1. Introduction

1.1. Natural products in anticancer therapy

The role of natural products as a source for remedies has been recognised since ancient times (de Pasquale, 1984). With the development of organic chemistry, synthetic products became the mainstream in modern health-care system. Despite major scientific and technological progress in combinatorial chemistry, however, drugs derived from natural products still make significant contributions to drug discovery today (da Rocha et al., 2001). An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (Cragg et al., 1997).

Tremendous chemical diversity makes natural products an attractive source for new therapeutic candidate compounds. Natural products provide greater structural diversity than standard combinatorial chemistry, so they offer major opportunities for finding novel low molecular weight leading structures that are active against a wide range of assay targets. Because less than 10% of the world's biodiversity has been tested for biological activity, many more useful natural leading compounds are awaiting discovery. The chemical novelty associated with natural products is greater than that of any other source: 40% of the chemical scaffolds in a published database of natural products (Dictionary of Natural Products, Chapman & Hamp; Hall) are absent from synthetic chemistry (Harvery, 2000).

Additionally, natural products that are biologically active in assays are generally small molecules with drug-like properties. In other words, they are capable of being absorbed and metabolised by the body. Moreover, natural products can be a more economical source of chemical diversity than the synthesis of equivalent numbers of diverse chemicals (Harvey, 1999).

The search for novel anti-tumour agents from natural sources continues through the collaboration of scientists world-wide in their search for novel bioactive compounds (Cragg et al., 1999). Experimental agents derived from natural products offer great opportunities to evaluate not only totally new chemical classes of anticancer agents, but also novel and potentially relevant mechanisms of action (Mukherjee et al., 2001).

Taxol is the most important natural product-derived medicine with anti-tumour activity discovered in recent years. It is a powerful anti-tumour drug that was first isolated from the bark of *Taxus brevifolia Nutt* (Hatae et al., 2000). Camptothecin, a plant alkaloid, was first isolated from *Camptotheca acuminata* by Wall et al. (1966). This compound has shown significant anti-neoplastic activity in various experimental tumour models. The cytotoxic effect of camptothecin has been shown to be targeted to the topoisomerase I (Rivory et al., 1995). Vinblastine and vincristine, anti-neoplastic vinca alkaloids, are isolated from the plant *Catharanthus roseus*. Both alkaloids are commonly used in western medicine for the treatment of breast, bladder and lung cancers, lymphomas, leukemias and various neoplastic

diseases (Hansen et al., 1972). Other prominent natural product-derived molecules include Navelbine, Etoposide, Teniposide, Topotecan and Irinotecan (Mukherjee et al., 2001)

1.2. Chemoprevention

Carcinogenesis is an abnormal alteration of differentiation, apoptosis, or both, which results from a prolonged and complex interaction between environmental stress and genetic factors (Hong et al., 1997). Using chemopreventive agents to block, reverse or prevent the development of cancers is a promising strategy for cancer therapy (Shureiqi et al., 2000). As cancer chemoprevention research has progressed, interest has turned to the investigation of natural products, a number of which have been found to be active in animal models lacking systemic toxicity (Rose et al., 1999).

The carcinogenesis process has historically been divided into three phases: initiation, promotion and progression. The complex process of multi-step carcinogenesis is subject to multiple levels of control (Manson et al., 2000a). The mechanism of chemopreventive agents is to block the initiation of carcinogenesis by the means of preventing DNA damage, which includes altering the profiles of both phase I and II drug metabolising enzymes, changing the rates of DNA repair and the scavenging of reactive oxygen and other free radical species. Chemopreventive agents suppress the promotion and progression of carcinogenesis by slowing down or reversing abnormal cell growth, even at quite late stages in the disease process. This can be achieved through the modulation of signalling pathways by inhibiting protein kinase or ornithase decarboxylase activity. It has been found that chemopreventive agents induce apoptosis through the inhibition of arachidonic acid metabolism, NF-kB activation, and mitogen activated protein kinase signalling cascades (Manson et al., 2000b).

Some natural products show a chemopreventive effect. For example, the use of green tea is associated with a lower risk of several types of cancer (Lin et al., 1999). The cancer chemopreventive effect of tea has been attributed to its major phytopolyphenols. The tea polyphenols comprise about one-third of the weight of the dried leaf, and they show profound biochemical and pharmacological activities including antioxidant activities, modulation of carcinogen metabolism, inhibition of cell proliferation, induction of cell apoptosis, and cell cycle arrest. *Panax ginseng* is a traditional Chinese medicine. It could be shown from a cohort and two case-control studies in Korea suggest that the intake of ginseng may reduce the risk of several types of cancer. When ginseng was tested in animal models, a reduction in cancer incidence and multiplicity at various sites was noted. *Panax ginseng* and its chemical constituents have been tested and their inhibiting capacity on tumour could be demonstrated (Shin et al., 2000).

1.3. Immunotherapy

Although there has been success in healing non-metastatic cancer, most forms of metastatic cancer are, in the long run, incurable with conventional treatment modalities such as surgery, radio- and chemotherapy. A major limitation of these modalities is the narrow therapeutic

window between killing neoplastic while preserving normal cells. In the search for more tumour-specific therapies that are less toxic to normal cells, immunologists have been able to develop a new approach to treating cancer, the immunotherapy. Several forms of immunotherapy are being rigorously explored in laboratories and tested in clinical trials, where they are showing promise as effective treatments for cancer (Rosenberg, 2001).

The various form of immunotherapy fall into these main categories: immune response modifiers, monoclonal antibodies and vaccine. Immune response modifiers are substances, either extrinsic or intrinsic to the body, which affect the immune response. The intrinsic group, known as biological response modifiers, includes interleukin-1 and interleukin-2, interferons, tumour necrosis factors, B cell growth factors and hematopoietic growth factors. These agents exert their influence at different stages of immune response. The first clear indication that immunological manipulation could cause the regression of established, invasive human cancers came from studies on the administration of interleukin-2 to humans with metastatic kidney cancer or melanoma (Rosenberg et al., 1998).

One group of extrinsic modifiers is referred to as immune potentiators. These include BCG, endotoxin and some extracts or components from plants that have been shown to modify the immune response and, under certain conditions, to cause the reduction of tumours or to slow their growth (Malmstrom, 2000). Lentinan, a polysaccharide extracted from shiitake, a kind of black mushroom, has been clinically applied as an anti-tumour and anti-metastatic drug, and has been reported to prevent both chemical and viral carcinogenesis. It is known that lentinan affects the tumorous vascular system, resulting in the induction of haemorrhagic necrosis which is dependent on T-cells in the tumour (Mitamura et al., 2000).

The identification of human cancer antigens has opened new approaches to the development of cancer vaccines. Tumour vaccines can enhance immunogenicity and stimulate the immune system to prevent the development of a tumour. There are two kinds of tumour vaccines. One is the tumour antigen peptide vaccine and other is the cellular tumour vaccine, both of which are produced by cell engineering techniques. Currently, two approaches are being used to enhance tumour immunogenicity: gene transfection to modify tumour cells, and antigen-presenting cells to present tumour antigens (Armstrong et al., 2001; Cunto-Amesty et al., 2001; Fong et al., 2000).

In addition, monoclonal antibodies are currently used alone or in association with radioactive substances and cytotoxic agents to enhance specificity at tumour cell killing. Antibody therapy might benefit from the use of a combination of antibodies directed against different target antigens and from the development of antibody constructs that can efficiently bind and activate tumour-directed immune effector cells (Ockert et al., 1999).

Currently, tumour immunotherapy studies are aimed at understanding the mechanisms that enable cancer to escape from immune attack. A renewed enthusiasm has been derived from the convergence of three separate channels of research: the identification of immunogenic tumour-associated antigens (TAA); an appreciation that professional antigen-presenting cells (APCs) must be activated to induce effective T cell immunity and that pattern and danger recognition receptors link innately to adaptive immunity via dendritic cells (DCs); and a

revisiting of suppressor or regulatory T lymphocytes and their ability to inhibit response against self tissue, including tumours (Smyth et al., 2001). Further laboratory research and extensive clinical testing will yield more definitive information on the amounts and mixtures of immunological substances that will prove most effective in battling cancer.

1.4. Traditional Chinese medicine (TCM) is a shortcut way

The philosophies underlying the practices are different, but both western medicine and TCM should be clinically effective in medical treatment. There is no doubt that TCM works. It has developed its own coherent theories with regard to the aetiology, diagnosis, and treatment of disease. A myriad of valuable clinical observations have occurred, some of which have provided the basis for some successful conventional medicines. For example, the Chinese herb Qing Hao (*Artemesia annua*) has been used in China for a thousand years to treat malaria. Through purification of the extract from the plant, Artemisinin (Qinghaosu) is now a very promising anti-malarial drug (Tran et al., 1996; Hien et al., 1993). Ephedrine, a widely used medication, was originally extracted from a plant used in TCM (Sheehan et al., 1992). The remarkable advantage of TCM is its abundant clinical experience and low toxicity, which has helped to develop shortcut ways to look for the bioactive components of TCM products.

The search for active ingredients and the investigation of functional mechanisms of the natural products used in TCM are becoming increasingly important. Plant or fungi parts have been used in primary anti-tumour treatments (Wasser et al., 1999; Graham et al., 2000). Several substances which are successful in tumour therapy, such as betulinic acid and indirubin, were recently isolated from products used in TCM, and their functional mechanisms were elucidated by molecular biological and cell biological methods (Pisha et al., 1995; Hoessel et al., 1999).

1.5. The biological functions of *Ganoderma lucidum* and *Polygonum cuspidatum*.

1.5.1. *Ganoderma lucidum*

Ganoderma lucidum is called "Lingzhi" in China. It is a basidiomycete, lamellaless fungus belonging to the family of polyporaceae. This medicinal mushroom has been widely used for the treatment of various diseases, including cancer, in China for more than 4000 years. Lately it has been accepted as one of the health-maintaining foods world-wide. There are many studies to date on the biological and medicinal functions of the extracts or the components of *Ganoderma lucidum.*

1.5.1.1. Immunomodulating activity

Ganoderma lucidum appears to have immunomodulating activity which is mainly due to protein or polysaccharide fraction. A protein, LZ-8, was isolated from *Ganoderma lucidum* and shown to have strong immunomodulatory activity. It has a mitogenic effect on both

human monocytes and human T cells (Kino et al., 1991). This effect is better termed "immunomodulatory" rather than "immunostimulatory" because it also prevents excess immune activity, as shown by increasing the survival time in two different models of allogeneic tissue transplantation (Murasugi et al., 1991; Van der Hem et al., 1995).

Wang et al. (2002) isolated a fucose-containing glycoprotein fraction from *Ganoderma lucidum* which induced the proliferation of spleen cells and the expression of cytokines IL-1β, IL-2 and IFN-γ. Another glycoprotein was isolated from *Ganoderma lucidum* which could stimulate the proliferation of spleen lymphocytes, and most of the activated cells in the mouse spleen lymphocytes were B cells. In addition, IL-2 production of lymphocytes was also increased. (Zhang et al., 2002).

The spore of *Ganoderma lucidum* also contains a polysaccharide which has demonstrated strong immunological activity in lymphocytes proliferation (Bao et al., 2002). A polysaccharide from fresh fruiting bodies of *Ganoderma lucidum* can stimulate human monocytes-macrophages and T lymphocytes, resulting in an increase of the concentrations of IL-1β, TNF-α and IL-6 that were 5.1, 9.8 and 29 times higher, respectively. The release of IFN-γ from T lymphocytes was also greatly promoted (Wang et al., 1997).

1.5.1.2. Anticancer activity

Ikekawa et al. (1968) first reported on the efficacy of soluble extracts from *Ganoderma lucidum* in inhibiting transplanted sarcoma 180 in mice. This host-dependent anti-tumour activity has been observed to be from the polysaccharide fraction of *Ganoderma lucidum* (Sasaki et al., 1971), and multiple similar studies subsequently confirm this observation (Maruyama et al., 1989; Zhang et al., 1994; Zhang et al., 1999; Zhang et al., 2000).

Ganoderma lucidum contains compounds called triterpenoids, which have direct cytotoxicity to tumour cells. Two triterpenoids, Lucidenic acid A and ganoderic acid E, showed significant cytotoxic activity against Hep G2 and P-388 tumour cells. (Wu et al., 2001). Six new highly oxygenated lanostane-type triterpenes were also found: ganoderic acid gamma, ganoderic acid delta, ganoderic acid epsilon, ganoderic acid zeta, ganoderic acid eta and ganoderic acid theta. All demonstrated inhibitory action against Meth-A and LLC tumour cell lines (Min et al., 2000).

Lin et al. (1991) reported that the steroid (ergosta-7,22-diene- 3 beta, 3 alpha, 9 alpha-triol) from the fruit bodies of *Ganoderma lucidum* exhibited potent inhibition of the growth of human epidermoid cancer KB cells and human hepatocellular carcinoma PLC/PRF/5 cells *in vitro*. One study showed that the extract from *Ganoderma lucidum* inhibited the growth of HeLa cells, and was capable of blocking the cell cycle at the transition from G1 to S phase and of inducing a marked decrease of intracellular calcium level (Zhu et al., 2000).

Administration of an extract of *Ganoderma lucidum* enhanced the recovery of body weight and the normalisation of the hemograms of mice before and after radiation by X-rays (Hsu et al., 1990). It was also found that a hot-water extract of the fruit body of *Ganoderma lucidum* demonstrated good radio-protective ability against DNA damage induced by hydroxy radicals

and UV irradiation *in vitro* (Kim et al., 1999a). These protective properties are associated with a reduction of side effects from radiotherapy in the tumour patient.

1.5.1.3. Anti-virus activity

Several biologically active triterpenes have been isolated from *Ganoderma lucidum* and proven effective as cytotoxic or antiviral. Ganoderiol F and ganodermanontriol were found to be active as anti-HIV-1 agents. Ganoderic acid B, ganoderiol B, ganoderic acid C1, 3 beta-5 alpha-dihydroxy-6 beta-methoxyergosta-7,22-diene, ganoderic acid alpha, ganoderic acid H and ganoderiol A were moderately active inhibitors against HIV-1 PR (el-Mekkawy et al., 1998). Other triterpenes, ganoderic acid beta, (24S)-lanosta-7,9(11)-diene-3 beta,24,25-triol (called lucidumol B), ganodermanondiol, ganodermanontriol and ganolucidic acid A showed significant anti-human immunodeficiency virus (anti-HIV)-1 protease activity (Min et al., 1998).

An acidic protein bound polysaccharide (APBP) was isolated from water soluble substances from *Ganoderma lucidum*, which exhibited more potent HSV-1 and HSV-2 antiviral activity. The antiherpetic activity of APBP seems to be related to its binding with HSV-specific glycoproteins responsible for attachment and penetration, and APBP impedes the complex interactions of viruses with cell plasma membranes. (Kim et al., 2000)

1.5.1.4. Other functions

Besides the effects mentioned above, extracts from *Ganoderma lucidum* also had other functions. It was reported that water extracts of *Ganoderma lucidum* reduced blood pressure and platelet aggregation (Kubo et al., 1983; Shimizu et al., 1985), the concentration of cholesterol in blood (Kanmatsuse et al., 1985). The extract of *Ganoderma lucidum* also has other functions, it is hepatoprotective (Kim et al., 1999b), and shows anti-microbial (Yoon et al., 1994), anti-inflammatory (Lin et al., 1993) and anti-allergic activities (Tasaka et al., 1988).

1.5.2. *Polygonum cuspidatum*

Polygonum cuspidatum Sieb. et Zucc. (Polygonaceae) is called "Huzhang" in China. The rhizome and root of the plant are the medicinal parts. Historically, the plant has been traditionally used for the treatment of skin burns, gallstones, hepatitis, inflammation, osteomyelitis and tumours in China. There have been many studies on the therapeutic effects of extracts from *Polygonum cuspidatum*.

1.5.2.1. Anticancer activity

Polygonum cuspidatum exhibits anticancer activity by acting as an antimutagen or as an inhibitor of protein-tyrosine kinase. The herb *Polygonum cuspidatum* and its active component emodin induced a dose-dependent decrease in the mutagenicity of benzo[a]pyrene (B([a]P), 2-amino-3-methylimidazo [4,5-f] quinoline (IQ) and 3-amino-1-methyl-5H-pyrido [4,3-b] indole (Trp-P-2). It was also found that both the plant extract and emodin acted as blocking and /or suppressing agents to reduce the direct mutagenicity of 1-nitropyrene (1-NP)

(Lee et al., 1991; Su et al., 1995). Three classes of protein-tyrosine kinase inhibitors, anthraquinone, stilbene, and flavonoid, were identified from *Polygonum cuspidatum* through bioassay-directed fractionation, and emodin displayed highly selective activity against two different oncogenes, the Src-Her-2/neu and ras oncogenes (Chang et al., 1996; Jayatilake et al., 1993; Jayasuriya et al., 1992).

1.5.2.2. Healing of burns

Polygonum cuspidatum also promotes healing of burns by enhancing immune system and cardiac functions. Administration of *Polygonum cuspidatum* restored impaired functions, such as the response to antigen signals, the proliferation capacity, IL-2 production and the antibody production ability of plasma cells to different degrees in severely burned mice (Luo et al., 1995; Luo, 1993). Severely burned animals survived longer while their neutrophil levels and neutrophilic adhesive rates remained near normal due to treatment with *Polygonum cuspidatum* (Wu et al., 1994; Wang et al., 1994). Wu et al. (1996) administered *Polygonum cuspidatum* to rats in the early stages of burn shock and found that plasma TNF levels remained normal, adhesive leukocytes remained nearly normal, disturbances in microcirculation were alleviated and injury to the lung was attenuated.

Another substance isolated from *Polygonum cuspidatum* is crystal No. 4, which appears to enhance cardiac and microcirculatory functions during burn shock. Administration of crystal No. 4 restored decreased heart functions, such as cardiac output, cardiac index, and stroke volume index, and restored pulse pressure to a normal level. It also decreased the number of adhesive white blood cells, the amount of open capillaries in muscles returned to near normal, allowing for an increased amount and velocity of blood flow; and the degree of tissue damage in the lung was alleviated (Zhao, 1989; Wu, 1992).

1.5.2.3. Other functions

The water extract of the rhizome of *Polygonum cuspidatum* demonstrated the strongest inhibition of xanthine oxidase, which plays a crucial role in gout (Kong et al., 2000). The extract of *Polygonum cuspidatum* also exhibited obvious effects on the duck hepatitis B virus and the human hepatitis B virus (Mi et al., 1997). It was additionally found that the extract from *Polygonum cuspidatum* appears to have antipyretic and analgesic activities, as studied in intact mice and rats (Lin et al., 1987). The extract appears to protect the gastric wall against stress ulcers, to slightly inhibit gastric secretion and to have no effect on blood pressure.

1. 6. The background of the work

A bioactive fraction (GLIS) was isolated from the fruiting body of the fungus *Ganoderma lucidum* using successive chromatographic steps. GLIS is a proteoglycan and has a carbohydrate:protein ratio of 11.5 : 1.

It was found that GLIS stimulated the proliferation of mouse spleen lymphocytes from normal and tumour-bearing mice. The lymphocytes of tumour-bearing mice are much more sensitive to this stimulus than that of normal mice. Most of the activated cells in mouse spleen

lymphocytes from normal or tumour-bearing mice by GLIS were B cells. After stimulation, the percentage of B cells in the lymphocytes from both normal and tumour bearing mice were increased several fold. The B cells were enlarged, expressed CD71 and CD25 on the cell surface and showed an increase in the secretion of immunoglobulin. Lymphocytes also showed a slightly increased production of IL-2, whereas the secretion of IL-4 was not influenced by GLIS. Furthermore, GLIS did not influence the intracellular $Ca2+$ concentration of lymphocytes; but it enhanced the expression of PKC in B cells (Zhang, 2000).

The anti-proliferative potential of the crude extract HZ from *Polygonum cuspidatum* against human tumour cell lines was evaluated in *in vitro*-test. HZ (500 µg/ml) inhibited the proliferation of SW620, MCF7, LS180, HT29 and HepG2 cells by 94%, 16%, 82%, 52%, 91%, respectively. HZ was further separated with DEAE chromatography to 7 fractions, the fraction HZ3 showing the strongest inhibitory capacity (Li, 1999).

1.7. Aim of this work

The aim of this work is to further investigate the immunostimulatory capacities of the active compound GLIS from *Ganoderma lucidum* and the anti-tumour capacities of the crude extract from *Polygonum cuspidatum*, which have shown promising activity in previous work.

The immunoactivation of macrophages and B cells by GLIS will be characterised and the mechanism stimulated by GLIS will be investigated. The chemical characterisation of GLIS will be further analysed.

The active compounds that induce apoptosis in tumour cells will be sought through bioassay-directed fractionation using different chromatographic steps from the aqueous fraction of *Polygonum cuspidatum*. The molecular mechanism of the apoptosis induced by the active compound will also be studied.