger immunomodulatory property then the butanolic, ethyl acetate or the remaining water fraction of same. The results of these experiments indicate that AM and CA may have a role not only in palliative care, but also in enhancing the effects of conventional oncology therapy. The stimulating activity on human lymphocytes deserves future investigation.

3.4. Cytokine studies from mice serum

3.4.1. Aim and experimental approach

It was reported earlier that AM is able to produce pro- and anti-inflammatory cytokines (Chu et al., 1988a; Chu et al., 1988b): IL-2, TNF-α (Hong et al., 2005), 6-8 weeks old BALB/c female mice were fed orally daily for 14 days with ethanolic extract of AM (50,100, 200 mg/BW kg). Blood samples were collected by cardiac puncture and the serum was analyzed for levels of IL-2, IL-6 and TNF-α.
3.4.2. Results

Table 5. Cytokine concentration after AM treatment in BALB/c mice

<table>
<thead>
<tr>
<th>TREATMENT DOSE (mg/Bw kg)</th>
<th>CYTOKINE CONCENTRATION (pg/ml)</th>
<th>IL-2</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>28.4 (±SD0.36)</td>
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<td>&lt; 3.8</td>
</tr>
<tr>
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<td>13.9 (SD±0.02)</td>
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<tr>
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<td></td>
<td>14.6 (SD±1.1)</td>
<td>24.2 (SD±0.14)</td>
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<td></td>
<td>19.2 (SD±0.11)</td>
<td>Not enough sample</td>
<td>&lt; 3.8</td>
</tr>
</tbody>
</table>

three readings for each group, duplicate samples (mean ±SD)

3.4.3. Discussion

IL-2 level fluctuated between the different AM treatment groups. There was no consistent IL-2 inducing activity observed.

We were able to detect a more then 70% increase in IL-6 serum level in animals which were treated with a double dose (50 versa 100 mg/BW kg). The limitation of our study was that we had insufficient blood sample from the control and 200 mg/BW kg treatment group.

TNF-α levels were undetectable < 3.8 pg/ml. The lack of detection might be due to the sensitivity of the test.

Our collaborator in USA (Gangemi, 2003) conducted tests with the ethanolic AM extract provided by us. Dr. Gangemi tested the inductive effect of AM on human lymphocytes in-vitro, measuring the levels of IL-6 and TNF-α cytokines. His findings were in line with our results: the ethanolic extract of AM augmented IL-6 production,
however seemed to reduce LPS-induced TNF-α levels in a dose dependent manner. The underlying mechanism remains to be determined.

3.5. *In-vitro* cytotoxicity studies

3.5.1. Aims and experimental approach

We sought a scientific evaluation of the cytotoxic effects of some Chinese herbs widely used in breast cancer patients at 2 TCM Clinics we surveyed. The herbs were identified through a quality-of-life (QOL) analysis using the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QLQ-C30 version 3.0) and the supplementary breast cancer module (QLQ-BR23). We compared the herbal prescription of the patients who had better scores on the symptoms scale (i.e. less fatigue, pain, dyspnoea, insomnia and appetite loss) and selected two of the most frequently used herbs, *Solanum nigrum* (SN) and *Scutellaria barbata* (SB) for their possible cytotoxic effects. First we tested them on MCF-7 to confirm the herb with the stronger cytotoxic property, then tested its effects on other human breast cancer cell lines: MDA-MB-231, T-47D and ZR-75-1.

3.5.2. Results

3.5.2.1. Results of cytotoxicity assays of *Scutellaria barbata* and *Solanum nigrum* on MCF-7, MDA-MB-231, T-47D, ZR-75-1 human breast cancer cell lines
The results from both SN and SB treatment groups were statistically significant (except SB 10 μl/ml and SN 40 μl/ml) in cell growth inhibition (p value < 0.005). SN has shown stronger cytotoxic activity.
SN appeared to be a stronger cytotoxic herb than SB.

The dose-response curve showed that the effect of SN declined after reaching a plateau (maximal effective dose) at approximately 30 µg/ml, while the SB curve did not plateau. ED$_{50}$ for SN was 7.76 µg/ml (A) and for SB > 40 µg/ml (B).

We continued our cytotoxic experiments with SN on other human breast cancer cell lines.
3.5.2.2. Results of cytotoxicity assays of the ethanol extract of *Solanum nigrum* on MCF-7, MDA-MB-231, T-47D and ZR-75-1 cell lines

Figure 19. Cytotoxic effect of the ethanol extract of SN on MCF-7, MDA-MB-231, T-47D and ZR-75-1 cell lines

The ED$_{50}$ values for SN treatment were: MCF-7 ED$_{50}$=7.76 µg/ml &lt; ZR-75-1 ED$_{50}$=24.32 µg/ml &lt; MDA-MB-231 ED$_{50}$=37.08 µg/ml &lt; T-47D ED$_{50}$=106.26 µg/ml.

MCF-7 and ZR-75-1 (ER$\alpha$, bcl-2+) cell lines were the most sensitive to SN, while MDA-MB-231 (ER $\alpha$-, bcl-2+) was less sensitive, T-47D (ER$\alpha$+, bcl-2-, prolactin +, androgen +) was the least inhibited by SN. Comparing the biochemical markers and genes expressed in the different cell lines, bcl-2 and p53 might have a role in the mechanism of cytotoxicity.
3.5.2.3. Results of DNA fragmentation in MCF-7 cells treated with *Solanum nigrum*

Figure 20. DNA fragmentation in MCF-7 cells (SN 4h)

![Graph showing DNA fragmentation in MCF-7 cells after 4 hours of treatment with *Solanum nigrum*. The x-axis represents concentration (µg/ml) ranging from 10 to 1000, and the y-axis represents absorbance (490-405 nm) ranging from 0.2 to 1.6. Two lines are shown, one for supernatant and one for lysate.](image1)

Figure 21. DNA fragmentation in MCF-7 cells (SN 8h)

![Graph showing DNA fragmentation in MCF-7 cells after 8 hours of treatment with *Solanum nigrum*. The x-axis represents concentration (µg/ml) ranging from 10 to 1000, and the y-axis represents absorbance (490-405 nm) ranging from 0.2 to 1.6. Two lines are shown, one for supernatant and one for lysate.](image2)
Figure 22. DNA fragmentation in MCF-7 cells (SN 12h)

Figure 23. DNA fragmentation in MCF-7 cells (SN 24h)
Preliminary ELISA DNA fragmentation study suggested that cell death in MCF-7 cell line occurred via apoptosis. Higher dosage (50-400) µg/ml with shorter incubation time (4, 8 h) and lower dosage (5-50 µg/ml) with longer incubation time (12, 24 h) were also tested. SN stimulated nucleosome release in a concentration- and time-dependent manner: (a) after 4 hours of incubation, the necrosis was more dominant, (b) after 8 hours of incubation, nucleosome release was present, (c) after 12 hours, the intensity of the nucleosome release reached a plateau.

3.5.2.4. Results of DNA fragmentation in MDA-MB-231 cells treated with Solanum nigrum

The supernatant (S1, blue) was analyzed for necrosis and the cellular lysate was further centrifuged. The supernatant of this cellular lysate (S2, pink) was used for apoptosis detection. The logarithmic curve was generated by measuring the nucleosome content of lysate S1 and S2 treated with SN after different incubation time.

Figure 24. Cytotoxic effect and cellular DNA fragmentation of the ethanol extract of SN on MDA-MB-231 after 2h incubation
After 2 hours of incubation of MDA-MB-231 cells with SN, the DNA fragment release was more prominent than the high molecular weight DNA release. After 4 hours, the nucleosome release reached a plateau and decreased after 6 hours.
Picture 9. MDA-MB-231 cells incubated with SN extract (100X)

<table>
<thead>
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<th>4 hours</th>
</tr>
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</tr>
<tr>
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<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
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<td><img src="image5" alt="Image" /></td>
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</tr>
<tr>
<td>80 µg/ml SN</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>
2 hours
SN 100µg/ml SN

4 hours
SN 200µg/ml

SN 400µg/ml

Positive control
Examination by light microscopy showed increased cellular damage in a time- and dose-dependent manner. The characteristics of apoptosis (cell shrinkage, nuclear condensation, apoptotic bodies, cell debris) were visible after 2 hours of incubation with higher than 80 µg/ml SN dose and after 4 hours of incubation with higher than 40 µg/ml SN dose.

3.5.3. **Discussion**

DNA fragmentation studies and microscopic examination revealed that incubation with SN extract caused apoptotic cell death in both caspase-3 negative MCF-7 and caspase-3 positive MDA-MB-231 human breast cancer cells *in-vitro*. The apoptosis occurred within 4 hours, after which secondary necrosis was more prominent. In the MCF-7 cells the apoptosis probably occurred in a caspase-3 independent manner. The distinctive morphological markers of apoptosis (membrane blebbing, cell shrinkage, condensation of chromatin, presence of apoptotic bodies) and the DNA fragmentation results were suggesting that the type of cell death could be apoptosis.

3.6. **In-vivo cytotoxicity studies**

3.6.1. **Aims and experimental approach**

Our previous *in-vitro* experiences showed that certain Chinese herbs, including SN, can cause cytotoxicity via apoptosis in human breast cancer cells. We found that the ethanolic extract of SN appears to have a dose-dependent cytotoxic effect on cancer cells *in-vitro*, with an average ED$_{50}$ of 7.75 µg/ml for MCF-7 and ED$_{50}$ of 37.08 µg/ml for MDA-MB-231.

Thus we investigated further whether SN crude extract could cause tumor regression in athymic mouse model xenografted with hormone-dependent MCF-7 (after priming
with estrogen) and hormone independent MDA-MB-231 human breast cancer cells. This model is ideal to investigate the actions of antitumor drugs (Gottardis et al. 1988; Tomayko et al., 1989; Mimnaugh et al., 1991; Hawkins et al., 2000).

3.6.2. Results

3.6.2.1. Acute toxicity test of *Solanum nigrum* in normal and nude BALB/c mice

Figure 27. Acute toxicity test of *Solanum nigrum* (water extract) in Swiss albino mice

The oral acute toxicity test showed that in Swiss albino mice LD$_{50}$ was higher than 5000 mg. There was no significant change in the weight, appearance, grooming and activity of the animals after a 2 week period of observation (Fig.29).

The i.p. administration of SN in nude mice appeared to be more toxic, LD$_{50}$ being around 2000 mg. The animals injected with SN doses higher than 2000 mg showed symptoms of tachycardia, psychomotor agitation, paralysis, circulatory and
respiratory depression and eventually death within 20 minutes of SN administration. Yen et al., 2001, reported that the toxicity could be due to the SN compound solasonine. This glycoalkaloid can be transformed into a secondary metabolite in the body, entering the blood-brain barrier causing disruption in essential life functions (Schwartz et al., 2005).
Nude mice treated with non-toxic dose had no change in their weight, appearance or behavior.

3.6.2.2. Anti-tumor activity of Solanum nigrum treated MCF-7 tumor bearing oophorectomized nude mice

Prior to SN-treatment, estradiol stimulated the growth of MCF-7 breast tumor implants (Pic.10,11,12) in BALB/c oophorectomized nude mice. Most of the tumors were continuously growing even after estrogen treatment had completed.
In this study we implanted (estradiol-primed) 15 mice with MCF-7 cells, chose 20 weeks as the cut off period to shorten the duration of suffering. 9/15 did not grow tumors after 20 weeks and but maintained their body weight (20-26 grams). 6/15 grew tumors larger then $8 \times \pi / 6 \text{ mm}^3$. 5 of these tumor bearing mice were treated with SN from the time when the tumor volume reached $8 \times \pi / 6 \text{ mm}^3$. 3 out of these 5 died and 2 survived. In one of these mice the tumor disappeared, while the other mouse maintained its body weight, though the tumor continued to grow. One negative control (no SN treatment), died within a week after the tumor volume reached $8 \times \pi / 6 \text{ mm}^3$.

The total mortality among the MCF-7 implanted mice was 6/15: 2/15 died without visible tumor but were cachexic. 3/15 died despite SN 300mg/kg BW treatment, 1/15 died without SN treatment. The mortality rate for tumor bearing mice was 60%.
Picture 10. Large solid MCF-7 tumor in estrogen-primed mouse #1

Picture 11. Two solid tumors grown from mouse #1
Mice treated with SN did maintain their original weight of 20-26 grams without any change in behavior. The tumor-bearing mice without SN treatment became cachexic, with bodyweights below 20 grams (Pic.13).

Picture 13. Cachexic mouse on the left compared to SN-treated mouse on the right
3.6.2.3. Estrogen-induced uterine cancer in oophorectomized BALB/c nude mice

We investigated tumor regression in athymic mouse model xenografted with hormone-dependent MCF-7 human breast cancer cells after priming with oestrogen. As mentioned previously, this model is ideal to investigate the actions of antitumor drugs.

Fifty inbred female oophorectomized BALB/c nude mice (Animal Research Centre, Canning Vale, Australia), 8 to 12 weeks old, received implants of 60 days release 17-\(\beta\)-oestradiol (0.72 mg) pellets (Innovative Research of America, Sarasota, Florida, USA).

However, incidental findings revealed that 0.012 mg daily (0.72 mg over 60 days) estrogen dose induced endometrial carcinoma (Pic.14) with an 11/50 total mortality rate (Fig.30). On post mortem examination (Pic.13), the mice had enlarged uteri with poorly differentiated cell infiltration. The average enlarged uterine diameter was 10 mm, compared to a normal of 2-3 mm.
Picture 14. Mouse uterine tissue H&E staining

Picture 15. Enlarged mouse uteri on post-mortem examination
3.6.2.3. Anti-tumor activity of *Solanum nigrum* treated MDA-MB-231 tumor bearing oophorectomized nude mice

3.6.2.4.1. Results of therapeutic *Solanum nigrum* treatment

In this study we implanted 15 mice with MDA-MB-231 cells. The cut-off for the observation period was 8 weeks post-implant since this type of tumor is very aggressive and spreads easily.

6/15 did not grow tumors and they maintained their body weight (20-25 grams). 7/15 grew tumors, out of which 5 had multiple metastases - 1 to the spine (Pic.16a,b,c,d), 1 to the orbit (Pic.17a,b,c) These mice received SN treatment – the tumors were growing though the body weight did not drop below 20 grams. 2 animals with tumors which did not received SN treatment became cachexic. 2 mice without measurable tumor but on SN treatment also became cachexic.

**Picture 16 (a, b, c). MDA-MB-231 metastasis to the spine**
Picture 17 (a, b, c). MDA-MB-231 metastasis to the orbit

Poorly differentiated nuclei (\(\cdot\)\(\cdot\)) and apoptotic bodies (\(\rightarrow\)\(\rightarrow\)) are marked with arrows on Picture 16(c) and 17(c).

It is shown on both Picture 16. and 17. the extensive damage the MDA-MB-231 implant caused – despite the SN-treatment. Picture 16. shows (a) spinal metastasis, (b) a paraffin section of this multi-lobular tumor, (c) H&E staining of a section of the above mentioned mass. On Picture 17. is shown (a) a large, left orbital, bony metastatic mass, (b) the paraffin section of the tumor site, (c) H&E staining of a tumor slide under light microscope (40x).

On both H&E slides is evident the presence of tumor cells with poorly differentiated nuclei. Some of the apoptotic bodies are marked with arrows on Picture 16(c).
3.6.2.4.2. Results of prophylactic *Solanum nigrum* treatment

In this study we chose 10 mice, cut-off period at 8 weeks. The grouping was the following: positive control Doxorubicin 5 mg/kg (n=3), negative control 10%DMSO in saline (n=3) and SN treatment (n=4). The mortality rate in the positive control group was 100% probably due to Doxorubicin-induced cardiac toxicity. In the negative control group 67% grew tumors, while 33% had no tumor. In the 500 mg/kg BW SN treatment group, 25% grew tumor, 75% had no tumor (Fig. 29). SN showed a 75% prophylactic rate against the growth of the tumor implants.

Figure 29. Prophylactic treatment of *Solanum nigrum* (ethanolic extract) on MDA-MB-231 cancer cell implanted BALB/c nude mice
3.6.2.5. Histopathological examination of *Solanum nigrum* induced apoptosis in MDA-MB-231 cells

Picture 18. SN extract *versa* saline-treated MDA-MB-231 tumors

SN-treated

![SN-treated](image)

saline-treated

![saline-treated](image)

The H&E stained paraffin MDA-MB-231 tumor sections on the left were treated with SN and shows several apoptotic bodies (black arrows) as a marker of apoptotic cell death.

On the right panel we can see mainly necrotic tissue and less apoptotic bodies.

3.6.3. Discussion

Our experiment with the MCF-7 induced nude mice showed that the growth of the implanted breast cancer cells was highly dependent on estrogen.
The results also revealed that the 0.012 mg daily (0.72 mg over 60 days) estrogen
dose was very high and induced endometrial carcinoma with a 11/50 total mortality
rate. This seems to suggest that mice models with estrogen-dependent human breast
cancer need to be hysterectomized in order to avoid the mortality rate from uterine
cancer.

We noted that cessation of estrogen at 60 days after pellet implant did not prevent
MCF-7 cells from growing tumors as occurred even at 10 days after estrogen
cessation.

SN treatment caused irreversible tumor regression where the tumor size was relatively
small.

In the cases where the mice grew multiple tumors, SN at 300 mg/bw kg was not
efficient in inhibiting the tumor growth, but the treated animals retained their original
body weight despite there being tumor growth, while untreated animals became
cachexic.

SN treatment could be used as prevention but the dose is not efficient for the
treatment of already existing aggressive tumors.

The acute toxicity test showed that the LD$_{50}$ for oral SN treatment is higher then 5000
mg which is within relative safety limits.
CHAPTER FOUR: SUMMARY AND OVERALL DISCUSSION OF THE RESULT
4.1 Summary of the results

In the clinical phase of our study we showed that most of our breast cancer patients responded favorably to herbal TCM. The two patients who were using radio, chemo, hormonal and herbal TCM therapy had much less dyspnoea, insomnia and diarrhea. The patient who was receiving radio-, hormonal therapy and herbal TCM had moderately less fatigue, while the two patients who were on herbal TCM treatment at the same time with Tamoxifen had much less constipation. When herbal TCM was the only treatment, the patient had a very significant decrease in fatigue, pain and insomnia without decreasing other functions or increasing disease symptoms.

Our experimental results showed that breast cancer patients benefited from herbal TCM use. The possible explanation for this could be that the prescribed herbal mixture with its different compounds had different actions: This might indicate that herbal TCM had reduced side effects of radio-, chemo- and hormonal therapy and could be especially useful in palliative cancer treatment.

In the laboratory phase of our study we were seeking scientific evidence for the therapeutic activity of respective herbs prescribed for breast cancer patients. The results indicate that both AM and CA have immuno-stimulatory effect. AM acts best in the presence of suboptimal dose of ConA, while CA caused significant splenocyte proliferation in the presence of optimal dose of ConA. The responder cells are probably T-cells, as there was increased splenocyte proliferation with the T-cell mitogen, ConA. The suppression of splenocytes in the presence of optimal doses of ConA with 10.0 and 1.0 μg/ml doses of AM might be due to the toxic effect of this extract. It appears that AM has a stronger immunomodulating property as it potentiated splenocyte activity at a lower dose than CA.
The ethanolic extract of AM displayed stronger immunomodulatory property than the butanolic, ethyl acetate or the remaining water fraction.

The results of the immunomodulating experiments suggest that AM and CA may have a role not only in palliative care, but also in enhancing the effects of conventional oncology therapy.

The stimulating activity on human lymphocytes deserve future investigation. The herbs could have a chemopreventive action by blocking carcinogen activation and suppressing malignant cell proliferation (Duvoix et al., 2005).

MCF-7 and ZR-75-1 cell lines (ERα+, bcl-2+) were the most sensitive to SN, MDA-MB-231 (ER α-, bcl-2+) was less sensitive, T-47D (ERα+, bcl-2-, prolactin +, androgen +) was the least inhibited by SN. Comparing the biochemical markers and genes expressed in the different cell lines, bcl-2 and p53 might have a role in the mechanism of cytotoxicity of SN.

The possible chemotherapeutic mechanisms of SN could involve blocking of tumor cell cycle progression and/or signal transduction pathways by bonding to TNFR1 or TNFR2 receptors, inhibiting the activities of transcription factors (NF-κB), increasing the production of nitric oxide thus triggering apoptosis (Lee et al., 2003; Son et al., 2003; Heo et al., 2004).

Preliminary ELISA DNA fragmentation study suggested that cell death in MCF-7 cell line occurred via apoptosis. Tested at higher dosages (50-400) μg/ml with shorter incubation time (4, 8 h) and lower dosages (5-50 μg/ml) with longer incubation time (12, 24 h), SN stimulated nucleosome release in a concentration- and time-dependent manner: a) after 4 hours incubation the necrosis was more dominant, b) after 8 hours incubation the nucleosome release was present, c) after 12 hours the intensity of the nucleosome release reached a plateau.
Further cellular DNA fragmentation studies and microscopic examination revealed that incubation with SN extract caused apoptotic cell death in MDA-MB-231 human breast cancer cells *in-vitro*. The apoptosis occurred within 4 hours, after which secondary necrosis was more prominent. The distinctive morphological markers of apoptosis were evident.

Our *in-vivo* experiment with the MCF-7 induced nude mice model showed that the growth of the implanted breast cancer cells was highly dependent on estrogen. Accidental observations also revealed that the 0.012 mg daily (0.72 mg over 60 days) estrogen dose was very high and induced endometrial carcinoma with a 22% total mortality rate. This seems to suggest that mice models with estrogen-dependent human breast cancer need to be hysterectomized in order to avoid mortality from uterine cancer.

We noted that cessation of estrogen at 60 days after pellet implant did not prevent MCF-7 cells from growing into tumors as this occurred even 10 days after estrogen cessation.

SN treatment caused irreversible tumor regression where the tumor size was relatively small.

In the cases where the mice grew multiple tumors, SN at 300 mg/BW kg was not efficient in inhibiting the tumor growth, but the treated animals retained their original body weight despite there being tumor growth, while untreated animals became cachexic.

SN treatment could be used in breast cancer prevention. It was however not efficient for the treatment of pre-existing aggressive tumors at the doses studied.

The acute toxicity test showed that the LD$_{50}$ for oral SN treatment (5000 mg) was within relative safety limits.
4.2 Overall discussion

The present work is an attempt to evaluate the efficacy of herbal TCM treatment in breast cancer. Scientific evaluation of TCM is extremely challenging. Several biomedical experiments have described many possible mechanisms behind the success of TCM herbal therapy in cancer: some plants/components have anti-oxidant, anti-angiogenesis, immunostimulator, cytotoxic, cytostatic effects. The molecular, genetic mechanisms behind these therapeutic effects are not clear. There is a need for scientific proof and clinical validation, including chemical standardization of complex herbal formulations, in-vitro and in-vivo biological assays, animal models and clinical studies (Yuan et al., 2000).

Future research and development in herbal TCM should adopt a clinical efficacy-driven strategy and plan randomized, double-blind placebo trials to evaluate their efficacy. However, the obstacle is how to generalize an individualized treatment? In our clinical case studies we described 6 patients whose QOL improved and we analyzed the prescribed medicinal herbs. This study has demonstrated in the laboratory that these herbs are effective individually, but our clinical findings suggests that the efficacy of TCM herbs is increased when they are used together, probably due to their synergistic interaction.

As herbal TCM is already in use for over two thousand years, the efficacy driven approach should have priority over the current mechanism centered approach (Tang, 2001).

TCM has a profound influence on the health care system in Singapore and should be integrated into supportive cancer care, combined with biomedical practices.
CHAPTER FIVE: CONCLUSION AND FUTURE STUDIES
5.1 Conclusion

The following results have been demonstrated:

1. Herbal TCM improved quality of life of the recruited breast cancer patients.

2. *Astragalus membranaceus* and *Curcuma aromatica* exhibited immunostimulatory effect in splenocytes.

3. *Solanum nigrum* had cytotoxic effects against both MCF-7 and MDA-MB-231 breast cancer cells *in-vitro* and *in-vivo*, and appeared to have a protective effect against the growth of metastatic breast cancer.

The findings of this study indicate that:

1. *Astragalus membranaceus* and *Curcuma aromatica* enhanced T lymphocyte proliferation and indirectly stimulated cytokine production.

2. *Solanum nigrum* induced cytotoxicity (in both estrogen-dependent and -independent human breast cancer cells) was shown to be by apoptosis.

5.2 Future studies

The results of this study show that *Solanum nigrum* may be useful in the prevention and/or treatment of invasive, non-hormone responsive breast cancer.

It would be worthwhile to identify the bioactive compound(s) of this herb, to further investigate the molecular mechanisms of its cytotoxic action and to evaluate the effect of the compound(s) on the apoptotic pathway.
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