國 立 交 通 大 學

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博 士 論 文

鑑定與解析咸豐草中的免疫調節物質

Identification and Characterization of Immunomodulatory Compounds from *Bidens Pilosa*

研 究 生：張書林

指導教授：吳東昆 博士

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中文摘要

咸豐草是一種民間常用來治療感染以及調節免疫的草藥植物，為了鑑定並解析咸豐草中的生物活性物質，首先，本研究利用以 Jurkat T 細胞為主的平台，篩選咸豐草萃取物中具有調節細胞激素 IFN-γ轉錄活性的有效成份；依循以生物活性為導向的分離純化原則(BGFI)，結果顯示，咸豐草中的兩個黃酮類純物質(centaurein 與 centaureidin)，具有提升 IFN-γ的轉錄活性的能力，進一步以 centaurein 進行分子機制的探討，發現 centaurein 對 IFN-γ上游的轉錄因子 T-bet，具有提升其基因轉錄活性以及基因表達的能力。由於已知 IFN-γ能活化巨噬細胞並幫助胞內菌(例如：李斯特菌)的清除，進一步將 centaurein 以腹腔注射投予 C57BL 老鼠，結果發現 centaurein 可提升 C57BL 老鼠血液中 IFN-γ的表現量，而藉由 IFN-γ的提升也可有效幫助巨噬細胞清除李斯特菌。在預防胞內菌的感染方面，實驗結果顯示 centaurein 可有效避免李斯特菌感染所造成的老鼠死亡情形。另外在治療老鼠受到李斯特菌感染的動物模式上，則發現不論單獨投予 centaurein 或將 centaurein 結合低劑量的抗生素(ampicillin)使用，都具有提升老鼠存活率的效果。

此外，本研究也利用體外誘導輔助型 T 細胞分化的平台，篩選咸豐草中對第一型輔助型 T 細胞(Th1)與第二型輔助型 T 細胞(Th2)的細胞分化具有調節能力的有效成份。依循以生物活性為導向的分離純化原則(BGFI)，實驗結果顯示，咸豐草的丁醇分層萃取物以及兩個聚乙炔類純物質(2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) and

3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**))，具有抑制第一型輔助型 T 細胞分化並促進第二型輔助型 T 細胞分化的能力。進一步以第一型輔助型 T 細胞(Th1)所主導的非肥胖型自體免疫糖尿病老鼠(NOD)為動物模型進行研究，結果發現，咸豐草的丁醇分層萃取物以及兩個聚乙炔類純物質，可明顯抑制 NOD 老鼠的發病情形，除了可有效避免尿糖以及血糖的升高，並且可幫助血中胰島素的正常分泌。

藉由本論文研究結果，除了建立以 T 細胞為主的模型，藉以篩選能調控細胞激素表達或具有調節第一型輔助型T細胞(Th1)與第二型輔助型T細胞(Th2)細胞分化的植物有效成份，更以咸豐草為例，成功鑑定並解析出咸豐草中具有免疫調節功能的純物質，本研究除了對民間使用咸豐草來治療感染以及糖尿病提供科學證據，更對植物藥的研究提供一套藥物開發平台。

Identification and Characterization of Immunomodulatory Compounds from *Bidens Pilosa*

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Abstract

B. pilosa has been claimed as an anti-infectious or immunomodulatory folk medicine. However, the pharmacological evidences for the traditional use of this plant are still poor. To characterize the immunomodulatory compounds from this plant, firstly, we screened plant constituents of *B. pilosa* which able to modulate IFN-γ production in T cells using luciferase reporter assays. Our data showed that two flavonoids from *B. pilosa*, centaurein and centaureidin, with the ability to up-regulate IFN-γ transcription, could be isolated using a BGFI procedure. Centaurein increased the IFN-γ expression in primary T and NK cells and the IFN-γ level in mice serum. Centaurein elevated the transcription of T-bet but not GATA-3, which is consistent with its effect on that of IFN-γ but not IL-4. The sera with elevated IFN-γ levels from the centaurein-treated mice could clear *Listeria* in macrophages. In vivo studies showed that centaurein protected mice against *Listeria* infection. Moreover, centaurein per se or in combination with antibiotics could treat *Listeria* infection.

We also aimed to identify the bioactive compounds with the ability to modulate

T cell differentiation. By using T cell differentiation assays, two polyacetylenic compounds, 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) and 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**), were identified from *B. pilosa* based on a BGFI fashion. These two polyacetylenic compounds could suppress the differentiation of naïve T helper (Th0) cells into Th1 cells but promote the differentiation of Th0 cells into Th2 cells. Furthermore, *in vivo* results showed that treatment with these compounds significantly prevented the diabetes progression in NOD mice.

These results suggest that the bioactive compounds from *B. pilosa* were identified with the screening methods and functionally characterized with *in vivo* mouse models. This approach may directly contribute to the ethnopharmacological effects of *B. pilosa* and furthermore, to facilitate drug discovery from plant sources.

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Abbreviations

AP-1	activator protein-1
BGFI	bioactivity-guided fractionation and isolation
CC50	cytotoxic concentration 50%
FACS	fluorescence-activated cell sorting
GATA-3	GATA binding protein-3
GFP	green fluorescence protein
h	hour
HPLC	high performance liquid chromatography
IC50	inhibitory concentration 50%
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
LT	lymphotoxin
min	minute
MTT	methylthiazoletetrazolium
NFAT	nuclear factor of activated T cells
NFκB	nuclear factor-κB
NOD	non-obese diabetes
PBS	phosphate-buffered saline
PMA	phorbol 12-myristate 13-acetate
PHA	phytohemagglutinin
TNF	tumor necrosis factor
T-bet	T-box expressed in T cells
TCR	T-cell receptor
Th	T helper cells
Th0	naïve Th
Th1	type I Th
Th2	type II Th
UV	ultraviolet

Chapter 1. Introduction

1.1 *Bidens pilosa*

Bidens pilosa L. is a common plant in the genus *Bidens* which contains approximately 230 species (Carr, 2009). It is a perennial herb widely grown in tropical and subtropical areas of the world. *B. pilosa* is glabrous or hairy and has green opposite leaves, which are serrate, lobed or dissected. It has white or yellow flowers and long narrow ribbed black achenes. *B. pilosa* can reach a height of 60 cm on average and 150 cm in favorable environment (Wiart, 2000) (Figure 1). Its variants include *B. pilosa* var. *radiata*, var. *minor*, var. *pilosa* and var. *bisetosa*. *B. pilosa* bears common names such as Spanish needles, beggar's ticks, devil's needles, cobbler's pegs, broom stick, pitchforks and farmers' friends because its achenes stick to people and animals (Wiart, 2000). *B. pilosa* is thought to originate from South America and spread across the world (Geissberger and Sequin, 1991). Due to its invasive tendencies, *B. pilosa* is often characterized as a weed (Carr, 2009). Minimal agricultural techniques are required for its cultivation.

Figure 1. Photographs of *B. pilosa* L. var. *radiata*.

1.1.1 Ethnomedical use of *B. pilosa*

Fresh or dried shoots and leaves of *B. pilosa* are utilized as vegetables, sauces and teas (Chih et al., 1995). Food and Agriculture Organization (FAO) of the United Nations once promoted the cultivation of *B. pilosa* in Africa because it is easy to grow, edible and safe (Division, 1997).

Either whole plant or different parts of *B. pilosa* has been used as a medicinal herb for a variety of diseases worldwide (Botsaris, 2007; Li, 2002; Wiart, 2000). For instance, it is used to treat infections in South Africa and Taiwan, stomach illness in Mexico, malaria and liver disorders in Brazil and diabetes in Taiwan and South America (Brandao et al., 1997; Marles and Farnsworth, 1995; Pereira et al., 1999; Ubillas et al., 2000). Aerial parts (leaves, flowers, seeds and stems) and whole plants (aerial parts plus roots) of *B. pilosa* are used as ingredients in folk medicines. It is frequently taken in the forms of dried powder, decoction, or tincture (Rendu et al., 1989). Despite lack of precise scientific evaluation, a dose of dried *B. pilosa* at 2 g per person, twice per day, is thought to be safe.

1.1.2 Chemical constituents of *B. pilosa* and their therapeutic functions

Compounds that were isolated from *B. pilosa* include polyynes (Brandao et al., 1997; Chang et al., 2000; Redl et al., 1994), flavonoids (Alcaraz and Jimenez, 1988;

Wang et al., 1997), phenylpropanoid (Chiang et al., 2005) and terpenes (Zulueta et al., 1995). *B. pilosa*. has been used in traditional medicine to treat stomach illnesses, liver disorders (Brandao et al., 1997; Chih et al., 1995; Geissberger and Sequin, 1991; Jager et al., 1996), cancers (Chang et al., 2001; Kumari et al., 2009; Kviecinski et al., 2008; Ong et al., 2008; Pereira et al., 1999; Sundararajan et al., 2006), viral infections, inflammation (Pereira et al., 1999), microbial infections (Khan et al., 2001), diabetes (Rucker et al., 1992; Ubillas et al., 2000), ulcers (Alvarez et al., 1999; Tan et al., 2000), allergy (Seelinger et al., 2008a), hypertension (Dimo et al., 2001; Dimo et al., 1999; Dimo et al., 2003; Dimo et al., 2002) and malaria (Andrade-Neto et al., 2004; Brandao et al., 1997; Kumari et al., 2009; Oliveira et al., 2004). *B. pilosa* plants also were shown to have chemopreventive (Chiang et al., 2004; Yuan et al., 2008; Zhong et al., 2007), anti-oxidant (Yang et al., 2006), prostaglandin-reducing (Jager et al., 1996), muscle-relaxing (Nguelefack et al., 2005) and immunomodulatory (Abajo et al., 2004) properties.

1.1.2.1 Anti-inflammatory compounds of *B. pilosa*

Phenylpropanoids, polyynes and flavonoids of *B. pilosa* have anti-inflammatory activities (Alcaraz and Jimenez, 1988; Chiang et al., 2005; Pereira et al., 1999). Ethyl caffeate, a phenylpropanoid compound isolated from *B. pilosa*, was shown to inhibit

NFκB activation and suppress the production of inflammatory mediators, iNOS, COS-2 and PGE2 *in vitro* or in animal studies (Chiang et al., 2005). Ethyl caffeate at 1 μg/ml significantly inhibit the expression of iNOS mRNA expression in LPS-treated macrophages (Chiang et al., 2005). 2-*O*-ß-glucosyltrideca-11(*E*)-en-3,5,7,9-tetrayn-1,2-diol, a polyyne of *B. pilosa*, showed anti-inflammatory and immunosuppressive activities based on lymphocyte proliferation assays and zymosan-induced arthritis mouse model (Pereira et al., 1999). A flavonoid of *B. pilosa*, quercetin 3-*O*-β-D-galactopyranoside, was shown to have anti-inflammatory activities (Alcaraz and Jimenez, 1988; Geissberger and Sequin, 1991). Luteolin, a flavonoid of *B. pilosa*, shows anti-inflammatory and anti-allergic activity (Kumazawa et al., 2006; Seelinger et al., 2008a).

1.1.2.2 Anti-tumoral compounds of *B. pilosa*

Solvent extracts of *B. pilosa*, particularly those of water and hexane fractions, show remarkable cytotoxicity to Hela, KB and leukemic cell lines (Chang et al., 2001; Sundararajan et al., 2006). Flavonoids, polyynes and fatty acids, isolated from *B. pilosa* were shown to be cytotoxic against a variety of tumor cells and/or vascular endothelial cells. The cytotoxicity of *B. pilosa* compounds is related to their anti-tumoral activity. Among the flavonoids, Luteolin (Hoffmann and Holzl, 1989;

Seelinger et al., 2008b) and butein (Yit and Das, 1994), were reported to induce cell apoptosis of different tumors. Polyynes such as 1,2-dihyroxytrideca-5,7,9,11-tetrayne , 1,3-dihydroxy-6(*E*)-tetradecene-8,10,12-triyne and 1,2–dihydroxy-5(*E*)-tridecene-7,9,11-triyne were isolated from *B. pilosa* and shown to inhibit angiogenesis (Wu et al., 2007; Wu et al., 2004), a key step to tumor growth and metastasis (Wu et al., 2007; Wu et al., 2004). Another polyyne, 1-Phenylhepta-1,3,5-triyne, were shown to have cytotoxicity to tumor cell lines (Alvarez et al., 1996). Cytotoxicity of *B. pilosa* polyynes is consistent with the fact that polyynes and/or their glucosides are anti-tumoral agents (Siddiq and Dembitsky, 2008). Besides, linoleic acid, a fatty acid of *B. pilosa* (Chang et al., 2000), was shown to have low cytotoxicity to carcinoma cells (Kadota et al., 2005).

1.1.2.3 Anti-diabetic compounds of *B. pilosa*

Crude extracts of *B. pilosa* were reported to treat type 2 diabetes (Hsu et al., 2009; Ubillas et al., 2000), which is caused by the defective insulin release and/or action in mouse models. *B. pilosa* extract improves type 2 diabetes as evidenced by decrease in blood glucose levels, islet destruction and the glycosylation of hemoglobulin A1c in db/db mice, a mouse model of type 2 diabetes (Hsu et al., 2009). Two polyynes, 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne

and 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne show anti-hyperglycemic effects on type 2 diabetes (Ubillas et al., 2000).

1.1.2.4 Anti-viral compounds of *B. pilosa*

Flavonoids, phenylpropanoids and long chain fatty acids of *B. pilosa* were reported to have anti-viral bioactivities. They target different steps of viral life cycle. Luteolin shows anti-viral bioactivities (Tewtrakul et al., 2003; Yi et al., 2004). Luteolin inhibits the integrase activity of human immunodeficiency virus (HIV) (Tewtrakul et al., 2003) and the entry of the severe acute respiratory syndrome coronavirus (SARS virus) into host cells (Yi et al., 2004). Dicaffeoylquinic acids, present in *B. pilosa* (Chiang et al., 2004), are selective inhibitors of HIV integrase (McDougall et al., 1998). *B. pilosa* also contains linoleic acid (Chang et al., 2000), which interferes with poliovirus replication via protease inhibition (Hwang et al., 2008).

1.1.2.5 Anti-protozoan compounds of *B. pilosa*

Crude extracts of *B. pilosa* were reported to be effective against protozoa such as *Leishmania* and *Plasmodium*. Butein, a flavonoid present in *B. pilosa* (Hoffmann and Holzl, 1989), shows anti-leishmanial activity (Nielsen et al., 1998).

1-Phenylhepta-1,3,5-triyne (Kumari et al., 2009), quercetin 3,3'-dimethyl ether 7-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (Brandao et al., 1998), quercetin 3,3'-dimethyl ether-7-*O*-β-D-glucopyranoside (Brandao et al., 1998) and 1-phenyl-1,3-diyn-5-en-7-ol-acetate (Brandao et al., 1997) were proposed as the bioactive compounds from the crude extracts of *B. pilosa.* for malaria, which is caused by *Plasmodium* (Oliveira et al., 2004).

1.1.2.6 Anti-microbial compounds of *B. pilosa*

Polyynes of *B. pilosa* show anti-microbial activities through direct killing of bacteria. 1-Phenylhepta-1,3,5-triyne, a polyyne of *B. pilosa* (Alvarez et al., 1996), was shown to be phototoxic against microbes (Wat et al., 1979; Wat et al., 1980). Further studies in the identification of bioactive compounds and mode of action are useful to understand the medicinal use of *B. pilosa* against bacteria.

1.2 The immune response

The physiologic function of the immune system is defense against foreign substances including pathogenic microorganisms and macromolecules such as proteins and polysaccharides. The immune responses can be divided into humoral and cell-mediated responses. The humoral immunity is mediated by the antibodies produced by B cells and caused the destruction of extracellular pathogens. Antibody functions as the effector of the humoral response by binding to antigen and facilitating its clearance from the body. However, the activation and differentiation of B cells into antibody-secreting plasma cells usually requires T cells (Charles A., 2005).

In the cell-mediated responses, various subpopulations of T cells recognize antigen presented on self-cells. Both activated T helper cells (Th) and T cytotoxic (Tc) cells serves as effector cells in cell-mediated responses. Th cells respond to antigen by producing various growth factors known collectively as cytokines which can activate various phagocytic cells and help them to phagocytose and kill microorganisms more effectively. Tc cells respond to antigen by developing into cytotoxic T lymphocytes (CTLs), which participate the killing of altered self-cells (e.g., virus-infected cells and tumor cells) (Charles A., 2005). Overall, T cells are key players in human immunity including cellular and humoral immunity. T-cell abnormalities and aberrant T cell cytokine profiles have been implicated in the immunodeficiencies, infectious diseases

and autoimmune disorders.

When a naïve T cell encounters its specific antigen for the first time, it is stimulated to differentiate into an effector T cell. Effector T cells can secrete cytokines (*e.g.*, IFN-γ and IL-4 *etc.*) which play an important role in activating B cells, macrophages and various other cells that participate in the immune response and eventually lead to the removal and destruction of the pathogens. Thus, T cells are normally the primary targets for immunity-related drugs. For instance, cyclosporine A can inhibit T cell activation and therefore, suppress graft rejection.

1.2.1 Biological actions of IFN-γ

T cells can produce immunomodulatory cytokines on encountering antigens or on activation. Among these cytokines, IFN-γ is a key cytokine produced by activated T cells. IFN-γ modulates a variety of immune responses including pathogen clearance, tumor eradication, T cell activation and inflammatory responses (Abbas et al., 1996; Swain, 1999) (Figure 2). The functions of IFN-γ are important in cell-mediated immunity against intracellular microbes. IFN-γ enhances the microbicidal function of macrophages through formation of nitric oxide and reactive oxygen intermediates (ROI). IFN-γ stimulates the expression of class I and class II MHC molecules and co-stimulatory molecules on antigen presenting cells. IFN-γ also activates cytotoxic T

cells and increases the cytolytic activity of NK cells. IFN-γ promotes the differentiation of naïve T cells to the Th1 subset and inhibits that of Th2 cells and induces B cells switching to certain immunoglobulin isotypes. The expression level of IFN-γ has been reported to be regulated by many nuclear factors such as T-bet, NFAT, NFκB, AP-1 and so on (Penix et al., 1996; Sica et al., 1997; Sweetser et al., 1998; Szabo et al., 2000). Accordingly, defects in IFN-γ pathway are associated with the susceptibility to disease caused by intracellular pathogens and some viruses (Dorman and Holland, 2000).

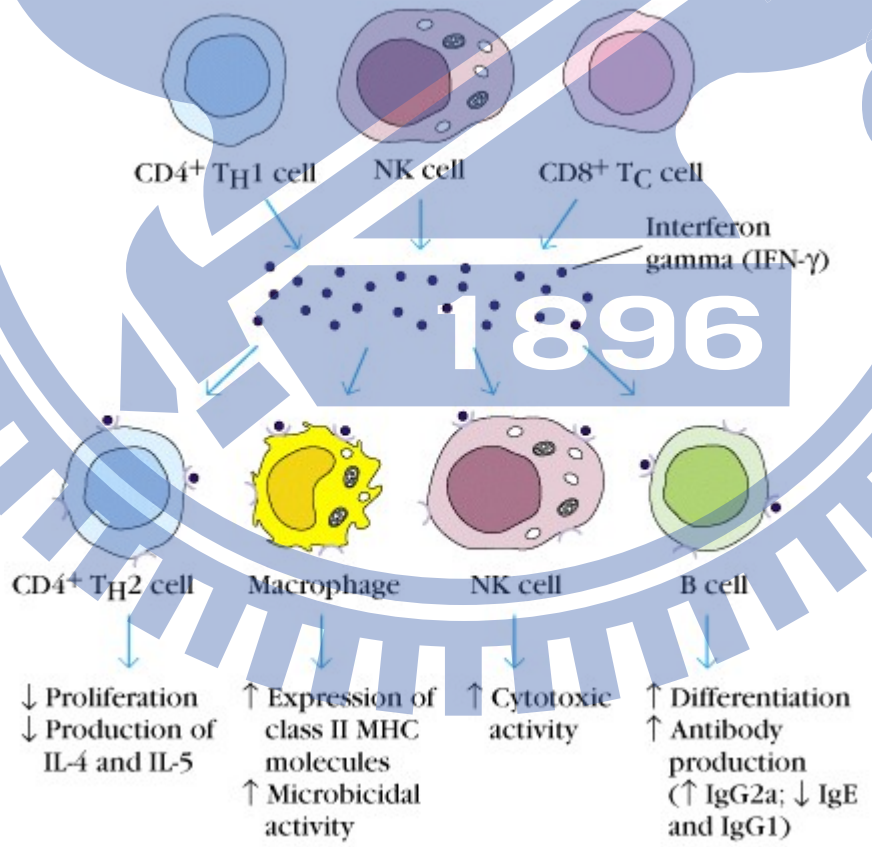

Figure 2. Immunomodulatory actions of IFN-γ.

1.2.1.1 Host immunity to intracellular pathogen infection

A characteristic of intracellular bacteria including *Listeria*, *Brucella*, *Legionella*, *Francisella* and *Mycobacterium* is their ability to survive and replicate within phagocytes or other cells and therefore escape from the host immune bacterial defense (Kaufmann, 1993). *Listeria* causes listeriosis in animals and humans. Once ingested, it can cross the intestinal barrier entering the blood and then invade the spleen and liver where the bacteria multiply (Figure 3) (Hamon et al., 2006). Finally, *Listeria*, released from both organs disseminates through blood to the nervous system, leading to bacteremia and meningitis or encephalitis respectively (Conlan and North, 1991).

Antibiotics (ampicillin, vancomycin, *etc.*) and immunotherapeutics (IFN-γ, IL-1, IL-2, *etc.*) are frequently used as anti-infective agents to combat *Listeria* and other intracellular bacteria (Calder, 1997; Haak-Frendscho et al., 1989; Jones et al., 1997; Kurtz et al., 1989; Roll et al., 1990). Unfortunately, antibiotic-resistant *Listeria* species are sometimes discovered (Facinelli et al., 1991; MacGowen et al., 1990). To overcome this antibiotic resistance, the development of immunomodulatory therapeutics for microbes such as *Listeria* is urgently needed because immunomodulatory therapeutics, in contrast with antibiotics, can not generate antibiotic-resistant bacteria (Buchwald and Pirofski, 2003).

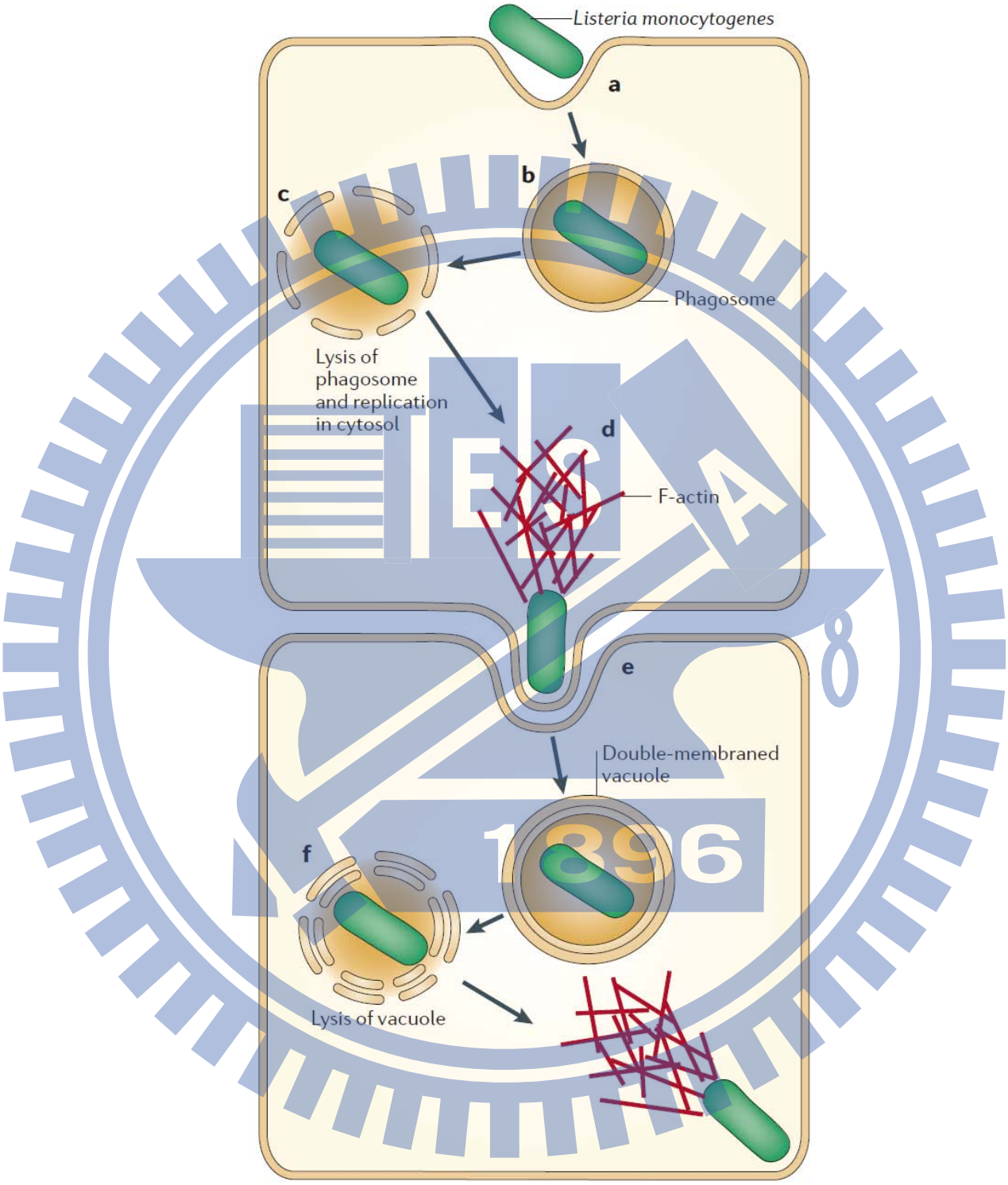

Figure 3. Schematic representation of the *Listeria monocytogenes* life cycle.

1.2.2 T helper cells differentiation

Upon encountering antigens, naïve $CD4^+$ T helper (Th0) cells can differentiate into two distinct subsets, namely type 1 T helper (Th1) cells and type 2 T helper (Th2) cells, as defined and categorized by their functions and cytokine profiles. Th1 cells are characterized by the secretion of Th1 cytokines including interferon gamma (IFN-γ), tumor necrosis factor (TNF)-α, interleukin (IL)-2, and lymphotoxin (LT). In contrast, Th2 cells are characterized by the secretion of Th2 cytokines (IL-4, IL-5, IL-13 and/or IL-10) (Murphy and Reiner, 2002). Cytokines have been demonstrated to be key players in the differentiation of Th0 cells into Th1 or Th2 cells. For examples, IL-12 can drive differentiation of Th0 into Th1 cells and IL-4 can drive differentiation of Th0 into Th2 cells (Abbas et al., 1996). Interestingly, Th1 cells and their cytokines such as IFN-γ enhance Th1 generation but inhibit Th2 generation, whereas Th2 cells and their cytokines such as IL-4 promote Th2 generation but inhibit Th1 generation. That is to say, Th1 cells and their cytokines antagonize the function of Th2 cells and their cytokines and vice versa.

1.2.2.1 T helper cells differentiation and autoimmune diseases

Under aberrant situations, a Th1/Th2 imbalance and various cytokines are thought to cause autoimmune diseases. For instance, Th1 cells and their cytokines (e.g.

IFN-γ) can exacerbate Th1-mediated autoimmune diseases such as non-obese diabetic (NOD) disease, rheumatoid arthritis and Crohn's disease but improve airway hypersensitivity, asthma and allergy. On the contrary, Th2 cells and their cytokines can antagonize Th1 cells, their cytokines and Th1-modulated disorders (Abbas et al., 1996). Therefore, skewing T cells differentiation or cytokine administration is frequently used to treat immune disorders.

Human type 1 insulin-dependent diabetes mellitus (IDDM) is caused by the autoimmune destruction of β cells in the pancreatic islets. Autoimmune diabetes is clinically diagnosed as leukocyte invasion into the pancreatic islets, called insulitis. These leukocytes include T cells ($CD4^+$ and $CD8^+$), B cells, macrophages and NK cells. However, compelling evidence has revealed that $CD4^+$ Th1 cells play a dominant role in the progression of diabetes in NOD mice model (Katz et al., 1995) (Toyoda and Formby, 1998). NOD mice can spontaneously develop a Th1 cell-mediated autoimmune diabetes with the destruction of pancreatic β cells. NOD mice with autoimmune diabetes exhibit many immunopathological features as human with type 1 insulin-dependent diabetes mellitus, and as such, serve as an ideal mouse model for IDDM research (Castano and Eisenbarth, 1990). During the progression of non-obese diabetes, leukocytes first infiltrate into the pancreatic islets, and then β cells are destroyed, leading to hypoinsulinemia and hyperglycemia.

There are two general strategies for halting the progression of IDDM. One is to suppress or eliminate the autoimmunity before it results in overt clinical disease. The other is the use of insulin replacement (DCCT Res. Group, 1993), β cell replacement (Ramiya et al., 2000) or a combination of immunosuppressants and β cell transplant (Stratta and Alloway, 1998) to treat this disease with insulin insufficiency or deficiency. The ways to suppress the autoimmunity against β cells are immunosuppressants, Th1/Th2 balance, or destruction/depletion of autoimmune cells (Christianson et al., 1993; Katz et al., 1995; Kurasawa et al., 1990). T cells seem to be a main target for autoimmune suppression or elimination (Harlan and von Herrath, 2005). For instance, immunosuppressants such as FK506 can be used to suppress T cell functions and therefore, to prevent or treat IDDM (Kurasawa et al., 1990). Depletion of $CD4^+$ or $CD3^+$ T cells using anti-CD4 or anti-CD3 antibodies have been demonstrated to be able to prevent IDDM (Koike et al., 1987; Miller et al., 1988). Besides, manipulation of the Th1/Th2 cell shift is thought to be an approach to treat autoimmune diseases (Adorini et al., 1996; Kroemer et al., 1996). Nevertheless, too few prophylactic or therapeutic drugs are now available for autoimmune disease such as IDDM.

1.3 Research summary

Natural products have been an important source of new medicines. It is estimated that ~28% of drugs commercially available come from plants or their derivatives (Newman and Cragg, 2007). However, cost-effective and time-saving screening methods for bio-active phytocompounds need to be established in order to facilitate drug discovery from plant sources. *B. pilosa* has been used to treat different categories of illnesses such as inflammatory disorders, immunological disorders, digestive disorders, infectious disease, cancers, diabetes and hypertension. In addition, it is also used for chemopreventive purposes. Some phytocompounds purified from *B. pilosa* have been claimed to possess anti-inflammatory, immunosuppressive, anti-bacterial and anti-malaria activities. However, these plant components are insufficient to explain the habitual use of *B. pilosa* as anti-infectious or immunomodulator agents in various regions of the world. Therefore, further characterization of bioactive immunomodulatory compound(s) from this plant is required.

Since IFN-γ is a potent immune modulator in macrophage activation and clearance for intracellular pathogens, we aimed to identify phytocompounds with the ability to modulate IFN-γ expression in immune cells (T cells or NK cells). We first screened plant constituents of *B. pilosa* which able to modulate IFN-γ expression using luciferase reporter assays (Figure 4). Our data showed that two flavonoids from

B. pilosa, centaurein and centaureidin, with the ability to up-regulate IFN-γ transcription, could be isolated using a bioactivity-guided fractionation and isolation (BGFI) procedure. Centaurein increased the IFN-γ expression in T and NK cells and the serum IFN-γ level in mice. We also examined the likely mechanism by which centaurein could up-regulate IFN-γ transcription and investigated the role of centaurein to protect against or treat an intracellular pathogen (e.g., *Listeria*). Centaurein elevated the transcription of T-bet but not GATA-3. In addition, centaurein effectively protected mice against *Listeria* infection. Moreover, centaurein per se or in combination with antibiotics could treat *Listeria* infection. Here, we identified and proved the concept that such immune modulator as centaurein can protect against or treat an intracellular pathogen in mice via an elevation of IFN-γ expression.

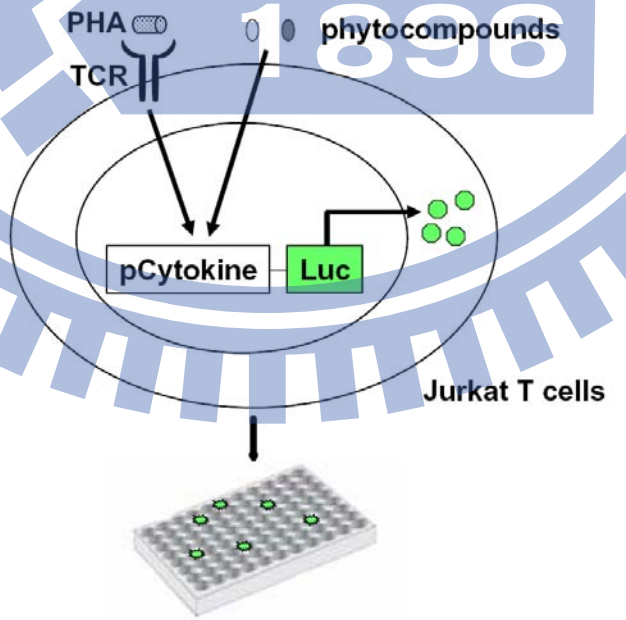

Figure 4. T cell-based transcriptional assay.

We also aimed to identify the bioactive compounds with the ability to modulate T cell differentiation. In this study, two polyacetylenic compounds, 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) and 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**), were identified from *B. pilosa* using T cell differentiation assays (Figure 5) based on a BGFI principle.

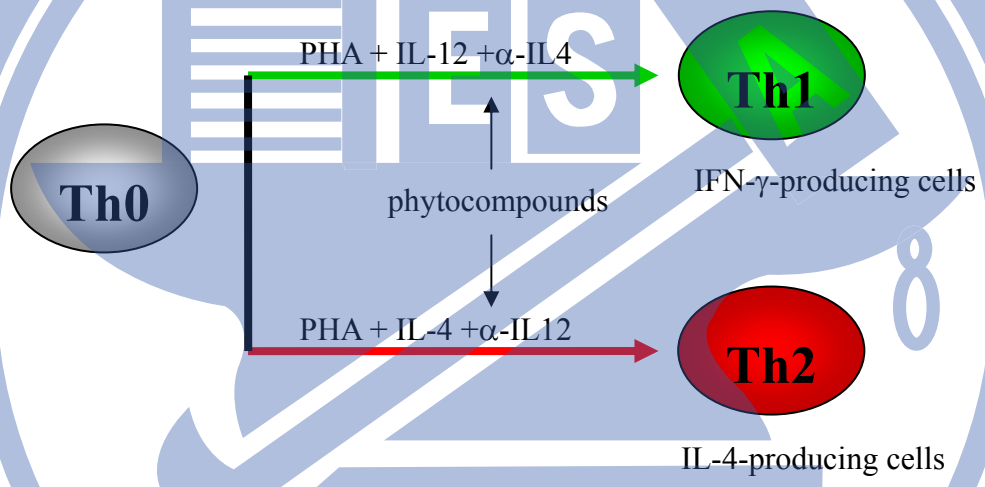

Figure 5. T cell differentiation assays.

Functional studies showed that these two polyacetylenic compounds could suppress the differentiation of naïve T helper (Th0) cells into Th1 cells but promote the differentiation of Th0 cells into Th2 cells. Since Th1 cells were reported to cause insulitis and diabetes in NOD mice. We reasoned that these compounds may *in vivo* prevent diabetes in NOD mice via down-regulation of Th1 cells or up-regulation of

Th2 cells, which antagonize Th1 cell function. We found that treatment with these compounds significantly prevented the onset of diabetes and maintained blood glucose levels in NOD mice. Here, we identified two polyacetylenes with the ability to prevent autoimmune diabetes plausibly via skewing the differentiation of T helper cells.

These results suggest that the bioactive compounds from *B. pilosa* could be identified with designated screening methods and functionally characterized with *in vivo* mouse models. It may directly contribute to the ethnopharmacological effects of *B. pilosa* and furthermore, to facilitate drug discovery from plant sources.

Chapter 2. Materials and Methods

2.1 Cells and animals

Jurkat cells (a T cell line) were obtained from American Type Culture Collection. *Listeria monocytogenes* (BCRC 15386) was obtained from Bioresource Collection and Research Center (Taiwan). Human cord blood cells were obtained from Taipei Medical University Hospital. C57BL/6J mice (National Laboratory Animal Center, Taiwan), IFN-γ-knockout mice on a C57BL/6J background (Jackson Laboratory, ME, USA) and NOD mice (Jackson Laboratory, ME, USA) were maintained and handled according to the guidelines of Academia Sinica Institutional Animal Care and Utilization Committee. Female or male mice with similar body weight, 6-to-8-week-old, were used in all our experiments.

2.2 Mediums, chemicals and reagents

RPMI 1640, DMEM medium and BHI broth were purchased from Gibco (Grand Island, NY, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), methanol, butanol, ethyl acetate, fetal bovine serum (FBS), penicillin, streptomycin, ampicillin, gentamicin, sodium pyruvate and phytohaemagglutinin (PHA) were purchased from Sigma (St. Louis, MO, USA).

2.3 T cell-based screening by using dual luciferase reporter assays

To determine the effect of plant extracts on IFN-γ promoter activity. Jurkat cells (10×10^6 cells), a leukemic T cell line obtained from American Type Culture Collection, in 0.6 ml RPMI 1640 medium were electroporated at 975 μF and 260 V

(Bio-Rad, Gene Pulser II) with luciferase reporter construct (10 μg of pIFN-γ-Luc plus 1 μg of pRL-TK). Plasmid pIFN-γ-Luc was composed of a 615-bp human IFN-γ promoter (-487 to +128 bp) fused with luciferase reporter gene. pRL-TK containing thymidine kinase promoter and *Renilla* luciferase reporter gene was purchased from Promega Corp. (WI, USA). After a 2-hr recovery, the cells were left stimulated with vehicle, PHA (1 μg/ml) or herbal fractions/compounds at the indicated concentrations for 24 hr. Cellular extracts were prepared using passive lysis buffer (Promega, WI, USA). Protein concentrations of the lysate were determined using Bradford reagent (Bio-Rad, CA, USA). Ten micrograms of cell lysate were subjected to the dual luciferase reporter assay, according to the manufacturer's instructions (Promega, WI, USA). The efficiency of transfection, as determined by *Renilla* luciferase activity (from pRL-TK gene expression) in the lysate, was used to normalize the activity of firefly luciferase (from the gene expression of pIFN-γ-Luc). The ratio of firefly luminescence to *Renilla* luminescence was then calculated for each lysate sample. Promoter activities of IFN-γ are presented as the normalized firefly luciferase activities in arbitrary units (AU).

2.4 Cell viability analysis using MTT assay

Cell viability was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl

tetrazolium bromide (MTT) colorimetric assay. Approximately 10^4 treated Jurkat cells, as described in section 2.3 were incubated with tetrazolium salt at 37 °C for 4 hours in culture medium. The insoluble products were then collected by centrifugation, dissolved in 100 µl DMSO and incubated at room temperature for 15 minutes. The reaction products formazan was quantified as measured at absorbance 560 nm. Viability of T cells after treatment with plant extracts or phytocompounds was calculated using the following formula: relative viability (%) = OD_{560} (treated cell culture)/OD_{560} (control, un-treated cell culture) x 100.

2.5 IFN-γ measurement in T cell stable clones

To generate T cell stable clones, 10×10^6 of Jurkat cells in 0.6 ml RPMI 1640 medium were electroporated at 975 µF and 260 V (Bio-Rad, Gene Pulser II) with pIFN-γ-Luc (10 µg) and pGEMT-Neo^r plasmid (10 µg) plasmids. After a 24-hr recovery, the cells were selected in RPMI 1640 medium supplemented with antibiotic G418 at 1.5 mg/ml for 3 wk. G418-resistant clones were obtained by a series of limiting dilutions and further analyzed for IFN-γ promoter activity using luciferase reporter assays. Selected Jurkat stable clones (A9, I8 and I9) were treated with vehicle (mock), PHA (positive control) at 1 µg/ml, or centaurein at 100 µg/ml. The IFN-γ promoter activity in AU is obtained by normalizing the firefly luciferase activity of

different treatments to that of the vehicle.

2.6 IFN-γ detection in splenocytes

Splenocytes from wild-type C57BL/6J mice use were collected and treated with PBS (vehicle control) or centaurein (100 μg/mL) for 24 h, followed by PMA/ionomycin treatment for 4 h plus GolgiPlug treatment (BD Biosciences, NJ, USA) for an additional 2 h. The cells were stained with CD4 (BioLegend, CA, USA), CD8 (Caltag, CA, USA) or NK (BioLegend, CA, USA) antibodies. Following intracellular staining with anti-IFN-γ antibody (BioLegend, CA, USA), the cells underwent fluorescence-activated cell sorting (FACS) analysis.

2.7 Transcriptional activity assay

T-bet is a crucial nuclear factor known to up-regulate IFN-γ expression but down-regulate IL-4 expression by sequestering GATA-3 from the binding of GATA-3 to the IL-4 (Hwang et al., 2005). To better understand the mechanism by which centaurein augments IFN-γ transcription, we tested whether centaurein can modulate these transcription factors. Jurkat cells (10×10^6) in 0.6 ml RPMI 1640 medium were electroporated at 975 μF and 260 V (Bio-Rad, Gene Pulser II) with luciferase reporter constructs (pT-bet-Luc (10 μg) or pGATA-3-Luc (10 μg) together with 1 μg of pRL-TK). Plasmid pT-bet-Luc containing a 1.5-kb human T-bet promoter (-1550 ~ -1 bp) linked to a luciferase reporter gene. Plasmid pGATA-3-Luc was composed of a

2.5-kb human GATA-3 promoter fused with luciferase reporter gene was a kind gift from Dr. Ho (Harvard school of public health, MA, USA) (Hwang et al., 2002). pRL-TK containing thymidine kinase promoter and *Renilla* luciferase reporter gene was purchased from Promega Corp. (WI, USA). After a 2-hr recovery, the cells were left stimulated with vehicle, PHA (1 μg/ml) or centaurein (100 μg/mL) for 24 hr. Following cell lysis, 10 μg of the cell lysate underwent dual luciferase reporter assays (Promega, WI, USA). The ratio of firefly luciferase activity to *Renilla* luciferase activity in each lysate was determined and presented in arbitrary units (AU).

2.8 Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Human umbilical $CD4^+$ T cells, purified with MACS (Miltenyi Biotech, Germany) according to the manufacturer's instructions, were treated with 1 μg/mL PHA, centaurein (100 μg/mL) or DMSO (vehicle control) for 24 h. Total RNAs were isolated with Trizol solution (Life Technologies, CA, USA) and converted into cDNAs with use of a first-strand cDNA synthesis kit (Amersham Biosciences, CA, USA). Total cDNAs were used as templates for PCR performed in the thermocycler at 95℃ for 1 min, 55℃ for 30 sec and 72℃ for 1 min for 27 cycles with following specific primer sets: IFN-γ, ACGAGATGACTTCGAAAAGCTG and TTTAGCTGCTGGCGACAGTTC; T-bet, CTAAAGCTCACAAACAACAAGG and

AGAAGCGGCTGGGAACAGGAT; GATA-3, GTCCTGTGCGAACTGTCAGA and TAAACGAGCTGTTCTTGGGG; IL-4, GCGATATCACCTTACAGGAG and TCAGCTCGAACACTTTGAATAT; and GAPDH, ACCACAGTCCATGCCATCAC and TCCACCACCCTGTTGCTGTA. PCR products were resolved in DNA gels and visualized with ethidium bromide, and the bands were quantitated using densitometery. Arbitrary units (AU) were obtained from the ratio of the signal of each band to that of GAPDH control.

2.9 IFN-γ detection in mice serum

To measure the serum IFN-γ level, C57BL/6J mice were intraperitoneally injected with centaurein at 20 μg. The sera were obtained in indicated time and IFN-γ concentration was determined using an ELISA kit (eBioscience, CA, USA).

2.10 *Listeria* detection in macrophages

The sera (1.5 mL) from C57BL/6J mice or IFN-γ-knockout mice, already treated with PBS or centaurein for 24 h, were concentrated 3-fold using SpeedVac® concentrators. For macrophage preparation, resident peritoneal macrophages from C57BL/6J mice were harvested by peritoneal lavage with 5 ml of ice-cold PBS, followed by centrifugation (Andrade et al., 2005). Peritoneal macrophages (2×10^5 cells) were incubated with 0.5 ml of the concentrated sera or a volume-matched mixture of anti-IFN-γ antibody (1 μg) and the serum of C57BL/6J mice with a 24-h injection of centaurein for 16 h. The cells were incubated with GFP-expressing *Listeria* (5×10^6 CFU), which was already transformed with plmo-GFP plasmid, for

30 min. After extensive washing, the cells were analyzed with FACS and fluorescent microscopy (0 h) or subjected to an additional 6-h incubation with gentamicin (40 μg/mL) and analyzed with FACS and fluorescent microscopy.

2.11 *Listeria* challenge

Listeria monocytogenes (BCRC 15386) was obtained from Bioresource Collection and Research Center (Taiwan). For prevention study, 6- to 8-week-old wild-type or IFN-$\gamma^{-/-}$ C57BL/6J mice were pretreated with vehicle, centaurein (a single dose at 10 or 20 μg/mouse) or ampicillin (1000 μg/mouse, 2 times per day for 3 days). After 24 h, mice were intraperitoneally injected with *Listeria* (1×10^6 CFU). For treatment study, 6- to 8-week-old C57BL/6J mice were intraperitoneally injected with *Listeria* (2×10^6 CFU). After 12 h, mice were treated with vehicle, centaurein (a single dose at 20 μg/mouse), ampicillin (5 or 30 μg/mouse, 2 times per day for 3 days) or a combination of centaurein (a single dose at 20 μg/mouse) and ampicillin (5 μg/mouse, 2 times per day for 3 days). The animals were then observed every day for determination of mortality.

2.12 T cell isolation, growth, differentiation and intracellular staining

Human umbilical cord blood $CD4^+$ Th0 cells were purified with a MACS column (Miltenyi Biotech, Germany) and grown in RPMI 1640 medium. Th0 cells

(0.5 x 10^6/ml) were incubated with RPMI medium containing Th0 cells (0.5x10^6 per ml) were incubated under Th1 condition containing PHA (2 μg/ml) plus IL-12 (2 ng/ml) and anti–IL-4 antibody (200 ng/ml) or Th2 condition containing PHA (2 μg/ml) plus IL-4 (10 ng/ml) and anti–IL-12 antibody (2 μg/ml). IL-2 (5 ng/ml) was added 48 h later. Plant extracts or compounds were incubated with differentiating helper T (Th) cells for 24 h on day 5. For intracellular cytokine staining, T cells were treated with PMA/ionomycin for 4 h plus Golgiplug (BD Biosciences, NJ, USA) for 2 h and then subjected to FACS (fluorescence activated cell sorter) analysis.

2.13 Mice and diabetes measurement

NOD mice from the Jackson Laboratory (ME, USA) were maintained in the institutional animal facility and handled according to the guidelines of Academia Sinica Institutional Animal Care and Utilization Committee. Marked diabetes in female NOD mice started from ~13 weeks of age and the cumulative diabetes incidence was ~60% of the total mouse population at 30 weeks of age. Urine glucose was monitored using Clinistix® (Bayer, PA, USA) at the indicated ages. Mice with 28 mM glucose or more in their urine for two consecutive weeks were considered to be diabetic. The concentration of blood glucose was monitored at the indicated intervals using Glucometer Elite® (Bayer, PA, USA). Blood insulin concentrations were

monitored using an ELISA kit (Crystal, IL, USA).

2.14 Statistical Analysis

Data from three independent experiments or more are pooled and expressed as an average of all the experiments with standard error of the mean (mean ± s.e.). For the survival data, the log-rank test was used to determine if a group was statistically significant from the control group. For the other experiments, the Student's *t*-test was performed to determine whether there was a significant difference between treatment groups and mock control groups. P (*) < 0.05 was considered to be statistically significant.

Chapter 3. Results

3.1 Screening strategy for the identification of bioactive compound(s) from plant

To explore immunomodulatory phytochemicals from plants, we developed T cell-based screening methods to analyze bioactive extracts, fractions or pure compounds in a bioactivity-directed manner (Figure 6). In brief, plant crude extracts were subjected to a T cell-based screening method. Effective crude extracts were further fractionated into different subfractions using various organic solvents based on bioactivity-guided fractionation and isolation. Different subfractions were subjected to the same screening method. Effective subfractions would continue to be purified via HPLC until effective compounds came out. Selected bioactive compounds were further examined their *in vivo* function in a mouse model.

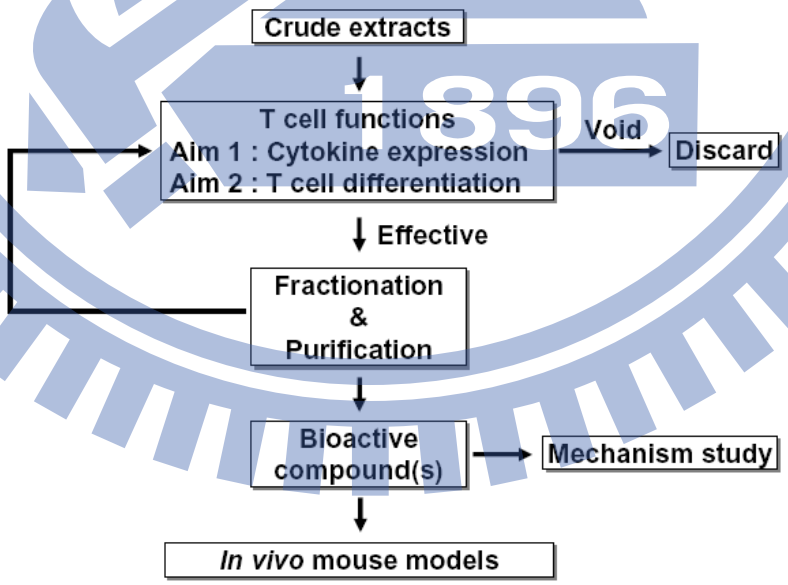

Figure 6. Screening strategy for the identification of bioactive compound(s) from plant crude extracts based on a BGFI method.

3.2 Establishment of an *in vitro* screening method, namely T cell-based transcription assay

In this study, Jurkat cells, a T cell line, transiently transfected with a plasmid construct containing a luciferase reporter gene driven by an IFN-γ promoter were used to evaluate whether crude extracts, fractions or pure compounds can affect transcription of IFN-γ gene, a hallmark of T cell activation. We henceforth refer to this method as T cell-based transcription assay. Luminometery and luciferase as a reporter gene have been broadly employed to assess eukaryotic gene expression. Moreover, the advantage of this method is of quick measurement and great sensitivity in comparison with colorimetry (New et al., 2003).

3.3 Two bioactive flavonoids, centaurein and centaureidin can be isolated from *B. pilosa* using the T cell-based transcription assay and stimulate IFN-γ transcription

We wanted to identify the bioactive compounds from immunomodulatory *B. pilosa*. To this end, we combined an IFN-γ promoter, luciferase reporter genes, and Jurkat T cells to analyze bioactive extracts, fractions or pure compounds in a BGFI manner. First of all, we found that *B. pilosa* hot water crude extracts at 500 μg/ml could induce a 2-fold increase in IFN-γ promoter activity (Figure 7). Subsequently, a

butanol subfraction from the *B. pilosa* hot water extract at 500 μg/ml increased IFN-γ promoter activity by 6-fold (Figure 8). This increase may be attributed to the enrichment of bioactive phytocompounds in the butanol fraction. Based on bioactivity-guided purification, two flavonoids, centaurein and centaureidin, from the *B. pilosa* butanol fraction was identified as bioactive compounds to modulate IFN-γ transcription. We found that centaurein at 100 μg/ml and centaureidin at 2 μg/ml could increase IFN-γ promoter activity to around 4-fold in contrast with 3,4-di-*O*-caffeoylquinic acid, isolated from *B pilosa* which served as a negative control (Figure 9).

PHA treatment is reported to cause T cell activation as well as T cell death, known as activation-induced cell death (Chwae et al., 2002). Our results showed that PHA treatment at 1 μg/ml induced IFN-γ production as well as cell death in T cells (Figure 9). Similarly, centaurein and centaureidin treatment at various concentration caused IFN-γ production as well as cell death in T cells. However, 3,4-di-*O*-caffeoylquinic acid did not have significant effect on IFN-γ production and T cell death (Figure 9). In contrast, over 60% of COS cells, a fibroblast cell line, were living when they were incubated with PHA, centaurein and centaureidin at the same concentrations (Figure 10), supporting the notion that PHA and the above flavonoids caused cell death in T cells via T cell activation but not cytotoxicity. Overall, these

results indicate that our T cell-based transcription assay platform is able to characterize bioactive phytocompounds from crude extracts based on T cell function.

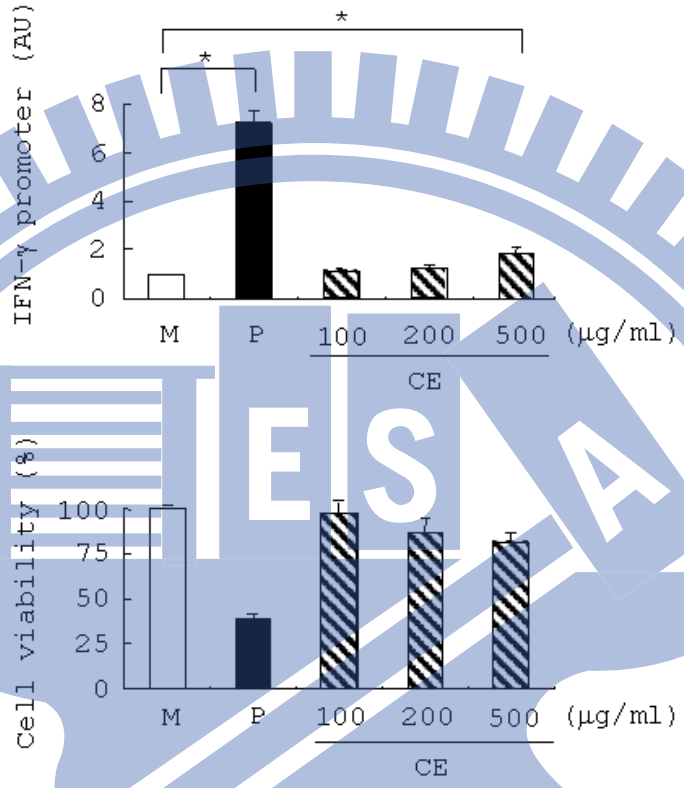

Figure 7. Crude extract of *B. pilosa* elevates IFN-γ promoter activity in Jurkat cells. Jurkat cells electroporated with pIFN-γ-Luc plus pRL-TK were left stimulated with vehicle (M), PHA (P) or hot water crude extracts (CE) of *B. pilosa* at 100, 200, and 500 μg/ml. The induction fold in arbitrary unit (AU) is obtained from the ratio of luciferase activity to that of *Renilla* luciferase (upper panel). The same cells (10^4) were tested for cell viability using MTT assay (lower panel). Data representative of three independent experiments were expressed as mean ± s.e. and (*) $p< 0.05$ is considered statistically significant.

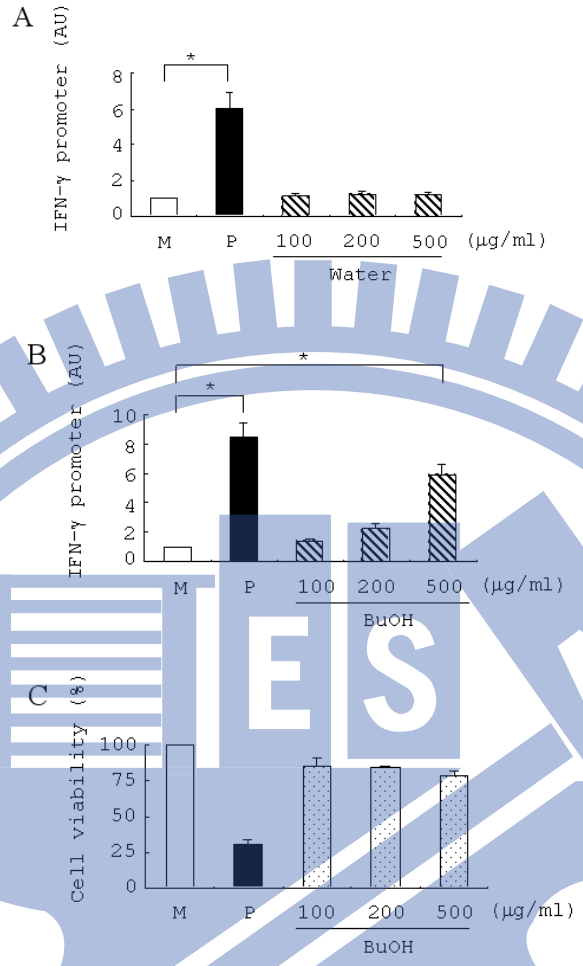

Figure 8. Butanol fraction of *B. pilosa* elevates IFN-γ promoter activity in Jurkat cells. As in **Figure 7**, Jurkat cells electroporated with pIFN-γ-Luc plus pRL-TK were left stimulated with vehicle (M), PHA (P), water fraction (Panel **A**), and butanol fraction (Panel **B**) of *B. pilosa* hot water crude extract at 100, 200, and 500 μg/ml. The induction fold in AU is obtained from the ratio of the firefly luciferase activity to the *Renilla* luciferase activity. (**C**) The same cells from butanol fraction were tested for cell viability using MTT assay. Data representative of three independent experiments were expressed as mean ± s.e. and (*) $p < 0.05$ is considered statistically significant.

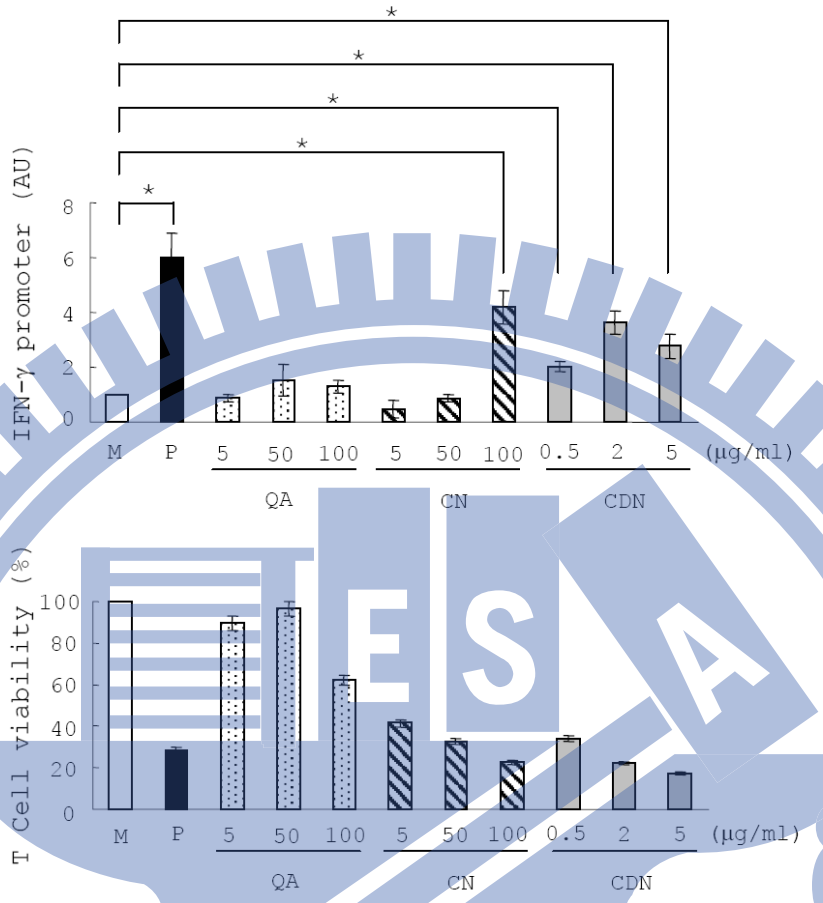

Figure 9. Centaurein and centaureidin but not 3,4-di-*O*-caffeoylquinic acid elevate IFN-γ promoter activity in Jurkat cells. Jurkat cells electroporated with pIFN-γ-Luc plus pRL-TK were left stimulated with vehicle (M), PHA (P), 3,4-di-*O*-caffeoylquinic acid (QA) and centaurein (CN) at 5, 50 and 100 μg/ml and centaureidin (CDN) at 0.5, 2, 5 μg/ml. The induction fold in AU is obtained from the ratio of the firefly luciferase activity to the *Renilla* luciferase activity (upper panel). The same cells were tested for cell viability using MTT assay (lower panel). Data representative of three independent experiments were expressed as mean ± s.e. and (*) $p < 0.05$ is considered statistically significant.

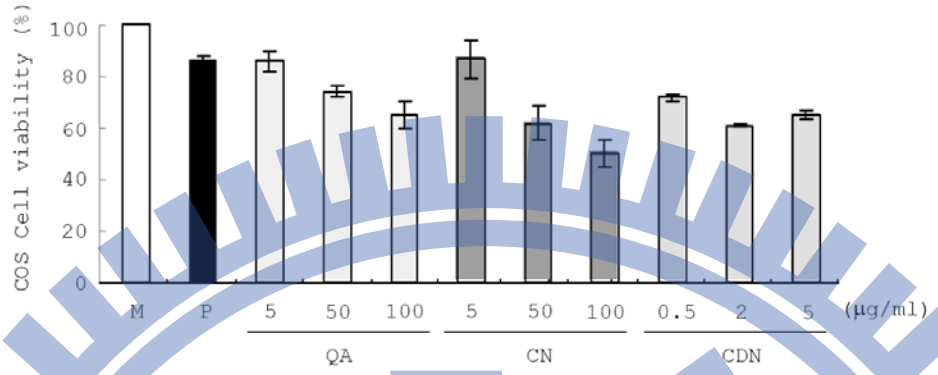

Figure 10. COS-7 cells were tested for cell viability using MTT assay. COS-7 cells were treated with vehicle (M), PHA (P), 3,4-di-*O*-caffeoylquinic acid (QA) and centaurein (CN) at 5, 50 and 100 μg/ml and centaureidin (CDN) at 0.5, 2, 5 μg/ml for 24 h. The cell viability was examined using MTT assay. Data representative of three independent experiments were expressed as mean ± s.e.

3.4 Centaurein up-regulates IFN-γ transcription in T cell stable clones

The aforesaid T cell-based transcription assay was performed using a transient transfection. To facilitate screening procedures using T cell-based transcription assays, we intended to employ T cell stable clones containing an IFN-γ promoter reporter construct to screen bioactive compounds from herbs. Here, three Jurkat stable clones (A9, I8, I9) were established. As expected, we demonstrated that centaurein at 100 μg/ml up-regulated IFN-γ expression in three different stable clones (Figure 11).

Therefore, T cell stable clones could be used to screen immunomodulatory phytocompounds. Interestingly, the clones obtained here seemed less responsive than transient transfectants after various stimulations.

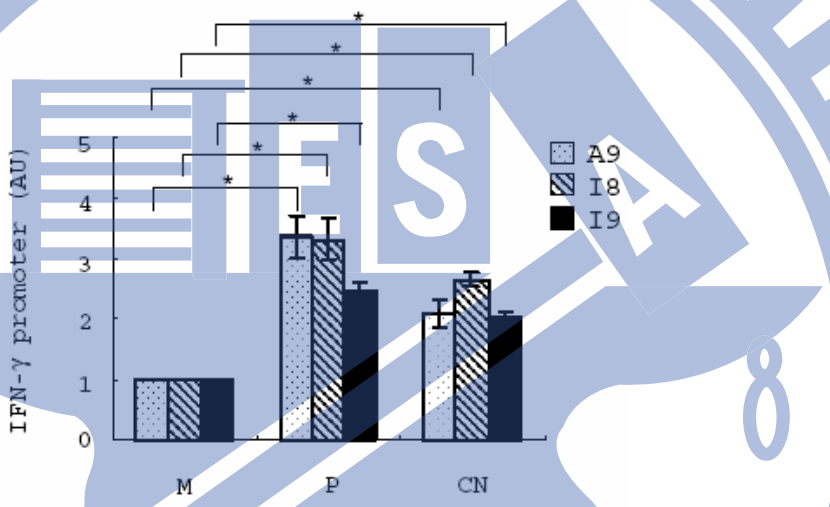

Figure 11. Centaurein elevates IFN-γ promoter activity in Jurkat stable clones.

Jurkat stable clones (A9, I8 and I9) were left stimulated with vehicle (M), PHA (P), or centaurein (CN, 100 μg/ml). The IFN-γ promoter activity in AU is obtained by normalizing the luciferase activity of different treatments to that of the vehicle (M). Data representative of three independent experiments were expressed as mean ± s.e.

3.5 Centaurein elevates IFN-γ expression in primary T cells and NK cells

To better understand the effect of centaurein on IFN-γ production in primary T cells as well as other immune cells, we incubated splenocytes with vehicle or centaurein and then assessed the IFN-γ production using flow cytometry. Centaurein augmented IFN-γ expression from 7% to 20% in $CD4^+$ T cells, from 21% to 41% in $CD8^+$ T cells and from 6% to 17% in NK cells (Figure 12). These results suggest that centaurein is a universal stimulator for these IFN-γ-producing cells.

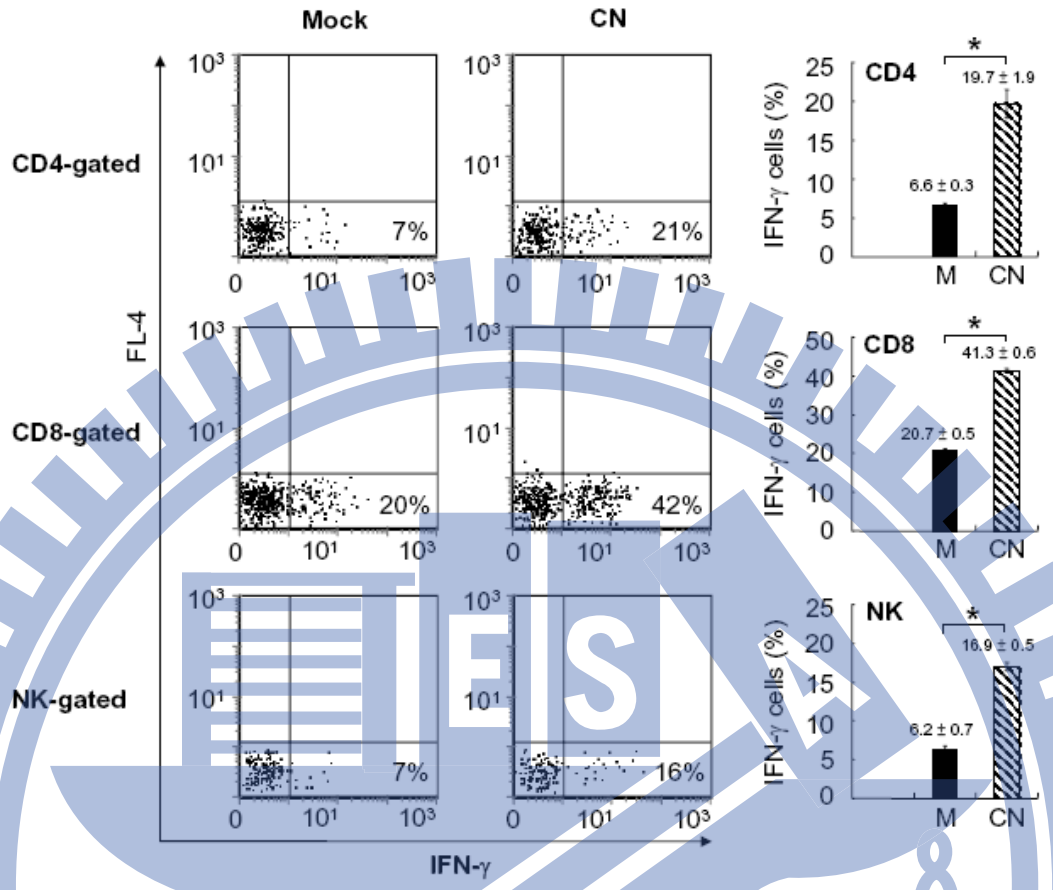

Figure 12. Centaurein stimulates IFN-γ production in T and NK cells. Mouse splenocytes were incubated with PBS (Mock) or centaurein at 100 μg/mL (CN) for 24 h, followed by PMA/ionomycin treatment for 4 h and GolgiPlug treatment for an additional 2 h. Cells stained with anti-IFN-γ-PE together with anti-CD4, anti-CD8 and anti-NK were subjected to FACS analysis. After cells were gated, the amount of IFN-γ-producing cells was counted. The FACS profiles are representative of 3 independent experiments (left panel). The histograms (right panel) show an average of 3 independent experiments with standard error of the mean (mean ± s.e.). Statistical analyses were performed using Student's *t*-test. *P* (*) < 0.05 was considered to be statistically significant.

3.6 Centaurein up-regulates the transcription of T-bet and IFN-γ but not GATA-3 and IL-4 in T cells

T-bet is a crucial nuclear factor which specifically regulates IFN-γ expression (Szabo et al., 2000). In contrast, GATA-3 can specifically regulate IL-4 expression. Since centaurein can induce IFN-γ expression, we next examined the effect of centaurein on the transcription of T-bet as well as GATA-3 using luciferase reporter assays. Our results showed that centaurein specifically enhanced T-bet transcription by 4-fold but had no significant effect on GATA-3 transcription in Jurkat cells (Figure 13). To further confirm this situation in primary cells, we treated human $CD4^+$ T cells with vehicle, PHA or centaurein and followed by RT-PCR analyses. Centaurein was found to increase the expression level of T-bet by ~5-fold. However, centaurein had a marginal effect or no effect on that of GATA-3 (Figure 14). Consistently, we found that centaurein treatment also enhanced the transcription of IFN-γ but not IL-4. In contrast, PHA at 1 μg/ml, used as a positive control, elevated the transcription of T-bet, IFN-γ and IL-4 (Figure 14). PHA at 1 μg/ml seemed not sufficient to induce GATA-3 transcription in primary $CD4^+$ T cells (Figure 14). These results suggest that centaurein can specifically up-regulate IFN-γ expression via control of T-bet.

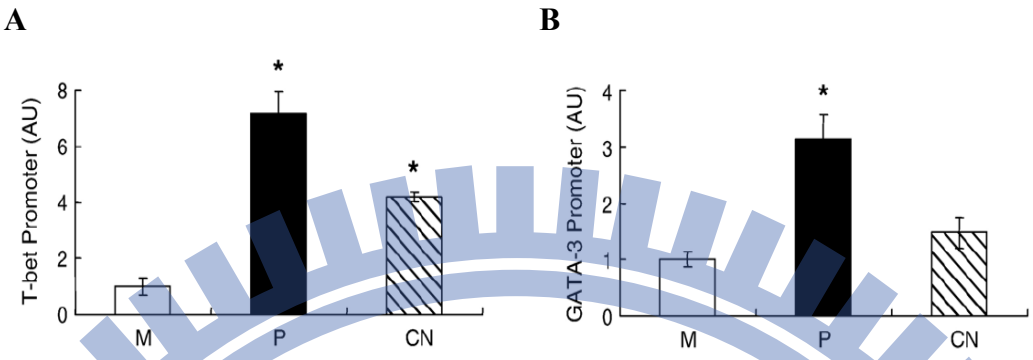

Figure 13. Centaurein elevates T-bet promoter activity in Jurkat cells. Jurkat cells electroporated with pT-bet-Luc (A) or pGATA-3-Luc (B) plus pRL-TK were left stimulated with vehicle (M), PHA (P, 1μg/ml) at or centaurein (CN, 100 μg/ml) for 24 h. The induction fold in AU is obtained from the ratio of the firefly luciferase activity to the *Renilla* luciferase activity. Data representative of three independent experiments were expressed as mean ± s.e. and (*) $p < 0.05$ is considered statistically significant.

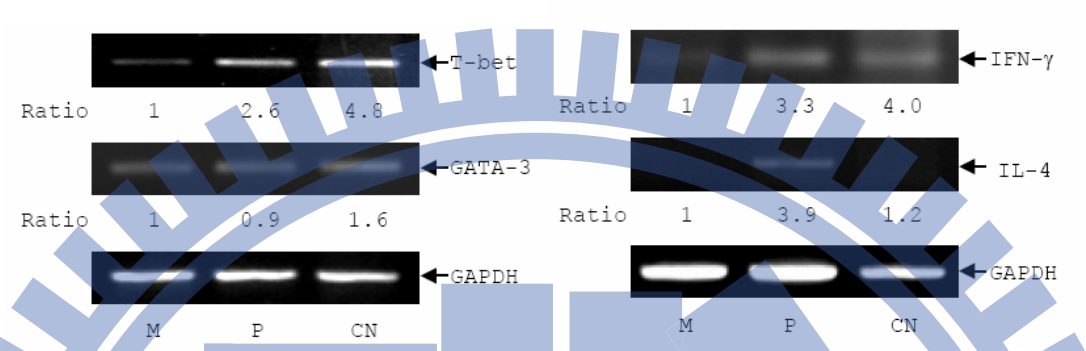

Figure 14. Centaurein increases IFN-γ and T-bet gene expression but not IL-4 and GATA-3 in T cells. Human umbilical T helper cells were treated with mock (M) or with 1μg/mL of PHA (P), and 100 μg/mL of centaurein (CN) for 24 h and were subjected to RT-PCR analysis using specific primers for T-bet, GATA-3, IFN-γ, IL-4 and GAPDH genes. PCR products were resolved on DNA gel and visualized with ethidium bromide and bands were quantitated using a scanning densitometer. Arbitrary units (AU) were obtained from the ratio of the signal of T-bet, GATA-3, IFN-γ or IL-4 bands to that of the GAPDH bands. The data are representative of three independent experiments.

3.7 Centaurein augments the serum IFN-γ level in mice

Centaurein stimulated IFN-γ production in the main IFN-γ-producing cells (T and NK cells). Thus, we wanted to evaluate this situation in mice. C57BL/6J mice were injected intraperitoneally with 20 μg of centaurein. ELISA assays revealed that centaurein augmented the serum IFN-γ level in mice, which peaked 24 h post-injection (Figure 15).

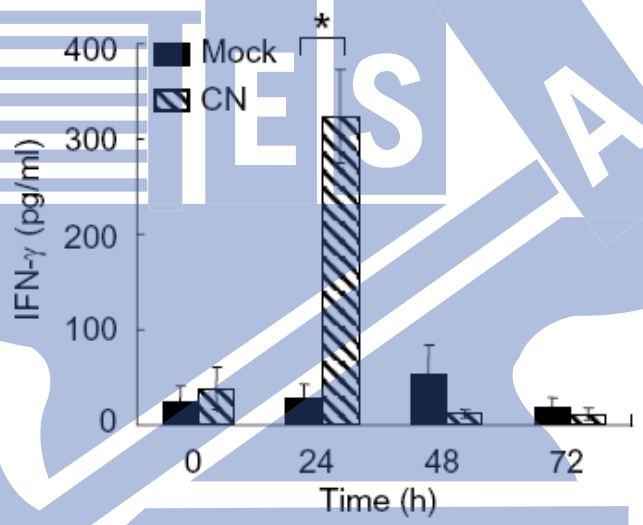

Figure 15. Centaurein elevates the serum levels of IFN-γ in mice. Blood samples from 3 mice per group were collected from C57BL/6J mice injected with PBS (Mock) or centaurein (CN, 20 μg/mouse) at 0, 24, 48, 72 h post-injection. The serum concentration of IFN-γ was measured using an ELISA kit. The bar graphs represent an average of three independent experiments with standard error of the mean (mean ± s.e.). $P< 0.05$ (*) is considered to be statistically significant based on Student's *t*-test.

3.8 Centaurein augments the serum IFN-γ and decreases *Listeria* level inside macrophages

IFN-γ is known to activate macrophages, resulting in *Listeria* clearance within macrophages. We next tested whether the sera from the centaurein-treated mice could activate the macrophage-mediated *Listeria* clearance. We treated mouse peritoneal macrophages with the aforesaid sera, followed by incubation with GFP-expressing *Listeria*. Green fluorescence was used as an indication to monitor the quantity of GFP-expressing *Listeria* inside macrophages. FACS analysis showed that no matter what serum was incubated with the macrophages for 30 min, GFP-expressing *Listeria* was detected inside 24% of macrophages (0 h, Figure 16A). Consistently, fluorescent images showed around 8 bacteria insides macrophages in each treatment (Figure 16B). These data suggest that serum treatment does not affect the initial entry of *Listeria* into macrophages.

We next evaluated *Listeria* clearance inside macrophages already treated with various serum treatment for an additional 6 h. Of note, the percentage of *Listeria*–infected macrophages, pretreated with the serum of control C57BL mice, was around 46%, similar to that of *Listeria*–infected macrophages, pretreated with the serum of centaurein-treated IFN-γ knockout mice (6 h, Figure 16A). In contrast, the percentage of *Listeria*–infected macrophages, pretreated with the serum of

centaurein-treated C57BL mice, decreased to 15%. However, that of *Listeria*–infected macrophages, pretreated with the serum of centaurein-treated C57BL mice in combination with IFN-γ-neutralizing antibody, was 34% (6 h, Figure 16A). Accordingly, fluorescent images indicated that the number of *Listeria* insides macrophages, pretreated with the control serum of C57BL mice, was 19 *Listeria*, similar to that of *Listeria* insides macrophages, pretreated with the serum of centaurein-treated IFN-γ knockout mice (6 h, Figure 16B). The number of *Listeria* insides macrophages, pretreated with the serum of centaurein-treated C57BL mice, was 1. However, the number of *Listeria* insides macrophages, pretreated with IFN-γ-neutralizing antibody and the serum of the centaurein-treated mice, was 15 (6 h, Figure 16B). Taken together, our data showed that sera with elevated IFN-γ levels from the centaurein-treated mice could clear *Listeria* in macrophages more efficiently than control sera.

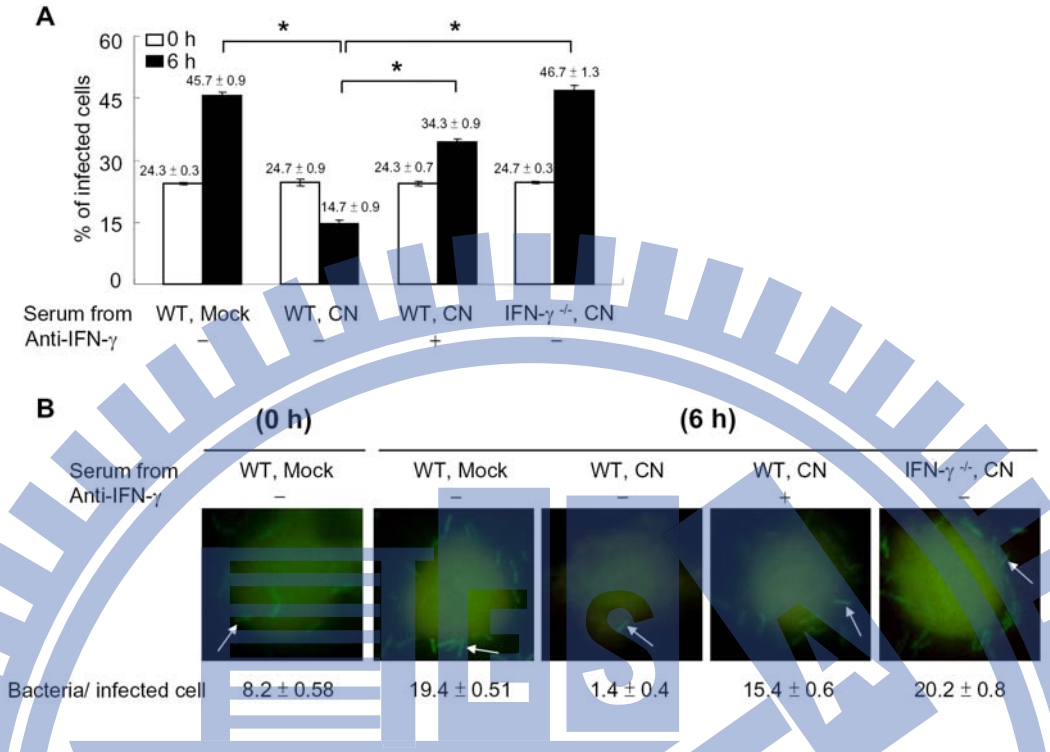

Figure 16. The effect of centaurein-treated serum on *Listeria* levels inside macrophages. Mouse resident peritoneal macrophages (2×10^5) were incubated with the serum of C57BL/6J mice with a 24-h injection of PBS (WT, Mock) or centaurein (WT, CN), a mixture of anti-IFN-γ antibody and the serum of C57BL/6J mice with a 24-h injection of centaurein (WT, CN + anti-IFN-γ) and the serum from IFN-γ knockout mice with a 24-h injection of centaurein (IFN-$\gamma^{-/-}$, CN). After a 16-h incubation with the sera, the cells were infected with GFP-expressing *Listeria* for 30 min. (A) Following extensive washing, the cells either started to undergo FACS analysis (0 h) or an additional 6-h culture (6 h), followed by FACS analysis. The histograms show an average of 3 independent experiments with error bar (mean ±

s.e.), indicating the percentage of macrophages with GFP-expressing *Listeria*. (B) The *Listeria* inside macrophages was visualized using fluorescent microscope. The average bacteria number per infected macrophage is expressed as mean ± s.e. White arrows show the place where *Listeria* is located. $P< 0.05$ (*) is considered to be statistically significant based on Student's *t*-test.

3.9 Centaurein protects mice against *Listeria* infection

Listeria monocytogenes is a useful model system to check immunity to intracellular bacteria. Since centaurein up-regulated IFN-γ production and thus, macrophage activation, we next examined if centaurein could protect mice against *Listeria* infection. Our data showed that a single dose of centaurein at 10 μg/mouse could partially protect (83%) mice from *Listeria* infection whereas control mice with only 13% of viability. Importantly, a single dosage of centaurein at 20 μg per capita could indeed protect mice against *Listeria* challenge. Thus, we demonstrated that centaurein could protect mice against *Listeria* infection in a dose-dependent fashion (Figure 17).

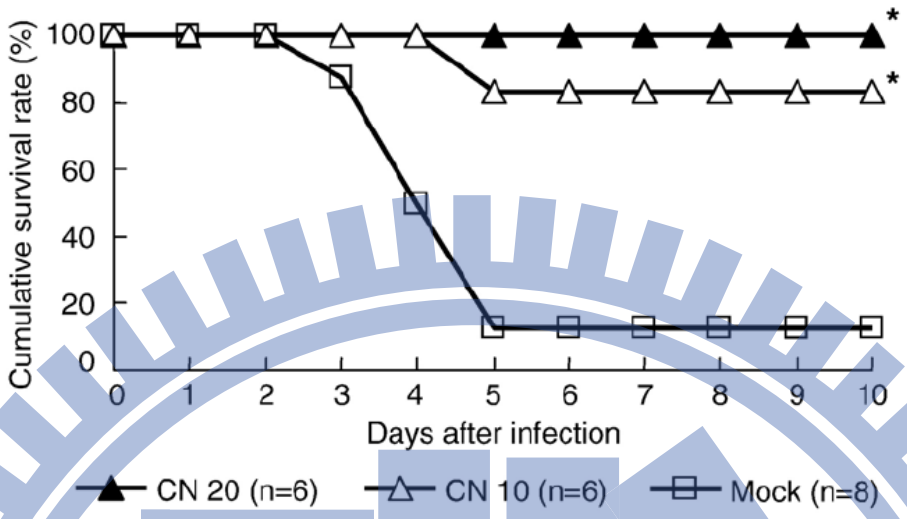

Figure 17. Centaurein protects C57BL/6 mice from *Listeria* infection. 6- to 8-week-old C57BL/6 mice were subjected to a single intraperitoneal (i.p.) injection of centaurein at 0 (Mock, □), 10 (CN 10, △), or 20 (CN 20, ▲) μg/mouse. After 24 hr, the mice were intraperitoneally challenged with *Listeria* (1×10^6 CFU). The cumulative survival rates of mice were determined. Mouse numbers per group are indicated in parenthesis. $P < 0.05$ (*) is significantly different from controls based on log-rank test.

3.10 IFN-γ gene disruption abolishes the centaurein-mediated *Listeria* prevention in mice

According to aforesaid data, we hypothesized that centaurein protected mice against *Listeria* infection via IFN-γ production. To test this hypothesis, we assessed

whether IFN-γ-knockout mice challenged with *Listeria* could be rescued by centaurein. Our results demonstrated that centaurein lost its ability to protect IFN-γ-knockout mice against *Listeria* infection. In contrast, ampicillin at 1000 μg/mL could protect against *Listeria* infection (Figure 18). Thus, these results showed that centaurein protects mice against *Listeria* infection through IFN-γ.

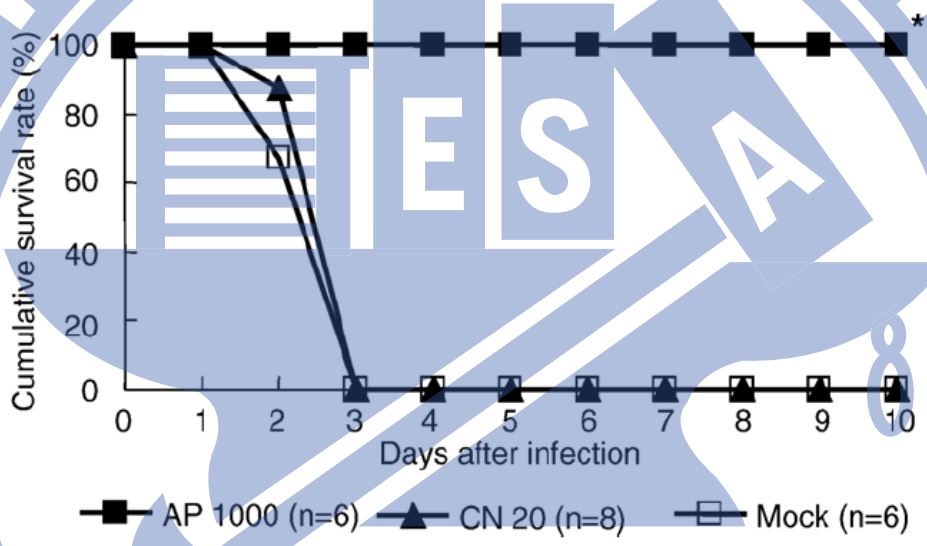

Figure 18. Centaurein cannot protect IFN-γ$^{-/-}$ C57BL/6 mice from *Listeria* infection. 6~8 week-old IFN-γ$^{-/-}$ C57BL/6J mice received a single intraperitoneal injection of PBS (Mock, □), centaurein at 20 μg (CN 20, ▲) or ampicillin at 1000 μg for 3 days, twice a day (AP 1000, ■). After 24 h, the mice were intraperitoneally challenged with *Listeria* (1×10^6 CFU). The cumulative survival rates of mice were determined. Mouse numbers per group are indicated in parenthesis. $P < 0.05$ (*) is significantly different from controls based on log-rank test.

3.11 Centaurein treats *Listeria* infection in mice alone or in combination with antibiotics

Since centaurein protected mice against *Listeria* infection, we next examined whether centaurein could be used to treat mice already infected with *Listeria*. Our data showed that centaurein treatment (20 μg/mouse) rescued 30% of the mice infected with a lethal dose of *Listeria* (2×10^6 CFU) (Figure 19). In contrast, ampicillin (5 μg ampicillin, 2 times per day for 3 days) rescued 50% of the mice that received the lethal dose of *Listeria*. Interestingly, a combination of ampicillin (5 μg/mouse, 2 times/day for 3 days) and centaurein (20 μg/mouse) rescued 70% of the mice that received the lethal dose of *Listeria*. Importantly, this combination, better than ampicillin or centaurein alone, had an additive effect on the mice already infected with *Listeria* (Figure 19). A high dosage of ampicillin (30 μg/mouse) fully treated *Listeria* infection (Figure 19) as published elsewhere (van Ogtrop et al., 1992).

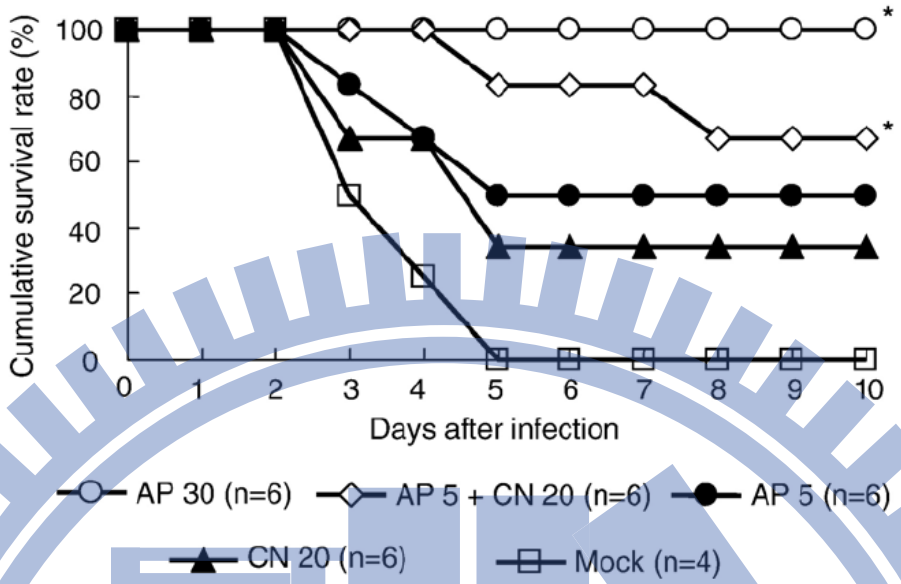

Figure 19. Centaurein per se or in combination with ampicillin can treat *Listeria* infection in mice. 6~8 week-old C57BL/6J mice were intraperitoneally challenged with *Listeria* on day 0. After 12 h, the mice received PBS (Mock, □), ampicillin at 5 (AP 5, ●) or 30 (AP 30, ○) μg, centaurein at 20 μg (CN 20, ▲), or ampicillin at 5 μg plus centaurein at 20 μg (AP 5 + CN 20, ◇). Ampicillin was given twice a day for 3 days, whereas centaurein was given in a single dose. The cumulative survival rates of mice were determined. Mouse numbers per group are indicated in parenthesis. $P < 0.05$ (*) is significantly different from controls based on log-rank test.

3.12 Establishment of an *in vitro* screening method, namely T cell differentiation assay

We aimed to identify the immunomodulatory compounds from *B. pilosa*. Here, we utilized the *in vitro* T cell differentiation assay to screen fractions or phytochemicals capable of modulating T cell differentiation with the principle of bioactivity-guided fractionation and isolation. In brief, human $CD4^+$ Th0 cells were isolated from umbilical cord blood and incubated under Th1 or Th2 conditions. To test the effect of plant extracts or phytochemicals on T cell differentiation, the differentiating helper T cells were treated with plant extracts or pure compounds for 24 h on day 5. The percentage of Th1 or Th2 population was determined by using intracellular cytokine staining and FACS analysis (Figure 5).

3.13 The butanol fraction but not water fraction of *B. pilosa* inhibits differentiation of Th0 cells into Th1 cells

Here, we set up the T cell differentiation assay as method to identify bioactive fractions or phytochemicals from *B. pilosa*. Firstly, the water and butanol fractions separated from the plant extract were subjected to the T cell differentiation assay. The butanol fraction showed a 50% inhibition (IC50) to the differentiation of Th0 into Th1 cells at 200 μg/ml and completely stopped the differentiation of Th0 into Th1 cells at

500 μg/ml (Figure 20). In contrast, the water fraction of *B. pilosa* did not affect Th1 cell differentiation from 0 to 500 μg/ml (Figure 20). These results indicated that the butanol fraction but not water fraction could inhibit the Th1 cell differentiation.

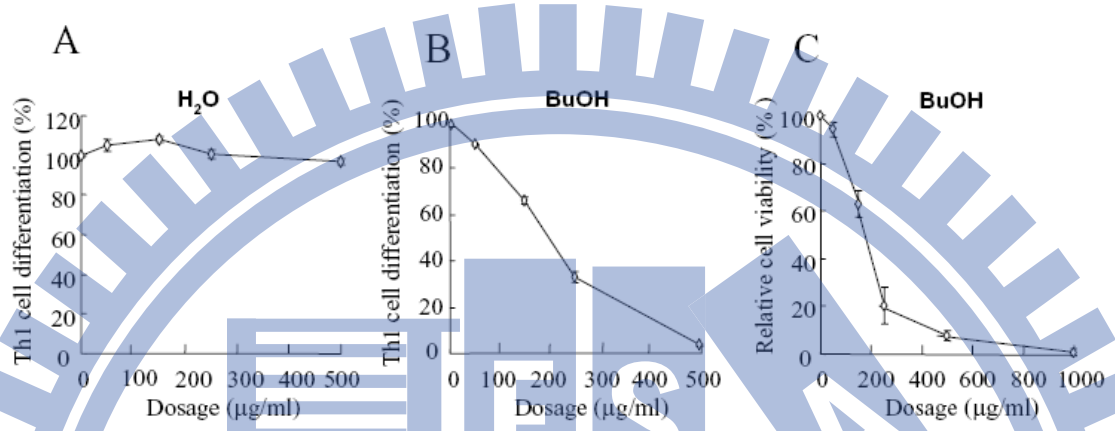

Figure 20. The effect of butanol and water fraction of *B. pilosa* on Th1 cell differentiation. (A) $CD4^+$ T cells were cultured under Th1 condition in the presence of water fraction of *B. pilosa* (H_2O) at the indicated concentrations (μg/ml). T cells were analyzed using FACS and the percentage (%) of Th1 cell differentiation was obtained from the ratio of the percentage of IFN-γ-producing cells treated with various concentrations of the water fraction to that without any treatment. (B) The same as A, except the butanol fraction (BuOH) was used at the indicated concentrations (μg/ml). (C) Relative cell viability. The percentage of surviving Th1 cells was determined in the presence of butanol fraction of *B. pilosa* (BuOH) at the indicated concentrations (μg/ml) using an MTT assay. Data representative of three independent experiments were expressed as mean ± s.e.

3.14 The butanol fraction of *B. pilosa* promotes the differentiation of Th0 cells into Th2 cells

Th1 and Th2 cell differentiation has been demonstrated to be cross-regulated (Abbas et al., 1996). Our data had demonstrated that a butanol fraction of *B. pilosa* could suppress the Th1 cells using FACS analysis; we next examined the efficacy of the butanol fraction in modulating Th2 cell differentiation. The butanol fraction at 50, 150 and 250 μg/ml increased the percentage of IL-4-producing cells (i.e., Th2 cells) from 11% to 28% (Figure 21A) whereas the butanol fraction at the same doses decreased that of IFN-γ-producing cells (i.e., Th1 cells) from 50% to 18% (Figure 21B). Our results indicated that the butanol fraction inhibited the differentiation of Th0 into Th1 cells but promoted that of Th0 into Th2 cells. This observation is in good agreement with the reciprocal antagonism of Th cell differentiation into distinct subsets. In summary, the butanol fraction from *B. pilosa* extract favors the generation of Th2 cells rather than Th1 cells, as evaluated using *in vitro* T cell differentiation assay.

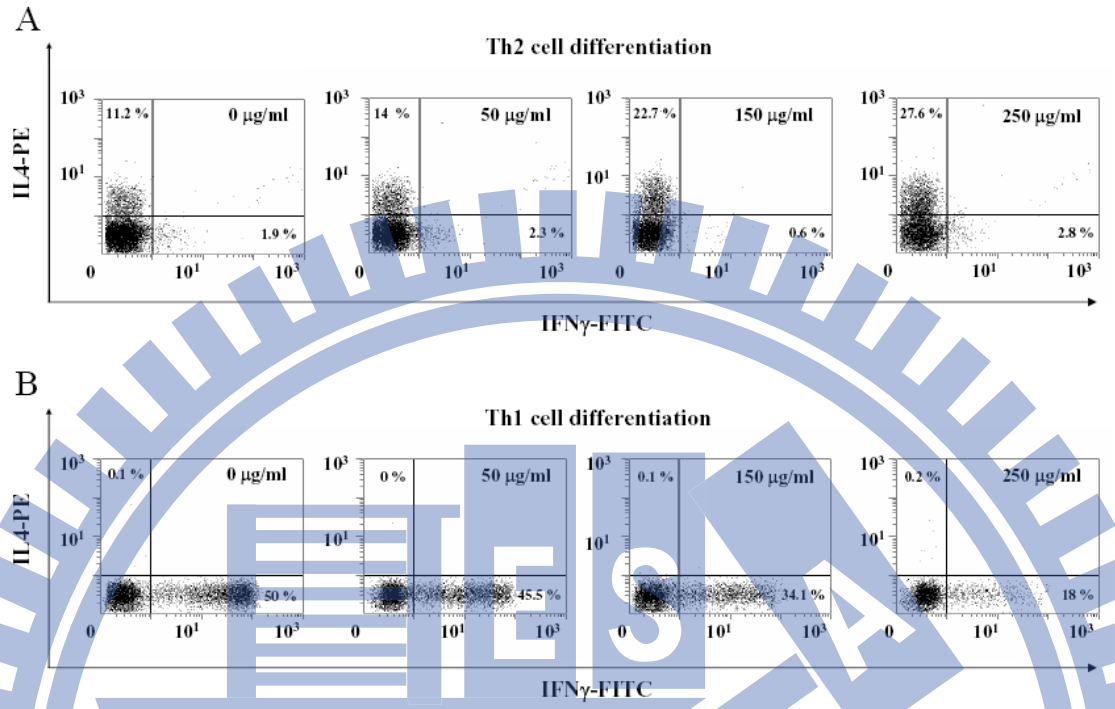

Figure 21. The butanol fraction of *B. pilosa* promotes Th2 cell differentiation but inhibits Th1 cells differentiation. (A) Human $CD4^+$ T cells were cultured under Th2 condition in the presence of the butanol fraction at 0, 50, 150 and 250 μg/ml. T cells were analyzed using FACS and the percentage of IL-4-producing cells was calculated. (B) Following the same treatment as described for Th1 cell differentiation (Figure 20), the percentage of IFN-γ-producing cells was calculated. Data are representative of three experiments.

3.15 Two polyacetylenic compounds, identified from butanol fraction of *B. pilosa*, promote the differentiation of Th0 cells into Th2 cells

Our data showed that a butanol fraction of *B. pilosa* promoted that of Th0 into Th2 cells but inhibited the differentiation of Th0 into Th1 cells using FACS analysis. We next tracked down and characterized the bioactive compounds with the efficacy on modulating T cell differentiation based on a BGFI principle. Here, two polyacetylenic compounds, 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) and 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**), were identified as bioactive constituents that capable of modulating T cell differentiation. Our data showed that the compound (**2**) at 5, 10 and 15 μg/ml increased the percentage of IL-4-producing cells (i.e., Th2 cells) from 11% to 15% (Figure 22A) whereas it at the same doses decreased that of IFN-γ-producing cells (i.e., Th1 cells) from 60% to 37% (Figure 22B). However, 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) showed, if any, only little inhibition (10%) of the differentiation of Th0 into Th1 cells and slight enhancement (8%) of the differentiation of Th0 into Th2 cells at the dose of 15 μg/ml (Figure 23). Taken together, our results display that this T cell differentiation method can be used as a highly efficient platform to distinguish bioactive phytochemicals from the crude extracts and lead to identification of bioactive phytocompounds.

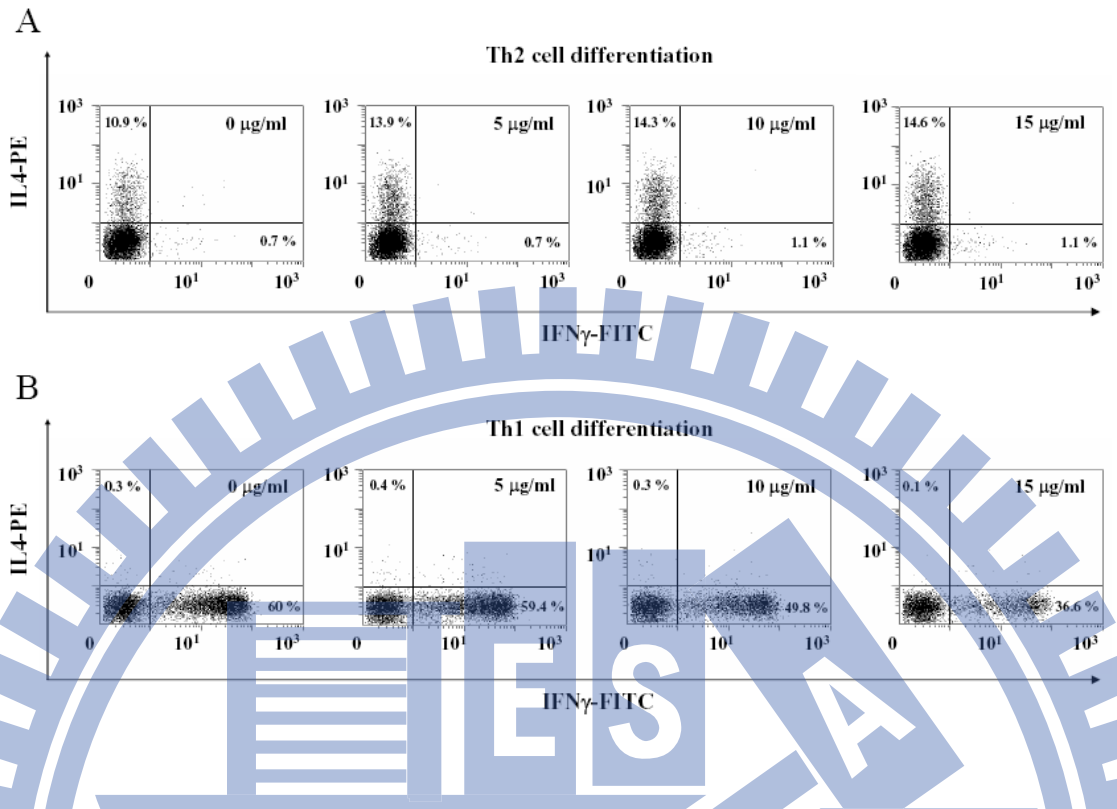

Figure 22. The effect of 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (2) in T helper cell differentiation. (A) Human $CD4^+$ T cells were cultured under Th2 condition in the presence of 3-ß-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne **(2)** at 0, 5, 10 and 15 μg/ml. T cells were analyzed using FACS and the percentage of IL-4-producing cells was calculated. (B) Following the same treatment as described for Th1 cell differentiation (Figure 20), the percentage of IFN-γ-producing cells was calculated. Data are representative of three experiments.

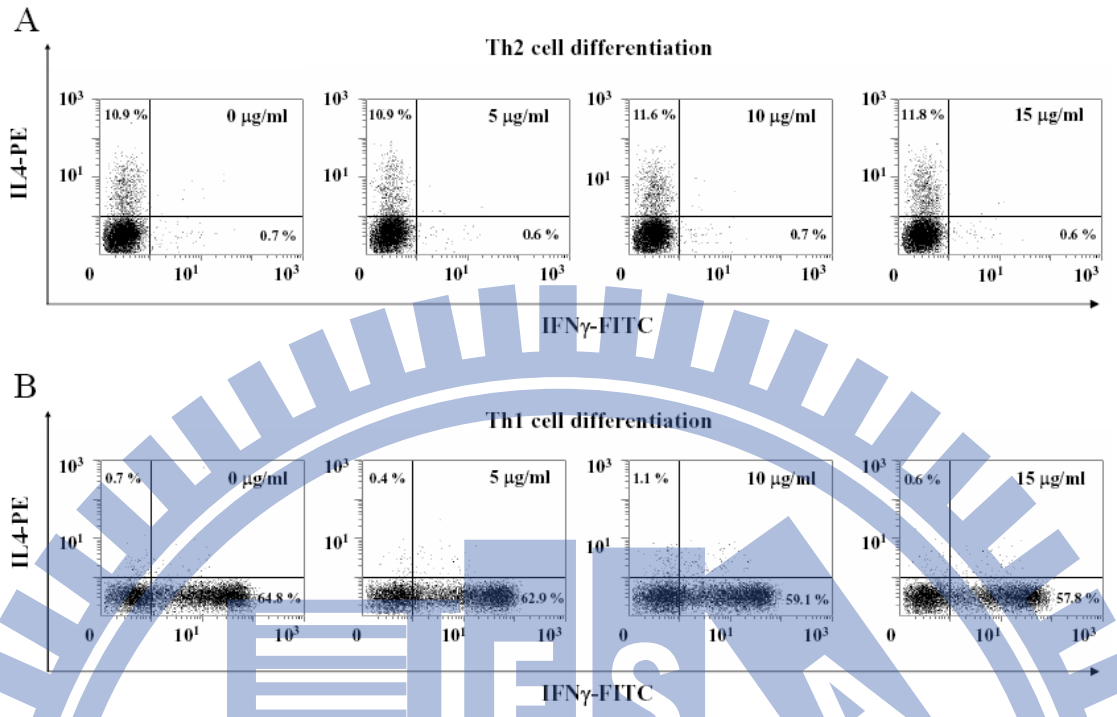

Figure 23. The effect of 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (1) in T helper cell differentiation. (A) Human $CD4^+$ T cells were cultured under Th2 condition in the presence of 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne **(1)** at 0, 5, 10 and 15 μg/ml. T cells were analyzed using FACS and the percentage of IL-4-producing cells was calculated. (B) Following the same treatment as described for Th1 cell differentiation (Figure 20), the percentage of IFN-γ-producing cells was calculated. Data are representative of three experiments.

3.16 The butanol fraction of *B. pilosa* prevents the onset of diabetes in NOD mice

Th1 cells were reported to cause insulitis and diabetes in NOD mice (Katz et al., 1995). Our *in vitro* data exhibited that the butanol fraction of *B. pilosa* could suppress the differentiation of Th0 into Th1 cells and preferentially promote that of Th0 into Th2 cells, implying a potential role of the butanol fraction in treating Th1-mediated autoimmune diseases. We reasoned that the butanol fraction may *in vivo* prevent diabetes in NOD mice via down-regulation of Th1 cells or up-regulation of Th2 cells, which antagonize Th1 cell function. To test this hypothesis, we utilized NOD mice as a Th1-mediated autoimmune disease mouse model to examine the effect of the butanol fraction in the diabetes progression. Our results indicated that mice with intraperitoneal (i.p.) injection using the butanol fraction at 3 mg/kg per dose had a lower diabetes incidence (33%) than control mice (56%), which has a similar incidence in the publication (Kai et al., 1993). Likewise, injection of NOD mice with the butanol fraction at 10 mg/kg could stop the initiation of the disease (0%). Thus, the butanol fraction treatment could protect NOD mice from developing diabetes in a dose-dependent manner (Figure 24). We also examined diabetes indicators such as blood glucose and insulin. We found that the butanol fraction treatment at 10 mg/kg prevented mice from hyperglycemia and hypoinsulinemia in comparison to control mice (Figure 25).

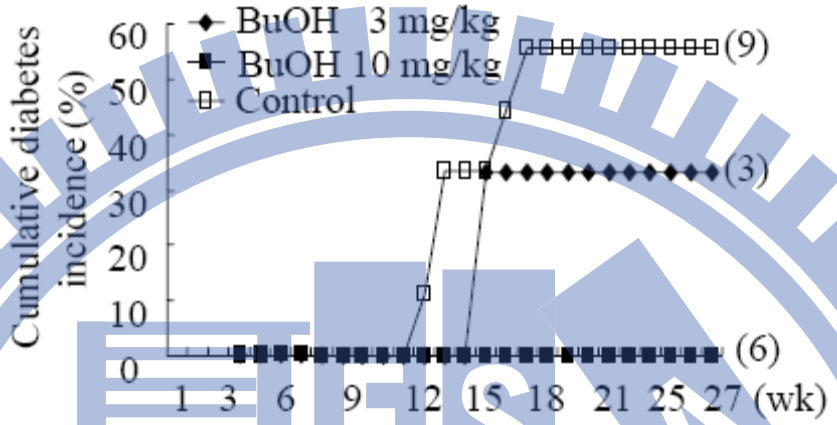

Figure 24. The effect of butanol fraction on the diabetes progression in NOD mice. Cumulative diabetes incidence in female NOD mice. Three groups of female NOD mice (mouse numbers per group are indicated in the parenthesis) received i.p. injections with the butanol fraction of *B. pilosa* at 3 mg/kg (BuOH 3 mg/kg), 10 mg/kg (BuOH 10 mg/kg) or PBS (Control) 3 times per week from 4 to 27 weeks of age. Urine glucose was monitored using Clinistix at the indicated ages. Mice with 28 mM glucose or more in their urine for two consecutive weeks were considered to be diabetic.

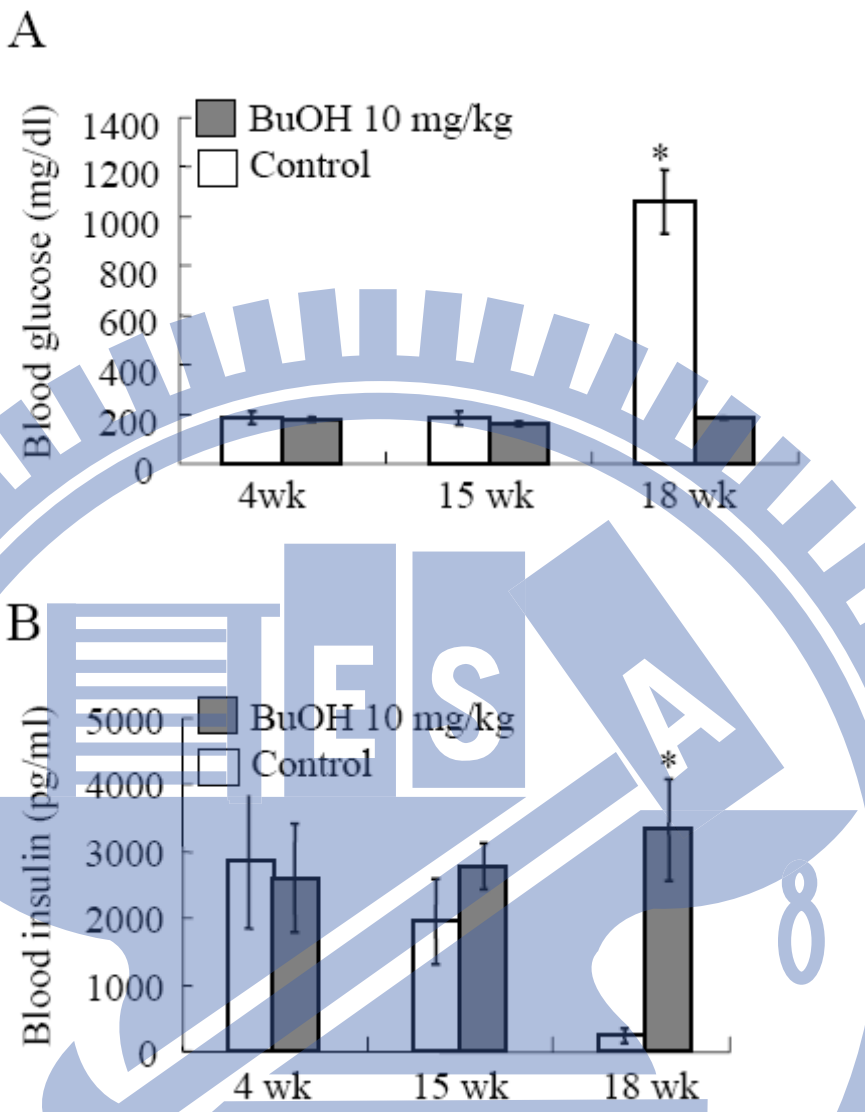

Figure 25. The effect of butanol fraction on blood glucose and blood insulin level in NOD mice. (A) The concentration of blood glucose (mg/dl) of the 10 mg/kg butanol fraction of *B. pilosa*-treated (BuOH 10 mg/kg) or control mice (4, 15 and 18 weeks of age) was determined using a glucometer. Data (mean ± s.e.) are representative of three experiments. (B) The same mice as indicated in panels A had their blood insulin concentrations (pg/ml) determined using an ELISA kit. Data (mean ± s.e.) are representative of three experiments and * : $P < 0.05$ by Student T test..

3.17 Two polyacetylenic compounds, identified from butanol fraction of *B. pilosa*, protect the diabetes onset in NOD mice.

We next determined if the bioactive phytocompounds identified from the the butanol fraction have similar effects on prevention of diabetes as the butanol fraction. We found that treatment with these two compounds significantly prevented the onset of diabetes in NOD mice (Table I).

Table I. Effect of the polyacetylenic compounds on diabetes prevention.

Compound **1** (n=3)	0% diabetic
Compound **2** (n=5)	0% diabetic
Control (n=6)	33% diabetic

NOD mice were i.p. injected 3 times per week from 10 to 13 weeks of age with different amounts of compound **1** (37 μg/kg) or compound **2** (45 μg/kg). The definition of diabetes is described in the legend of Figure 24. n : mouse number.

Chapter 4. Discussion

4.1 Bioactives from *B. pilosa*

Natural products have been the most productive source of leads for the drug development. More than 80% of drug substances were natural products or inspired by a natural compound. It's about 50% of the drugs approved from 1994 to 2007 are based on natural products.

B. pilosa has been claimed as an anti-infectious or immunomodulatory folk medicine. Here, we first evaluated the immune efficacy of *B. pilosa*, as evidenced in the up-regulation of IFN-γ, a potent cytokine in many immunomodulatory aspects. We have effectively identified two bioactive flavonoids, centaurein and centaureidin, from *B. pilosa* with the ability to stimulate IFN-γ expression using a BGFI method. It was reported that centaurein and centaureidin were synthesized in *B. pilosa* or other plants (Chiang et al., 2004). However, their biological functions remained unknown. In this study, we, for the first time, manifested that centaurein and its aglycone, centaureidin, were able to modulate IFN-γ transcription. Centaurein was used to further study how both flavonoids could stimulate IFN-γ transcription. Our results showed that centaurein could activate the transcription activity of T-bet. Therefore, we postulated that centaurein mediated IFN-γ expression through nuclear factor T-bet. Our data manifested that centaurein or centaureidin can *in vitro* boost IFN-γ production. Their

in vivo function in immune modulation (e.g., pathogen clearance) was further verified using *Listeria* infection mouse models.

We here proved the concept that a combination of IFN-γ promoter, luciferase as a reporter gene, and T cells can be used to screen immunomodulatory phytochemicals from the *B. pilosa* plant, traditionally used as a folk medicine to improve immunity and infections. Although the use of luciferase as a reporter gene in biological assays is not a brand-new idea, yet it is relatively rapid, sensitive, cost-effective, and feasible for robotization (New et al., 2003). The effective dose of centaurein used here to stimulate the IFN-γ production is relative high (100 μg/ml). In contrast, centaureidin, an aglycone of centaurein, at 2 μg/ml has similar effect as centaurein at 100 μg/ml on IFN-γ stimulation. Of note, we proved that compounds with a low or high potency were able to be identified in our experimental setups.

On the other hands, our results showed that two polyacetylenic compounds and a butanol fraction of *B. pilosa* extract modulated T cell differentiation. Meanwhile, the butanol fraction also lowered diabetic incidence, whilst maintaining normal levels of blood sugar, insulin produced by β cells in NOD mice. To our knowledge, this is the first report so far to demonstrate that the butanol fraction of *B. pilosa* can effectively prevent IDDM, as evaluated using a NOD mouse model. One possible scenario for the suppression of IDDM may be that the butanol fraction and polyacetylenic

compounds inhibit the generation of Th1 cells and promotes that of Th2 cells infiltrating into the islets of NOD mice as the fraction and compounds do *in vitro*.

Intriguingly, *B. pilosa* has been used as herbal medicines to treat diabetes without scientific proof (Dimayuga and Agundez, 1986). A mixture of 2-β-$_D$-glucopyranosyloxy-1-hydroxy-5(E)-tridecene-7,9,11-triyne (**1**) and 3-β-$_D$-glucopyranosyloxy-1-hydroxy-6(E)-tetradecene-8,10,12-triyne (**2**) from *B. pilosa* have been demonstrated to have blood sugar lowering effect in type II diabetes (db/db) mice, partly ascribed to the anorexic effect of both polyacteylenic glucosides (Ubillas et al., 2000). However, the mechanism by which both polyacetylenic glucosides affect diabetes in the type II diabetes mice is not elucidated.

4.2 *Listeria* infection

The annual incidence of listeriosis in humans is rare, ~ 0.4 to 7.4 per million people (Calder, 1997). But among those who develop listeriosis, the death rate is high (~25 to 30%) (Rouquette and Berche, 1996). Antibiotic drugs are currently used for *Listeria* infection. However, antibiotic-resistant *Listeria* strains have been increasingly reported (Yamaoka et al., 1998; Yamaoka et al., 2000). Therefore, it is necessary to develop new therapeutics for treating *Listeria* infection.

Several herbal medicines have been reported to have a protective effect against

Listeria infection in mice. For example, the crude extract of a Chinese medicine, Bu-Zhong-Yi-Qi-Tang, up-regulates IFN-γ production and, therefore, eradicates *Listeria* infection in mice (Yamaoka et al., 1998; Yamaoka et al., 2000; Yamaoka et al., 2001). Some polysaccharides isolated from the *Echinacea purpurea* plant protect mice against *Listeria* infection (Steinmuller et al., 1993). However, the detailed pharmacological mechanisms and/or the bioactive compounds in the above systems remain to be elucidated.

Centaurein was previously isolated from a plant (*B. pilosa*) with a folk tradition of anti-bacterial use (Chiang et al., 2004). Here, we, for the first time, found that NK and T cells increase IFN-γ production in response to centaurein. This IFN-γ increase was also observed in mice. The fact that centaurein up-regulates T-bet expression suggests a molecular mechanism by which centaurein mediates IFN-γ expression via an IFN-γ regulator, T-bet. In this study, we confirm that centaurein can protect against or treat *Listeria* infection in mice via up-regulation of IFN-γ and macrophage activation.

IFN-γ can activate the macrophage-mediated killing of intracellular pathogens. Both IFN-γ and macrophage activation are pivotal for *Listeria* eradication in cell and animal models (Hubel et al., 2002). IFN-γ alone or in conjunction with antimicrobial agents, is also reported to clinically treat patients infected with an intracellular

microbe, *Mycobacteria* (Hubel et al., 2002). Of note, IFN-γ showed a promising effect on the adjunctive treatment of multidrug-resistant *Mycobacteria* in patients (Hubel et al., 2002). We report that centaurein alone or in combination with antibiotics protects against and treats *Listeria* infection via up-regulation of IFN-γ. In addition, the anti-bacterial susceptibility test showed that the minimal inhibitory concentration of centaurein for *Listeria* is over 200 μg/ml, indicating that centaurein itself did not show any significant bacteriocidal or bacteriostatic activity against *Listeria* because of its high minimal inhibitory concentration. On the contrary, centaurein can prevent and treat *Listeria* infection indirectly via boosting immune responses (IFN-γ production and macrophage activation).

Our results are encouraging for the use of centaurein protecting against and treating antibiotic-resistant intracellular bacteria via enhanced IFN-γ production. Similar approaches can be used to develop immune modulators and prophylactics/therapeutics for infectious pathogens.

4.3 Autoimmune diseases

Autoimmune disease is the third largest category of illness in the developed countries—behind cardiovascular disease and cancer. It was estimated that over 20 million people are afflicted with autoimmune diseases and that a conservative medical expenditure covering those autoimmune diseases is 21 billion US dollars per year. However, few drugs have so far been developed for autoimmune diseases compared to other diseases. Treatments for autoimmune diseases rely on immunosuppressants or immune modulators. Immunosuppressants such as cyclosporine A can shut down the immune system and prevent inflammation. However, treatment with immunosuppressants may carry a risk of infectious disease or cancers (Kai et al., 1993). Immune modulators, which skew T cell differentiation, have been used to treat T cell-mediated disorders. For instance, IL-4 and IL-10 are used for treatment of Th1-mediated diseases (Kawamoto et al., 2001; Ko et al., 2001). However, a strategy which skews production of Th1 cells into Th2 cells may have adverse effects, such as the induction of Th2-mediated autoimmune diseases.

Our study is designed to identify immunomodulatory plant extracts and phytocompounds using *in vitro* T cell differentiation method in combination with NOD mouse model. Here, we have successfully screened out a butanol fraction of *B. pilosa* and its subsequent two compounds which can suppress Th1 differentiation but

promote Th2 differentiation from Th0 cells. Our results from NOD mouse model also demonstrated that the butanol fraction and compounds can prevent the progress of Th1-mediated diabetes. Our results are encouraging the potentially therapeutic use of butanol fraction and bioactive compounds in Th1-mediated autoimmune diseases.

Chapter 5. Conclusions and future perspectives

Our results demonstrated that both T cell-based luciferase reporter assay and T helper differentiation assay can be used to identify immunomodulatory phytochemicals from the *B. pilosa*. These screening methods may be further improved and developed into a high throughput platform for evaluating and screening other immunomodulatory herbs, fractions and compounds.

We demonstrate that centaurein protects against or treats *Listeria* infection through a regulation of IFN-γ expression. However, in our studies, the beneficial therapeutic effect of centaurein was only based on healthy young mice and/or IFN-γ knockout mice with serious innate immunodeficiency. Additional experiments in evaluating the efficacy of centaurein in partially immunocompromised mice, such as dexamethasone-treated mice, needs be considered. The use of healthy mice and mice with innate or acquired immunodeficiency to evaluate the therapeutic effect of centaurein on *Listeria* infection help draw the conclusion on the efficacy of centaurein in *Listeria* elimination and may be more like humans susceptible to *Listeria* infection.

Our data showed that centaurein increased the IFN-γ production in T and NK cells. T-bet is required for IFN-γ production in T cells and NK cells (Szabo et al., 2002; Townsend et al., 2004). However, T-bet was reported not to be required for host resistance to *Listeria* infection (Way and Wilson, 2004). Our results showed that

centaurein augments IFN-γ expression in cells and mice. Such an increase accompanies T-bet up-regulation. Therefore, our results strongly suggest that centaurein elevates IFN-γ production via control of T-bet. IFN-γ was reported to induce T-bet expression (Lighvani et al., 2001), raising the possibility that the up-regulation of T-bet is an indirect consequence of centaurein inducing IFN-γ. However, this should not be the case because centaurein still up-regulates T-bet transcription in Jurkat cells, in which IFN-γ production is defective. The regulation of IFN-γ expression involves a complicated mechanism mediated by various nuclear factors. More studies utilizing the T-bet knock out mice or primary cells from T-bet knock out strain would provide additional evidences to ascertain the detail mechanism of the action of centaurein on *Listeria* infection and position the involvement of T-bet in the production of IFN-γ in response to centaurein.

In additional, PHA, a T cell stimulant, can activate T cells to produce cytokines like IFN-γ and then cause T cell death. This phenomenon is known as activation-induced cell death (Chwae et al., 2002). We also observed that similar to PHA, centaurein and centaureidin in some cases induced IFN-γ transcription as well as apoptosis in T cells. However, PHA and both flavonoids at the same dose showed a marginal effect on the cell death of a non-T cell line, COS cells, suggesting that the significant effect of the above compounds on T cell death was partially ascribed to

activation-induced cell death. How both flavonoids can cause T cell activation-induced death needs to be further examined.

The concentrations of centaurein used in our studies are 100 μg/ml in cells and 10 to 20 μg/mouse in mice. Additionally, our data showed that centaureidin, an aglycone of centaurein, could increase IFN-γ production 30 times more than centaurein. Therefore, there is great potential for use of centaurein or its derivatives to treat infectious diseases.

Identification of bioactive pure compounds from *B. pilosa* can help us elucidate the mechanism by which *B. pilosa* can prevent non-obese diabetes. Here, we demonstrated that the butanol fraction of *B. pilosa* can ameliorate type I diabetes probably via controlling T cell differentiation into Th2 cells. Indeed, we have identified two pure compounds (compound **1** and compound **2**) which can prevent diabetes development in NOD mice although compound **2** is more potent than compound **1** in T cell differentiation. More studies are warranted to ascertain the detailed mechanism of the action of these compounds on IDDM.

B. pilosa was documented to treat other categories of diseases. For instance, *B. pilosa* extracts was shown to decrease acid/pepsin secretion (Alvarez et al., 1999) and inhibit ulcers (Tan et al., 2000). Besides, its extract was shown to have anti-hypertensive effects in rats (Dimo et al., 2001; Dimo et al., 1999), inhibit the

vasocontriction by blocking the Ca^{2+} influx into the cells (Dimo et al., 1998; Nguelefack et al., 2005) and slow cardiac pump (Dimo et al., 2003). However, no specific compound responsible for the above categories of diseases of *B. pilosa* has been identified up to date. Bioactivity-directed fractionation and isolation approach can be taken to understand the active compounds of *B. pilosa* for gastrointestinal and cardiovascular diseases.

Chapter 6. References

Abajo, C., Boffill, M. A., del Campo, J., Alexandra Mendez, M., Gonzalez, Y., Mitjans, M., and Pilar Vinardell, M. (2004). *In vitro* study of the antioxidant and immunomodulatory activity of aqueous infusion of *Bidens pilosa*. J Ethnopharmacology *93*, 319-323.

Abbas, A. K., Murphy, K. M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787-793.

Adorini, L., Gregori, S., Magram, J., and Trembleau, S. (1996). The role of IL-12 in the pathogenesis of Th1 cell-mediated autoimmune diseases. Ann N Y Acad Sci *795*, 208-215.

Alcaraz, M. J., and Jimenez, M. J. (1988). Flavonoids as anti-inflammatory agents. Fitoterapia *59*, 25-38.

Alvarez, A., Pomar, F., Sevilla, and Montero, M. J. (1999). Gastric antisecretory and antiulcer activities of an ethanolic extract of *Bidens pilosa* L. var. *radiata* Schult. Bip. J Ethnopharmacol *67*, 333-340.

Alvarez, L., Marquina, S., Villarreal, M. L., Alonso, D., Aranda, E., and Delgado, G. (1996). Bioactive polyacetylenes from *Bidens pilosa*. Planta Med *62*, 355-357.

Andrade-Neto, V. F., Brandao, M. G., Oliveira, F. Q., Casali, V. W., Njaine, B., Zalis, M. G., Oliveira, L. A., and Krettli, A. U. (2004). Antimalarial activity of *Bidens pilosa* L. (Asteraceae) ethanol extracts from wild plants collected in

various localities or plants cultivated in humus soil. Phytotherapy Research *18*, 634-639.

Andrade, R. M., Portillo, J. A., Wessendarp, M., and Subauste, C. S. (2005). CD40 signaling in macrophages induces activity against an intracellular pathogen independently of gamma interferon and reactive nitrogen intermediates. Infect Immun *73*, 3115-3123.

Botsaris, A. S. (2007). Plants used traditionally to treat malaria in Brazil: the archives of Flora Medicinal. J Ethnobiol Ethnomed *3*, 18.

Brandao, M. G., Krettli, A. U., Soares, L. S., Nery, C. G., and Marinuzzi, H. C. (1997). Antimalarial activity of extracts and fractions from *Bidens pilosa* and other *Bidens* species (Asteraceae) correlated with the presence of acetylene and flavonoid compounds. J Ethnopharmacol *57*, 131-138.

Brandao, M. G., Nery, C. G., Mamao, M. A., and Krettli, A. U. (1998). Two methoxylated flavone glycosides from *Bidens pilosa*. Phytochemistry *48*, 397-400.

Buchwald, U. K., and Pirofski, L. (2003). Immune therapy for infectious diseases at the dawn of the 21st century: the past, present and future role of antibody therapy, therapeutic vaccination and biological response modifiers. Curr Pharm Des *9*, 945-968.

Calder, J. A. (1997). *Listeria* meningitis in adults. Lancet *350*, 307-308.

Carr, N. (2009). Encyclopaedia Britannica Online (Chicago, IL, U.S.A., Enclyclopaedia Brittanica, Inc.).

Castano, L., and Eisenbarth, G. S. (1990). Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. Annu Rev Immunol *8*, 647-679.

Chang, J. S., Chiang, L. C., Chen, C. C., Liu, L. T., Wang, K. C., and Lin, C. C. (2001). Antileukemic activity of *Bidens pilosa* L. var. *minor* (Blume) Sherff and Houttuynia cordata Thunb. Am J Chin Med *29*, 303-312.

Chang, M., Wang, G., Kuo, Y. H., and Lee, C. K. (2000). The low polar constituents from *Bidens pilosa* L. var. *minor* (Blume) Sheriff. J Chinese Chemical Society *47*, 1131-1136.

Charles A., J. J., Paul Travers, Mark Walport, Mark J. Shlomchik, ed. (2005). Immunobiology, 6th Edition.

Chiang, Y. M., Chuang, D. Y., Wang, S. Y., Kuo, Y. H., Tsai, P. W., and Shyur, L. F. (2004). Metabolite profiling and chemopreventive bioactivity of plant extracts from *Bidens pilosa*. J Ethnopharmacol *95*, 409-419.

Chiang, Y. M., Lo, C. P., Chen, Y. P., Wang, S. Y., Yang, N. S., Kuo, Y. H., and Shyur, L. F. (2005). Ethyl caffeate suppresses NF-kappaB activation and its downstream inflammatory mediators, iNOS, COX-2, and PGE2 in vitro or in mouse skin. Br J Pharmacol *146*, 352-363.

Chih, H. W., Lin, C. C., and Tang, K. S. (1995). Anti-inflammatory activity of Taiwan folk medicine "ham-hong-chho" in rats. American J Chinese Medicine *23*, 273-278.

Christianson, S. W., Shultz, L. D., and Leiter, E. H. (1993). Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. Diabetes *42*, 44-55.

Chwae, Y. J., Chang, M. J., Park, S. M., Yoon, H., Park, H. J., Kim, S. J., and Kim, J. (2002). Molecular mechanism of the activation-induced cell death inhibition mediated by a p70 inhibitory killer cell Ig-like receptor in Jurkat T cells. J Immunol *169*, 3726-3735.

Conlan, J. W., and North, R. J. (1991). Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. J Exp Med *174*, 741-744.

DCCT Res. Group (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. . N Engl J Med *329*, 977-986.

Dimayuga, R. E., and Agundez, J. (1986). Traditional medicine of Baja California Sur (Mexico). I. J Ethnopharmacol *17*, 183-193.

Dimo, T., Azay, J., Tan, P. V., Pellecuer, J., Cros, G., Bopelet, M., and Serrano, J. J. (2001). Effects of the aqueous and methylene chloride extracts of *Bidens pilosa* leaf on fructose-hypertensive rats. J Ethnopharmacol *76*, 215-221.

Dimo, T., Nguelefack, T. B., Kamtchouing, P., Dongo, E., Rakotonirina, A., and Rakotonirina, S. V. (1999). [Hypotensive effects of a methanol extract of *Bidens pilosa* Linn on hypertensive rats]. C R Acad Sci III *322*, 323-329.

Dimo, T., Nguelefack, T. B., Tan, P. V., Yewah, M. P., Dongo, E., Rakotonirina, S. V., Kamanyi, A., and Bopelet, M. (2003). Possible mechanisms of action of the neutral extract from *Bidens pilosa* L. leaves on the cardiovascular system of anaesthetized rats. Phytotherapy Research *17*, 1135-1139.

Dimo, T., Rakotonirina, S., Kamgang, R., Tan, P. V., Kamanyi, A., and Bopelet, M. (1998). Effects of leaf aqueous extract of *Bidens pilosa* (Asteraceae) on KCl- and norepinephrine-induced contractions of rat aorta. J Ethnopharmacol *60*, 179-182.

Dimo, T., Rakotonirina, S. V., Tan, P. V., Azay, J., Dongo, E., and Cros, G. (2002). Leaf methanol extract of *Bidens pilosa* prevents and attenuates the hypertension induced by high-fructose diet in Wistar rats. J Ethnopharmacol *83*, 183-191.

Division, F. a. N. (1997). Agriculture food and nutrition for Africa - A resource book for teachers of agriculture (Rome, Publishing Management Group, FAO Information Division).

Dorman, S. E., and Holland, S. M. (2000). Interferon-gamma and interleukin-12 pathway defects and human disease. Cytokine Growth Factor Rev *11*, 321-333.

Facinelli, B., Giovanetti, E., Varaldo, P. E., Casolari, P., and Fabio, U. (1991). Antibiotic resistance in foodborne *Listeria*. Lancet *338*, 1272.

Geissberger, P., and Sequin, U. (1991). Constituents of *Bidens pilosa* L.: do the components found so far explain the use of this plant in traditional medicine? Acta Trop *48*, 251-261.

Haak-Frendscho, M., Young, K. M., and Czuprynski, C. J. (1989). Treatment of mice with human recombinant interleukin-2 augments resistance to the facultative intracellular pathogen *Listeria monocytogenes*. Infect Immun *57*, 3014-3021.

Hamon, M., Bierne, H., and Cossart, P. (2006). *Listeria monocytogenes*: a multifaceted model. Nat Rev Microbiol *4*, 423-434.

Harlan, D. M., and von Herrath, M. (2005). Immune intervention with anti-CD3 in diabetes. Nat Med *11*, 716-718.

Hoffmann, B., and Holzl, J. (1989). Chalcone Glucosides from *Bidens pilosa*. Phytochemistry *28*, 247-249.

Hsu, Y. J., Lee, T. H., Chang, C. L., Huang, Y. T., and Yang, W. C. (2009). Anti-hyperglycemic effects and mechanism of *Bidens pilosa* water extract. J Ethnopharmacol *122*, 379-383.

Hubel, K., Dale, D. C., and Liles, W. C. (2002). Therapeutic use of cytokines to modulate phagocyte function for the treatment of infectious diseases: current status of granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and interferon-gamma. J Infect Dis *185*, 1490-1501.

Hwang, E. S., Choi, A., and Ho, I. C. (2002). Transcriptional regulation of GATA-3 by an intronic regulatory region and fetal liver zinc finger protein 1. J Immunol *169*, 248-253.

Hwang, E. S., Szabo, S. J., Schwartzberg, P. L., and Glimcher, L. H. (2005). T helper cell fate specified by kinase-mediated interaction of T-bet with GATA-3. Science *307*, 430-433.

Hwang, Y. C., Chu, J. J., Yang, P. L., Chen, W., and Yates, M. V. (2008). Rapid identification of inhibitors that interfere with poliovirus replication using a cell-based assay. Antiviral Research *77*, 232-236.

Jager, A. K., Hutchings, A., and van Staden, J. (1996). Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. J Ethnopharmacol *52*, 95-100.

Jones, E. M., Howe, R. A., and MacGowan, A. P. (1997). Treatment of *listeria* meningitis. Lancet *350*, 1034.

Kadota, Y., Ibuki, T., Goto, K., Matsumoto, Y., and Ueoka, R. (2005). Marked Inhibitory Effects of Extracts from Barley-Koji Miso Encapsulated in Hybrid Liposomes on the Growth of Human Stomach Tmor Cells in Vitro. Chemistry Letters *34*, 370-371.

Kai, N., Motojima, K., Tsunoda, T., and Kanematsu, T. (1993). Prevention of insulitis and diabetes in nonobese diabetic mice by administration of FK506. Transplantation *55*, 936-940.

Kaneko, M., Kawakita, T., Yamaoka, Y., and Nomoto, K. (2001). Development of the susceptibility to oral tolerance induction in infant mice administered a herbal drug, Hochu-ekki-to (Bu-Zhong-Yi-Qi-Tang). Int Immunopharmacol *1*, 219-227.

Katz, J. D., Benoist, C., and Mathis, D. (1995). T helper cell subsets in insulin-dependent diabetes. Science *268*, 1185-1188.

Kaufmann, S. H. (1993). Immunity to intracellular bacteria. Annu Rev Immunol *11*, 129-163.

Kawamoto, S., Nitta, Y., Tashiro, F., Nakano, A., Yamato, E., Tahara, H., Tabayashi, K., and Miyazaki, J. (2001). Suppression of T(h)1 cell activation and prevention of autoimmune diabetes in NOD mice by local expression of viral IL-10. Int Immunol *13*, 685-694.

Khan, M. R., Kihara, M., and Omoloso, A. D. (2001). Anti-microbial activity of *Bidens pilosa*, *Bischofia javanica*, *Elmerillia papuana* and *Sigesbekia orientalis*. Fitoterapia *72*, 662-665.

Ko, K. S., Lee, M., Koh, J. J., and Kim, S. W. (2001). Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. Mol Ther *4*, 313-316.

Koike, T., Itoh, Y., Ishii, T., Ito, I., Takabayashi, K., Maruyama, N., Tomioka, H., and Yoshida, S. (1987). Preventive effect of monoclonal anti-L3T4 antibody on development of diabetes in NOD mice. Diabetes *36*, 539-541.

Kroemer, G., Hirsch, F., Gonzalez-Garcia, A., and Martinez, C. (1996). Differential involvement of Th1 and Th2 cytokines in autoimmune diseases. Autoimmunity *24*, 25-33.

Kumari, P., Misra, K., Sisodia, B. S., Faridi, U., Srivastava, S., Luqman, S., Darokar, M. P., Negi, A. S., Gupta, M. M., Singh, S. C., and Kumar, J. K. (2009). A promising anticancer and antimalarial component from the leaves of *Bidens pilosa*. Planta Med *75*, 59-61.

Kumazawa, Y., Kawaguchi, K., and Takimoto, H. (2006). Immunomodulating effects of flavonoids on acute and chronic inflammatory responses caused by tumor necrosis factor alpha. Current Pharmaceutical Design *12*, 4271-4279.

Kurasawa, K., Koike, T., Matsumura, R., Takabayashi, K., Tomioka, H., Ito, I., and Yoshida, S. (1990). The immunosuppressant FK-506 prevents progression of diabetes in nonobese diabetic mice. Clin Immunol Immunopathol *57*, 274-279.

Kurtz, R. S., Young, K. M., and Czuprynski, C. J. (1989). Separate and combined effects of recombinant interleukin-1 alpha and gamma interferon on antibacterial resistance. Infect Immun *57*, 553-558.

Kviecinski, M. R., Felipe, K. B., Schoenfelder, T., de Lemos Wiese, L. P., Rossi, M. H., Goncalez, E., Felicio, J. D., Filho, D. W., and Pedrosa, R. C. (2008). Study of the antitumor potential of *Bidens pilosa* (Asteraceae) used in Brazilian folk medicine. J Ethnopharmacology *117*, 69-75.

Li, T. S. C. (2002). Chinese and Related North American Herbs (New York, New York, U.S.A., CRC Press).

Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., Nguyen, B. V., Gadina, M., Sher, A., Paul, W. E., and O'Shea, J. J. (2001).

T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. Proc Natl Acad Sci U S A *98*, 15137-15142.

MacGowen, A. P., Reeves, D. S., and McLauchlin, J. (1990). Antibiotic resistance of *Listeria monocytogenes*. Lancet *336*, 513-514.

Marles, R. J., and Farnsworth, N. R. (1995). Antidiabetic plants and their active constituents. Phytomedicine *2*, 137-189.

McDougall, B., King, P. J., Wu, B. W., Hostomsky, Z., Reinecke, M. G., and Robinson, W. E., Jr. (1998). Dicaffeoylquinic and dicaffeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. Antimicrobial Agents & Chemotherapy *42*, 140-146.

Miller, B. J., Appel, M. C., O'Neil, J. J., and Wicker, L. S. (1988). Both the Lyt-2+ and L3T4+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice. J Immunol *140*, 52-58.

Murphy, K. M., and Reiner, S. L. (2002). The lineage decisions of helper T cells. Nat Rev Immunol *2*, 933-944.

New, D. C., Miller-Martini, D. M., and Wong, Y. H. (2003). Reporter gene assays and their applications to bioassays of natural products. Phytother Res *17*, 439-448.

Newman, D. J., and Cragg, G. M. (2007). Natural products as sources of new drugs over the last 25 years. J Nat Prod *70*, 461-477.

Nguelefack, T. B., Dimo, T., Mbuyo, E. P., Tan, P. V., Rakotonirina, S. V., and Kamanyi, A. (2005). Relaxant effects of the neutral extract of the leaves of *Bidens pilosa* Linn on isolated rat vascular smooth muscle. Phytotherapy Research *19*, 207-210.

Nielsen, S. F., Christensen, S. B., Cruciani, G., Kharazmi, A., and Liljefors, T. (1998). Antileishmanial chalcones: statistical design, synthesis, and three-dimensional quantitative structure-activity relationship analysis. J Medicinal Chemistry *41*, 4819-4832.

Oliveira, F. Q., Andrade-Neto, V., Krettli, A. U., and Brandao, M. G. (2004). New evidences of antimalarial activity of *Bidens pilosa* roots extract correlated with polyacetylene and flavonoids. J Ethnopharmacol *93*, 39-42.

Ong, P. L., Weng, B. C., Lu, F. J., Lin, M. L., Chang, T. T., Hung, R. P., and Chen, C. H. (2008). The anticancer effect of protein-extract from *Bidens alba* in human colorectal carcinoma SW480 cells via the reactive oxidative species- and glutathione depletion-dependent apoptosis. Food & Chemical Toxicology *46*, 1535-1547.

Penix, L. A., Sweetser, M. T., Weaver, W. M., Hoeffler, J. P., Kerppola, T. K., and Wilson, C. B. (1996). The proximal regulatory element of the interferon-gamma promoter mediates selective expression in T cells. J Biol Chem *271*, 31964-31972.

Pereira, R. L., Ibrahim, T., Lucchetti, L., da Silva, A. J., and Goncalves de Moraes, V. L. (1999). Immunosuppressive and anti-inflammatory effects of

methanolic extract and the polyacetylene isolated from *Bidens pilosa* L. Immunopharmacology *43*, 31-37.

Ramiya, V. K., Maraist, M., Arfors, K. E., Schatz, D. A., Peck, A. B., and Cornelius, J. G. (2000). Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med *6*, 278-282.

Redl, K., Breu, W., Davis, B., and Bauer, R. (1994). Anti-inflammatory active polyacetylenes from *Bidens campylotheca*. Planta Med *60*, 58-62.

Rendu, F., Daveloose, D., Debouzy, J. C., Bourdeau, N., Levy-Toledano, S., Jain, M. K., and Apitz-Castro, R. (1989). Ajoene, the antiplatelet compound derived from garlic, specifically inhibits platelet release reaction by affecting the plasma membrane internal microviscosity. Biochem Pharmacol *38*, 1321-1328.

Roll, J. T., Young, K. M., Kurtz, R. S., and Czuprynski, C. J. (1990). Human rTNF alpha augments anti-bacterial resistance in mice: potentiation of its effects by recombinant human rIL-1 alpha. Immunology *69*, 316-322.

Rouquette, C., and Berche, P. (1996). The pathogenesis of infection by *Listeria monocytogenes*. Microbiologia *12*, 245-258.

Rucker, G., Kehrbaum, S., Sakulas, H., Lawong, B., and Goeltenboth, F. (1992). Acetylenic glucosides from *Microglossa pyrifolia*. Planta Med *58*, 266-269.

Seelinger, G., Merfort, I., and Schempp, C. M. (2008a). Anti-oxidant, anti-inflammatory and anti-allergic activities of luteolin. Planta Med *74*, 1667-1677.

Seelinger, G., Merfort, I., Wolfle, U., and Schempp, C. M. (2008b). Anti-carcinogenic effects of the flavonoid luteolin. Molecules *13*, 2628-2651.

Sica, A., Dorman, L., Viggiano, V., Cippitelli, M., Ghosh, P., Rice, N., and Young, H. A. (1997). Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. J Biol Chem *272*, 30412-30420.

Siddiq, A., and Dembitsky, V. (2008). Acetylenic anticancer agents. Anticancer Agents in Medicinal Chemistry *8*, 132-170.

Steinmuller, C., Roesler, J., Grottrup, E., Franke, G., Wagner, H., and Lohmann-Matthes, M. L. (1993). Polysaccharides isolated from plant cell cultures of *Echinacea purpurea* enhance the resistance of immunosuppressed mice against systemic infections with *Candida albicans* and *Listeria monocytogenes*. Int J Immunopharmacol *15*, 605-614.

Stratta, R. J., and Alloway, R. R. (1998). Pancreas transplantation for diabetes mellitus: a guide to recipient selection and optimum immunosuppression. BioDrugs *10*, 347-357.

Sundararajan, P., Dey, A., Smith, A., Doss, A. G., Rajappan, M., and Natarajan, S. (2006). Studies of anticancer and antipyretic activity of *Bidens pilosa* whole plant. Africa Health Sciences *6*, 27-30.

Swain, S. L. (1999). Helper T cell differentiation. Curr Opin Immunol *11*, 180-185.

Sweetser, M. T., Hoey, T., Sun, Y. L., Weaver, W. M., Price, G. A., and Wilson, C. B. (1998). The roles of nuclear factor of activated T cells and ying-yang 1 in activation-induced expression of the interferon-gamma promoter in T cells. J Biol Chem *273*, 34775-34783.

Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G., and Glimcher, L. H. (2000). A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell *100*, 655-669.

Szabo, S. J., Sullivan, B. M., Stemmann, C., Satoskar, A. R., Sleckman, B. P., and Glimcher, L. H. (2002). Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. Science *295*, 338-342.

Tan, P. V., Dimo, T., and Dongo, E. (2000). Effects of methanol, cyclohexane and methylene chloride extracts of *Bidens pilosa* on various gastric ulcer models in rats. J Ethnopharmacol *73*, 415-421.

Tewtrakul, S., Miyashiro, H., Nakamura, N., Hattori, M., Kawahata, T., Otake, T., Yoshinaga, T., Fujiwara, T., Supavita, T., Yuenyongsawad, S., *et al.* (2003). HIV-1 integrase inhibitory substances from *Coleus parvifolius*. Phytotherapy Research *17*, 232-239.

Townsend, M. J., Weinmann, A. S., Matsuda, J. L., Salomon, R., Farnham, P. J., Biron, C. A., Gapin, L., and Glimcher, L. H. (2004). T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity *20*, 477-494.

Toyoda, H., and Formby, B. (1998). Contribution of T cells to the development of autoimmune diabetes in the NOD mouse model. Bioessays *20*, 750-757.

Ubillas, R. P., Mendez, C. D., Jolad, S. D., Luo, J., King, S. R., Carlson, T. J., and Fort, D. M. (2000). Antihyperglycemic acetylenic glucosides from *Bidens pilosa*. Planta Med *66*, 82-83.

van Ogtrop, M. L., Mattie, H., Sekh, B. R., van Strijen, E., and van Furth, R. (1992). Comparison of the antibacterial efficacies of ampicillin and ciprofloxacin against experimental infections with *Listeria monocytogenes* in hydrocortisone-treated mice. Antimicrob Agents Chemother *36*, 2375-2380.

Wang, J., Yang, H., Lin, Z. W., and Sun, H. D. (1997). Flavonoids from *Bidens pilosa* var. *radiata*. Phytochemistry *46*, 1275-1278.

Wat, C. K., Biswas, R. K., Graham, E. A., Bohm, L., Towers, G. H., and Waygood, E. R. (1979). Ultraviolet-mediated cytotoxic activity of phenylheptatriyne from *Bidens pilosa* L. J Nat Prod *42*, 103-111.

Wat, C. K., Johns, T., and Towers, G. H. (1980). Phototoxic and antibiotic activities of plants of the Asteraceae used in folk medicine. J Ethnopharmacol *2*, 279-290.

Way, S. S., and Wilson, C. B. (2004). Cutting edge: immunity and IFN-gamma production during *Listeria monocytogenes* infection in the absence of T-bet. J Immunol *173*, 5918-5922.

Wiart, C. (2000). Medicinal plants of Southeast Asia, Vol 1 (Selabgor Darul Ehsan, Malaysia, Pelanduk Publications).

Wu, L. W., Chiang, Y. M., Chuang, H. C., Lo, C. P., Yang, K. Y., Wang, S. Y., and Shyur, L. F. (2007). A novel polyacetylene significantly inhibits angiogenesis and promotes apoptosis in human endothelial cells through activation of the CDK inhibitors and caspase-7. Planta Med *73*, 655-661.

Wu, L. W., Chiang, Y. M., Chuang, H. C., Wang, S. Y., Yang, G. W., Chen, Y. H., Lai, L. Y., and Shyur, L. F. (2004). Polyacetylenes function as anti-angiogenic agents. Pharm Res *21*, 2112-2119.

Yamaoka, Y., Kawakita, T., Kishihara, K., and Nomoto, K. (1998). Effect of a traditional Chinese medicine, Bu-zhong-yi-qi-tang on the protection against an oral infection with *Listeria monocytogenes*. Immunopharmacology *39*, 215-223.

Yamaoka, Y., Kawakita, T., and Nomoto, K. (2000). Protective effect of a traditional Japanese medicine, Bu-zhong-yi-qi-tang (Japanese name: Hochu-ekki-to), on the restraint stress-induced susceptibility against *Listeria monocytogenes*. Immunopharmacology *48*, 35-42.

Yamaoka, Y., Kawakita, T., and Nomoto, K. (2001). Protective effect of a traditional Japanese medicine Hochu-ekki-to (Chinese name: Bu-zhong-yi-qi-tang), on the susceptibility against *Listeria monocytogenes* in infant mice. Int Immunopharmacol *1*, 1669-1677.

Yang, H. L., Chen, S. C., Chang, N. W., Chang, J. M., Lee, M. L., Tsai, P. C., Fu, H. H., Kao, W. W., Chiang, H. C., Wang, H. H., and Hseu, Y. C. (2006).

Protection from oxidative damage using *Bidens pilosa* extracts in normal human erythrocytes. Food & Chemical Toxicology *44*, 1513-1521.

Yi, L., Li, Z. Q., Yuan, K. H., Qu, X. X., Chen, J., Wang, G. W., Zhang, H., Luo, H. P., Zhu, L. L., Jiang, P. F., *et al.* (2004). Small molecules blocking the entry of severe acute respiratory syndrome coronavirus into host cells. J Virology *78*, 11334-11339.

Yit, C. C., and Das, N. P. (1994). Cytotoxic effect of butein on human colon adenocarcinoma cell proliferation. Cancer Lett *82*, 65-72.

Yuan, L. P., Chen, F. H., Ling, L., Dou, P. F., Bo, H., Zhong, M. M., and Xia, L. J. (2008). Protective effects of total flavonoids of *Bidens pilosa* L. (TFB) on animal liver injury and liver fibrosis. J Ethnopharmacology *116*, 539-546.

Zhong, M. M., Chen, F. H., Yuan, L. P., Wang, X. H., Wu, F. R., Yuan, F. L., and Cheng, W. M. (2007). Protective effect of total flavonoids from *Bidens bipinnata* L. against carbon tetrachloride-induced liver injury in mice. J Pharmcy & Pharmacology *59*, 1017-1025.

Zulueta, C. A., Zulueta, A., Tada, M., and Ragasa, C. Y. (1995). A diterpene from *Bidens pilosa*. Phytochemistry *38*, 1449-1450.

Appendix I.

List of publications

1. **Shu-Lin Chang**, Cicero Lee-Tian Chang, Yi-Ming Chiang, Rong-Hong Hsieh, Chii-Ruey Tzeng, Tung-Kung Wu, Huey-Kang Sytwu, Lie-Fen Shyur, Wen-Chin Yang. Polyacetylenic compounds and butanol fraction form Bidens pilosa can modulate the differentiation of helper T cells and prevent autoimmune diabetes in non-obese diabetic mice. (2004) Planta Med. 70: 1045-1051. (**SCI, IF=2.289**)
2. **Shu-Lin Chang**, Yi-Ming Chiang, Cicero Lee-Tian Chang, Hsu-Hua Yeh, Lie-Fen Shyur, Yueh-Hsiung Kuo, Tung-Kung Wu and Wen-Chin Yang. Flavonoids, centaurein and centaureidin, from Bidens pilosa, stimulate IFN-γ expression. (2007) J Ethnopharmacol. 112: 232-236. (**SCI, IF=2.260**)
3. **Shu-Lin Chang**, Hsu-Hua Yeh, Yu-Shiun Lin, Yi-Ming Chiang, Tung-Kung Wu and Wen-Chin Yang. The effect of centaurein on interferon-γ expression and Listeria infection in mice. (2007) Toxicol Appl Pharmacol. 219: 54-61. (**SCI, IF=4.722**)

Appendix II.

List of presentations at international scientific meetings

1. **Shu-Lin Chang**, Lee-Tian Chang, Yi-Ming Chiang, Tung-Kung Wu, Wen-Chin Yang. (2004) Polyacetylenic compounds and butanol fraction from *Bidens pilosa* can modulate the differentiation of helper T cells and prevent autoimmune diabetes in NOD mice. The 3th International Congress on Immunosuppression. (San Diego, USA. Dec. 8-11.)
2. **Shu-Lin Chang**, Hsu-Hua Yeh, Yi-Ming Chiang, Cicero Lee-Tian Chang, Yu-Shiun Lin, Wen-Chin Yang. (2006) Centaurein can eradicate *Listeria* from mice via an up-regulation of Interferin-γ Expression. The 46th International Conference on Antimicrobial Agents and Chemotherapy. (San Francisco, USA. Sep. 27-30.)