Antitumor Activities of Extracts and Fractions from *Wedelia trilobata*

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Abstract

Nowadays more and more people concern about the healthcare-related natural products, especially the pharmacological properties of medicinal herbs and edible plants. Antitumor is one of the numerous pharmacological effects that have raised public interest. In this study, *Wedelia trilobata* was extracted and fractionated, and the antitumor activites of the fractions were investigated using *in vitro* and *in vivo* assays.

After screening the antitumor activity of water extracts of four Compositae species, *Wedelia trilobata* was chosen for detailed investigation in this project. Hot water extraction, ethanol precipitation and further fractionation were performed. The ethanol supernatant (WT4) was fractionated by a macroporus resin column. Four fractions, WT4-1, WT4-2, WT4-3 and WT4-4, with decreasing polarity were collected. The most active fraction WT4-4 was further fractionated by silica gel column. Then four fractions, WT4-4 A, WT4-4 B, WT4-4 C and WT4-4 D, with increasing polarity were collected. WT4-4 A was found to be the most effective fraction. A purified component (crystal) was collected from WT4-4 A and was determined to be sesquiterpene lactone with the chemical structure $C_{23}H_{32}O_9$ by

X-ray crystallography.

The extracts, fractions and crystal were subjected to *in vitro* antitumor assay. WT 4-4 significantly inhibited the proliferation of HL-60, K-562, S180, HepG2 and MCF-7, with IC_{50} of 4 μg/ml, 6.5 μg/ml, 8 μg/ml, 24 μg/ml and 36 μg/ml respectively. WT 4-4 showed no significant toxicity against normal human liver cell line L-02 and normal monkey kidney cell line Vero up to 50 μg/ml and 6.25 μg/ml respectively. IC_{50} of the further purified WT4-4 A on proliferation of HL-60, K-562 and S180 was 3 μg/ml, 3.4 μg/ml and 2.8 μg/ml respectively. The IC_{50} of the sesquiterpene lactone on proliferation of HL-60 was 2.2 μg/ml. DNA from WT4-4 treated HL-60, K-562 and S-180 did not show DNA laddering pattern on DNA agarose gel electrophoresis.

Since WT 4-4 could significantly inhibit the growth of S-180 cell line, *in vivo* antitumor assay using Sarcoma 180 tumor-bearing BALB/c mice was also carried out. After 10 consecutive days of intraperitoneal injection of WT4-4 at 3.2 mg/kg, there was 62.95% inhibition on tumor growth. WT4-4 did not have significant effect on body weight change.

論文摘要

現代人越來越關注天然保健產品，尤其有食用療效的植物和草藥當中的藥理特性。天然產品的抗癌功效更是眾多被受注目的療效之一種。本研究以三裂葉蟛蜞菊爲樣本，提取和分離當中的抗癌成份，進行一連串體內和體外的抗癌測試。

四種不同菊科植物的水提液經初步抗癌測試後，三裂葉蟛蜞菊的活性較顯著，故被選爲本研究之樣本，進行更深入的提純分析及活性測試。醇沉後的上清液(WT4)經大孔樹脂分餾後取得四個部份：WT4-1、WT4-2、WT4-3 及 WT4-4，極性由強至弱。而當中抗癌效果最佳的 WT4-4 則再以硅膠分餾，取得四個部份: WT4-4 A、B、C 及 D，極性由弱至強，而 WT4-4 A 爲最有效部位，並於當中取得純品結晶。經 X-射綫衍射檢測後其化學結構被測定爲 $C_{23}H_{32}O_9$， 屬倍半萜烯內酯。

於提取過程所得的各部位均進行體外抗癌測試，WT4-4 有效地抑制 HL-60、K-562、S-180、HepG2 及 MCF-7 等腫瘤細胞株之生長，其 IC_{50} 分別爲 4 μg/ml、6.5 μg/ml、8 μg/ml、24 μg/ml 及 36 μg/ml。WT4-4 於 50 μg/ml 對正常人類肝臟細胞(L-02)並無毒性，於 6.25 μg/ml 或以下對正常猴腎細胞(Vero)並未產生毒性。而被更進一步純化的 WT4-4 A 比 WT4-4 更能抑制 HL-60、K-562 及

S-180 之生長，其 IC_{50} 分別爲 3 μg/ml、3.4 μg/ml 及 2.8 μg/ml。於 WT4-4 取得的純品結晶對 HL-60 之抗癌能力較 WT4-4 A 高一些，其 IC_{50} 爲 2.2 μg/ml。經 WT4-4 一同體外培養三天的 HL-60、K-562 及 S-180 之 DNA 並沒有於 DNA 電泳中顯示 DNA 梯級式樣。

除此以外，在動物實驗中，一連十天腹腔注射 WT4-4 (3.2 mg/kg)，能抑制 Balb/c 小鼠 Sarcoma 180 肉瘤的生長達 62.95%，但並未影響腫瘤 Balb/c 小鼠體重之正常增長。

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ATCC	American type culture collection
BCRB	Breast cancer resistance protein
CCl-4	Carbon tetrachloride
Cdk	Cyclin-dependent kinase
CPT	Camptothecin
CPT-11	Irinotecan
DI	*Dendranthema indium*
DL	Daltons lymphoma
DM	*Dendranthema morifolium*
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOX	doxorubicin
ECA	Ehrlich ascites carcinoma
FAD	Food and Drug Administration
HCC	Hepatocellular carcinoma
HCPT	10-hydroxycamptothecin
HPLC	High performance liquid chromatography
i.p.	Intraperitoneal
IMDM	Iscove's modified dulbecco's medium
MEM	Minimal essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide

PBS	Phosphate buffered saline
PCD	Programmed cell death
PI	Propidium iodide
pRb	Retinoblastoma protein
PS	Phosphatidylserine
SGOT	Serum glutamate oxaloacetic transminase
SGPT	Glutamate pyruvic transminase
ST1481	7-t-butoxyiminomethylcamptothecin
T-1	Nuclear enzyme topoisomerase I
TCM	Traditional Chinese medicine
TM	*Taraxacum mongolicum*
TPA	12-tetradecanoylphorbol 13-acetate
TPT	Topotecan
WT	*Wedelia trilobata*

Chapter 1

Introduction

Recently, interest in medicinal value and clinical use of natural products has been in tremendous surge and many commercialized natural health-related products are widely accepted by the public. Actually, both 'whole plant' and 'plant derived drug' are common in modern medicine, especially those plant derived medicine (Robbers *et al.*, 1996). Among the bioactivities in natural products, antitumor is one of that have raised people's interest. Although there are now many drugs or radiotherapy for cancer treatment, the need for better and more variety of drugs for cancer patients is still desirable. It is because current chemotherapy or radiotherapy has many side effects such as loss of hair and body weight or development of resistance, although there are improvements (Jong and Donovick, 1989). So, searching for new antitumor drug from a rich source, plants, is still in great needs.

Herbal therapy has a long history in China and India (Bedi and Shenefelt, 2002) and it is regaining the prominent position in many developing and developed countries nowadays (Li, 2000; Li, 2002). Moreover, plants have become a natural source for modern drug, as there are about 40% of modern pharmaceuticals coming from natural origin (Samuelsson, 1999). Examples of successful plant-derived antitumor drugs include camptothecin from *Camptotheca acuminata* and taxol from *Taxus brevifolia* (Lin *et al.*, 2002).

In searching bioactive components, Compositae being the largest flowering plant Family which comprises more then 23,000 known species is a big source (Bremer *et al.*, 1994). Many Compositae plants are traditionally used as medicinal herbs and different bioactivities have been reported, for examples, some are used to treat infection and headaches (Smith, 1911), whereas others are found to have hepatoprotective activity (Lin *et al.*, 1994) and antitumor activity (Kupchan and Bauerschmidt, 1971; Roussakis *et al.*, 1984). Both *in vitro* and *in vivo* antitumor activities of Compositae species have been reported. For examples, the methanolic extract of *Emilia sonchifolia* has significant *in vitro* cytotoxic effect on DL, ECA and L-929 cancer cell lines with EC_{50} at 1.5 mg/ml, 1 mg/ml and 15 μg/ml respectively. It also significantly reduced DL solid tumor *in vivo* through oral administration

(Shylesh and Padikkala, 2000). Although there are no single standardized procedures to study *in vivo* antitumor effect, and there are different parameters used to illustrate the effect, for examples increased in life span and reduced in tumor size, the basic method is similar. Firstly, the selected tumor cell line was inoculated into the selected animal models for tumor formation. Secondly, the tumor-bearing animal models would receive the tested sample for several dosages and finally are sacrificed and the parameters used to illustrate the antiumor effect would be measured (Kaneda *et al.*, 1997; Shylesh and Padikkala, 2000; Wang *et al.*, 2000). Even though the basic method is more or less the same, the period for each step and route of administration may be different. This is also the case for *in vitro* antitumor assay.

When people concerns about antitumor effect of a natural product, they also are interested in mechanisms behind the drug effect. An antitumor drug may exert the effect through many pathways. Cell cycle control and apoptosis are two common means. For examples, taxol cause cell cycle arrestment in G2/M phase in cancer cells in order to block the cell division (Horwitz, 1992), and aqueous extract of *Salvia miltiorrhiza* induced apoptosis with DNA fragmentation in cancer cell line (Liu *et al.*, 2000). Cell cycle arrestment can be illustrated by flow cytometry using the sample treated cancer cells (Soni *et al.*, 2000; Towle *et al.*, 2001) while DNA fragmentation,

hallmark of apoptosis, can be illustrated by DNA gel electrophoresis of the DNA from sample treated cancer cells (Cotter and Martin, 1996; DeBlois *et al.*, 2000).

In this project, the antitumor activity of four Compositae species namely *Dendranthema indicum*, *Dendranthema morifolium*, *Taraxacum mongolicum* and *Wedelia trilobata* were screened using *in vitro* assay. *Wedelia trilobata* was chosen for further investigation because it's extracts had strong activity and it is easily available on campus. It belongs to the family Asteraceae and tribe Heliantheae, is introduced into Hong Kong from South America and now widely spread as decorative plant.

Wedelia is a large genus, and compounds usually found in various *Wedelia* species include flavonoid, terpenoid and lactone (Bohlmann *et al.*, 1984; Block *et al.*, 1998). Moreover, sesquiterpene lactones, which are also frequently found in Compositae, possess many biological activities (Marles *et al.*, 1995).

Folkloric use of *Wedelia* species can be found in many countries to treat swelling, headaches, fevers, infections and pathologies of the respiratory tract. Other than folkloric use, they were also reported to have hepatoprotective (Gopalakrishnan *et al.*, 1989), hypoglycaemic (Novaes *et al.*, 2001), antimicrobial (Taddei and Rosas-Romero, 1999), antifeedant and antifungal (Miles *et al.*, 1990; Miles *et al.*,

1993) activities. *Wedelia trilobata* has also traditionally been used to relief discomfort symptoms of colds and flu, hepatitis, indigestion, and infections (Roque *et al.*, 1987; Miles *et al.*, 1990; Lin *et al.*, 1994). Although antitumor activity has been reported in several Compositae species (Shylesh and Padikkala, 2000; Chatterjee *et al.*, 1999; Bedi and Shenefelt, 2002), bioactivity of *Wedelia trilobata* was seldom investigated. The antitumor and related bioactivity of *Wedelia trilobata* is deserved further investigation.

In this thesis, the antitumor activity of different fractions and components isolated from *Wedelia trilobata* was investigated using both *in vitro* and *in vivo* assays. The *in vitro* cytotoxic effect on normal cell lines was investigated by MTT assay method, and the *in vivo* side effect was expressed as change in body weight in the sample treated tumor-bearing mice group as compared to untreated normal group. The effect of the active fraction on DNA of tumor cell lines was investigated by DNA agarose gel electrophoresis.

Chapter 2

Literature Review

2.1 Medicinal Plants and Herbs

The use of herbal therapy originated in China and India and has a history of thousands of years (Bedi and Shenefelt, 2002). In China, the first recorded use of medicinal plants dates back to 2800 BC and the legendary use was even earlier (Borchers *et al.*, 1997). While in Indian, Ayurvedic medicine dates back to 3000 BC (Bedi and Shenefelt, 2002). The medical use of plants and herbs is still common nowadays. In 1999, the Chief Executive of Hong Kong Special Administrative Region of the People's Republic of China, Mr. Tong Chee Hwa, declared that he would like to put lots of efforts to develop Hong Kong as a world centre for Traditional Chinese Medicine (TCM) (Li, 2002).

Nowadays, people and mass media worldwide more and more concern the

use of medicinal plants or herbs and natural health products, both for their pharmaceutical properties in health and cosmetic use. In China, Egypt, Indian, and other developing countries, people are still widely employing the medicinal plants as part of medical practice (Li, 2000). Moreover, eighty percent of the world's population is still using traditional medicine (Li, 2002), either due to the high cost of modern medical services, limited source of modern medicine or increasing concepts of health enhancement.

Other than Asian countries, there are also explosive growth in the use of medicinal plants and related remedies over the past few decades in Western countries. Only in the United States, there is approximately 15 percent annual increase in the consumption of medicinal herbs (Marwick, 1995). A consumer survey for the year 1996 reported that during the previous year, about one-third of all adult Americans were estimated to have used a herbal product and these people were estimated to spend about US$3.24 billion per year on herbs (Klepser and Klepser, 1999; Neldner, 2000). So, it is not surprising that, there has been a great interest in medicinal plants and herbs, and in both North America and Europe, natural products have become a multibillion-dollar industry (Li, 2000).

2.1.1 Antitumor activities

Cancer, being the third major cause of mortality, is the largest single cause of death in both men and women in the world, causing over 6 million deaths per year (Yang *et al.*, 2000). Radiotherapy and chemotherapy being the major traditional cancer therapy, cause many adverse side effects, for example vomit and loss of hair. Many anticancer drugs have been developed and used clinically nowadays, however, development of resistance to anticancer drugs has been observed. Therefore, new safe drugs are in great needs.

Plants and natural products, being the invaluable source of drug discovery, possessing various biological activities, for examples, antiviral (Vivian *et al.*, 2001), antioxidant (VanderJagt *et al.*, 2002) and anti-inflammatory (Motohiko *et al.*, 2001), have been vastly searched and investigated. The antitumor activity is also of great interest. Some successful examples in discovering antitumor, cancer chemoprotective activities or antitumor drug derived from plants, are camptothecin, taxol, magnolol (Lin *et al.*, 2002) and resveratrol (Hsieh and Wu, 1999; Park *et al.*, 2000).

2.1.1.1 Successful examples – Camptothecin

Compounds occurring in nature usually possess wide range of biological

activities, and most of them are secondary metabolites. Camptothecin (CPT) is one of the most famous natural products derived from plants which possess antiutumor effects on a wide range of tumors, for examples, breast cancer, carcinoma of stomach, rectum, colon and bladder, and chronic leukaemia (Li, 2002). It is also clinically used in the United States.

Camptothecin, being a promising new class of antitumor drugs over the last decade, was initially extracted from stembark of *Camptotheca acuminata,* Xi Shu, a native Chinese tree, and the chemical structure was identified by Wall *et al.* (1966).

CPT is a pentacyclic indole alkaloid, with molecular weight of 348.111, and formula $C_{20}H_{16}N_2O_4$ with unique structure involving terminal closed α-hydroxy-δ-lactone in ring E (Wall and Wani, 1996). The lactone E-ring of CPT molecule is probably the site responsible for the antitumor activity (Keir *et al.*, 2001).

However, natural camptothecins share a common property (Fig. 2.1). The α-hydroxy lactone ring is intrinsic instable and undergoes hydrolysis in alkaline pH, pH>8.0, for example in physiological conditions, to give the open-ring, water soluble carboxylate form with decreased *in vivo* and *in vitro* antitumor activity. But in acidic medium, pH<5, the carboxylate form undergoes relactonization to become the more active lactone form CPT, which is extremely water-insoluble but soluble in

dimethylsulfoxide (DMSO) (Wall *et al.*, 1966; Ahmed *et al.*, 1998; Adams *et al.*, 2000; Oguma, 2001). Since naturally occur CPT is highly labile in nature, present in small amounts and water insoluble, in the past few decades, a lot of efforts have been applied to produce more stable CPT analogues with equal or higher antitumor activity and higher solubility.

CPT and its analogues for examples, irinotecan (CPT-11), topotecan (TPT), 10-hydroxycamptothecin (HCPT) and 7-t-butoxyiminomethylcamptothecin (ST1481), are reported to have good antitumor effect on wide range of tumors.

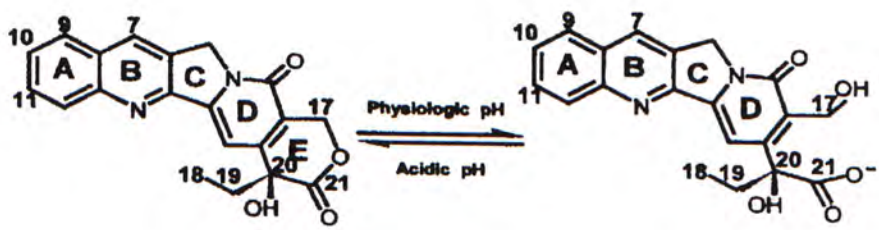
CPT-11 and TPT demonstrated significant antitumor effect in clinical trials (Adams *et al.*, 2000). CPT-11 is proved and clinically available to treat colorectal cancer while TPT to treat ovarian and small-cell lung cancers (Keir *et al.*, 2001; Oguma, 2001). In Japan, CPT-11 became available since 1994 while in the United States, it is used to treat advanced colorectal adenocarcinoma (Okuno *et al.*, 2000). CPT-11 is also reported to be effective in human breast cancer *in vitro* and *in vivo*, through apoptotic pathway (Zhang, 1997; Liu and Zhang, 1998).

HCPT showed *in vivo* and *in vitro* antitumor effects against human breast cancer cell lines MCF-7 and MDA-MB-468 (Liu and Zhang, 1998) while lipophilic ST1481, was reported to posses antitumor activity against colon tumor and able to

overcome the breast cancer resistance protein (BCRP) - associated resistance in colon carcinoma cell line HT29/MIT *in vitro* and *in vivo* (Perego *et al.*, 2001).

CPT and its analogues are well known to be effective through the inhibition of nuclear enzyme topoisomerase I (T-I), an enzyme responsible for different cellular DNA transaction such as replication, transcription, and recombination (Hsiang *et al.*, 1985). Moreover, the antitumor activity of CPT was maximized in the S-phase of the cell cycle and recognized as S phase-specific agents (Del Bino *et al.*, 1991). Although the active lactone E-ring of CPT is labile in physiological pH, the reaction is reversible and many solid tumors produce lactic acid in anaerobic glycolysis that flavours the relactonization of CPT to its active form (Adams, 2000).

Figure 2.1 Structure of lactone and carboxylate form of camptothecin (Adopted from Adams, 2000).



active lactone form of CPT

inactive carboxylate form of CPT

2.1.1.2 Successful examples – Taxol

In the last decade, taxol, a diterpenoid plant product, is one of the most famous antitumor agents introduced in clinical oncology (Svojanovsky *et al.*, 1999). Taxol, same as CPT, is a secondary metabolite in plant. This antitumor compound was discovered and first isolated as an active constituent from the bark of *Taxus brevifolia* (Pacific yew) in Washington state in 1971 (Wani *et al.*, 1971). *T. brevifolia* is a slow growing tree mainly found in the coastal areas of Washington state of the United States (Wall and Wani, 1996).

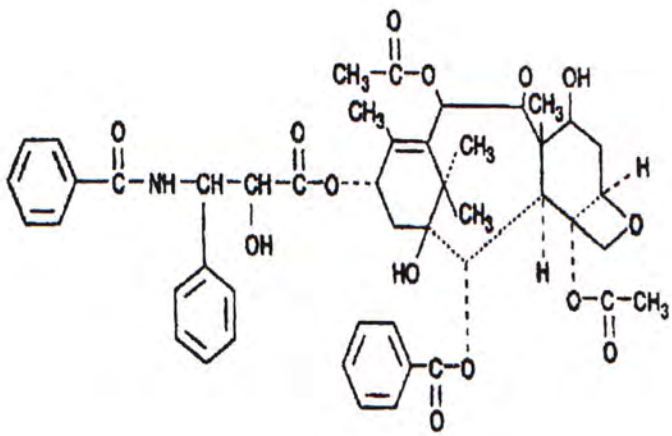
The chemical structure of taxol is very complex (Fig. 2.2), consisting of a taxane ring system linked to a unique four-membered oxetane ring, which does not occur in any other natural taxanes, and an ester side chain (Wani *et al.*, 1971). The side chain of taxol is probably responsible for it's antitumor effect because analogues of taxol without the side chain are not cytotoxic (Horwitz, 1992).

Taxol was reported to exhibit profound antineoplastic effects against a wide range of human tumors, for examples, breast, ovarian, head and neck, small cell and non-small-cell lung cancers and metastatic melanomas (Rowinsky *et al.*, 1992; Kohn *et al.*, 1994; Rowinsky and Donehower, 1995). According to its activity in human clinical trials, the U.S. Food and Drug Administration (FAD) has approved taxol for

use against ovarian cancer (Rao *et al.*, 1995).

When deducing the antitumor mechanism of taxol, it is very logical to suggest that the unique diterpenoid structure would relate to a novel mechanism. In 1970s the mechanism of taxol antitumor effect was actually described as unique among plant alkaloids (Svojanovsky *et al.*, 1999). The cytotoxicity effect of taxol is to induce the formation of highly stable microtubules, which are vital to mitosis. Since these microtubules are so stable, they resist depolymerization to tubulin, cause cells arrestment in the late G2/M phase of cell cycle, block the mitotic stage and so inhibit cell division (Horwitz, 1992).

Figure 2.2 Structure of taxol (Adopted from Parekh and Simpkins, 1997).



2.2 Compositae and its Traditional Functions

Compositae (Asteraceae) consists of herbs, shrubs, or trees (although less common), and is the largest family of flowering plants. They share the same characteristic of having reduced flowers and organized into an involucrate pseudanthium in the form of a head or capitulum. Many Compositae flowers are very attractive and beautiful, so people used them as ornamental plants around the world.

According to a comparatively recent subfamilial classification instead of an older tribal classification, Compositae comprises over 1500 genera and around 23,000 known species. They are arranged in three subfamilies, namely Asteroideae, Cichorioideae and Barnadesioideae, and seventeen tribes. Table 2.1 shows a brief overview of classification of Compositae, including the number of subtribes, genera and species. Each year, there are still more than 10 genera being described, therefore, it is far to achieve a stable and cladistic classification of Compositae (Bremer *et al.*, 1994).

Being the largest family of flowering plants, traditionally Compositae are also widely used as medicinal herbs in many countries with different functions. Therefore it is a valuable and tremendous source for scientists to explore and search for bioactive natural products.

Although there are many species belonging to Compositae, Chinese regards the therapeutical actions of different varieties of Compositae plants practically same, i.e. they can "benefit the blood and circulation, preserve vitality and flowers are prescribed in colds, headaches, and inflamed eyes" (Smith, 1911). The white variety is claimed to be effective to prevent hair from falling out and keep in black, preventing turning to gray. 'Chrysanthemum wine' produced by soaking chrysanthemum flowers in wine is considered to be good for digestion, circulatory and nervous systems. The wild variety is used to treat retained menses, enlarged glands and wash infected and cancerous sores (Smith, 1911).

More specific examples of traditional use of Compositae such as *Wedelia chinensis*, Hwang-hua-mih-tsay, is a Taiwanese folk medicine, used to treat swelling (Lin *et al.*, 1994). *Achillea millefolium*, commonly called yarrow, is folkloric used to reduce fever and induce sweating (Tozyo, 1994). Roots of *Ratibida mexicana* is crushed and used by Tarahumara Indians to treat rheumatism of legs (Calera *et al.*, 1995).

Table 2.1 Classification of the Asteraceae, with number of subtribes, genera, and species (Adapted from Bremer *et al.*, 1994).

	Subtribes	Genera	Species
Asteraceae	82	1,535	23,000
Barnadesioideae	--	9	92
Barnadesieae	--	9	92
Cichorioideae	25	391	6,700
Mutisieae	2	76	970
Cardueae	4	83	2,500
Lactuceae	11	98	1,550
Vernonieae	6	98	1,300
Liabeae	--	14	160
Arctoteae	2	16	200
Asteroideae	57	1,135	16,200
Inuleae	--	38	480
Plucheeae	--	28	220
Gnaphalieae	5	181	2,000
Calenduleae	--	8	110
Astereae	3	174	2,800
Anthemideae	12	109	1,740
Senecioneae	3	120	3,200
Helenieae	8	110	830
Heliantheae	10	189	2,500
Eupatorieae	16	170	2,400

2.3 Antitumor activity of Compositae

Other than folkloric use, scientific publications on different bioactivities of Compositae are also available. For examples, hepatoprotective (Lin *et al.*, 1994), antioxidatant (Chu *et al.*, 1999) and antinociceptive effects (Block *et al.*, 1998).

One of the bioactivities that raised people's interest is the antitumor activity. It is also reported that many Compositae plants have this bioactivity (Kupchan and Bauerschmidt, 1971; Roussakis *et al.*, 1984). Some recent publications about antitumor activity of Compositae include *Emilia sonchifolia* (Shylesh and Padikkala, 2000), Silymarin (Chatterjee *et al.*, 1999; Bedi and Shenefelt, 2002), *Achillea millefolium*, yarrow (Tozyo *et al.*, 1994) and *Parthenium hysterophorus* (Mukherjee and Chatterjee, 1993).

2.3.1 *Emilia sonchifolia*

E. sonchifolia, a herbaceous plant found in Asia, is used as folklore medicine in India and China. It was reported to have cytotoxic effect on Daltons lymphoma (DL), Ehrlich ascites carcinoma (EAC) and mouse lung fibroblast (L-929) cells.

In the *in vitro* cytotoxicity assay, the cell viability was determined by trypan blue exclusion method. The methanolic extract of *E. sonchifolia* exhibited significant

cytotoxic effect towards DL, ECA and long term cytotoxicity towards L-929 with EC_{50} at 1.5 mg/ml, 1 mg/ml and 15 μg/ml respectively. But no cytotoxicity was found on normal human lymphocytes (Shylesh and Padikkala, 2000).

In the *in vivo* antitumor assay, effect against both ascites and solid tumor were investigated. Oral administration of the *E. sonchifolia* methanolic extract significantly increased the life span of EAC bearing mice, with 241 percent increase for mice received extract 24 h after tumor transplantation and 129 percent increase for mice received extract 10 days after tumor transplantation. The extracts also significantly reduced the DL solid tumor development in mice, from 4.25 cm^3 tumor volume to 1.25 and 2.5 cm^3 for mice received extract 24 h after tumor transplantation and mice received extract 10 days after tumor transplantation respectively (Shylesh and Padikkala, 2000).

Other than antitumor activity, extracts of *E. sonchifolia* were also reported to possess antioxidant and anti-inflammatory activity (Shylesh and Padikkala, 1999; Muko and Ohiri, 2000)

2.3.2 Silymarin

Silymarin is a naturally occurring polyphenolic flavonoid isolated from

Silybum marianum, commonly known as milk thistle. Conventionally, silymarin is used for liver disease, with profound antioxidant effect, and is clinically used in Europe and Asia as an antihepatotoxic agent. Recent research conducted by Chatterjee *et al.* (1999) found that, silymarin like many other antioxidants, was an anti-tumor promotor agent, especially effective in protection against stage I tumor promotion. Stage I tumor promotion is an initial step, in which, initiated cell is converted to a dormant tumor cell (Chatterjee *et al.*, 1999; Bedi and Shenefelt, 2002).

In the *in vivo* assay, silymarin applied topically to SENCAR mouse before 12-O-tetradecanoylphorbol 13-acetate (TPA) application resulted in a highly significant protection against chemically induced skin tumor promotion. The protective effects of silymarin was observed at the dose 3, 6, 12 mg per application in terms of (1) tumor incidence, with 25%, 40%, and 75% protection respectively; (2) tumor multiplicity (cumulative number of tumors per group or number of tumors per mouse), with 76%, 84%, and 97% protection respectively; and (3) tumor volume, with 76%, 94%, and 96% protection respectively. Moreover, in the *in vitro* assay, the DNA synthesis of human epidermoid carcinoma A431 cells was also inhibited by the silymarin (Chatterjee *et al.*, 1999).

2.4 *Wedelia* species

Wedelia is a large genus of the family Compositae and belongs to the largest subfamily of Compositae, Asteroideae, tribe Heliantheae, subtribe Ecliptinae (Herz and Kulanthaivel, 1984). Many species of *Wedelia* are used in the folk medicine to cure a large variety of diseases in many countries, for examples, swelling, headaches, fevers, infections and pathologies of the respiratory tract (Roque *et al.*, 1987; Miles *et al.*, 1990; Lin *et al.*, 1994).

2.4.1 Hepatoprotective effect

Hepatoprotective effect is one of the bioactivities exhibited by several *Wedelia* species. *Wedelia calendulacea* was found to have antihepatotoxic activity, which counteracted the effects induced by hepatotoxin carbon tetrachloride (CCl-4), such as lowering the increase of liver weight, hepatic lipid peroxidation and serum alkaline phosphatase (Gopalakrishnan *et al.*, 1989), The alcoholic extract of whole plant also elevated the bile flow in rats, which implied the stimulation of liver secretory capacity (Sharma *et al.*, 1989).

Wedelia chinensis also possessed hepatoprotective effect. It is a Taiwan folk medicine, used to cure swelling. In East and Southeast Asia, leaves are also used as a

tonic and useful folk medicine in treating coughs, headaches, skin disease, and baldness (Lily, 1980). The crude water extract of *Wedelia chinensis* at the dose of 300 mg/kg, p.o. at 2, 6, 10 hours could reduce the increase of serum glutamate oxaloacetic transminase (SGOT) and glutamate pyruvic transaminase (SGPT) levels in acute hepatitis mice and rats which were induced by three well known hepatotoxins: CCl-4, acetaminophen for mice and D(+)-galactosamine for rats. Recently in Taiwan, it has been used as a remedy for hepatitis (Lin *et al.*, 1994).

2.4.2 Hypoglycemic effect

Wedelia paludosa is a Brazilian medicinal plant. The extracts of this plant could significantly lower the blood glucose level in alloxan-induced diabetic rats by intragastical administration, implying that *W. paludosa* could be used as an adjuvant agent in treating diabetes (Novaes *et al.*, 2001).

2.4.3 Antimicrobial activity

The n-hexane extract of *Wedelia trilobata* exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria, including *Bacillus subtilis*, *Mycobacterium smegmatis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*,

which are Gram-positive; and *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella* group C, *Salmonella paratyphi*, and *Shigella sonnei*, which are Gram-negative. The ethyl acetate extract, however was active against *Salmonella* group C only (Taddei and Rosas-Romero, 1999).

2.4.4 Antinociceptive activity

The hydroalcoholic extract of *Wedelia paludosa*, a Brazilian medicinal plant, which exhibited hypoglycemic effect, also possessed another bioactivity, the antinociceptive activity. Intraperitoneally administration of several fractions obtained from the hydroalcoholic extract of *Wedelia paludosa* at 3 mg/kg significantly reduced the number of abdominal constrictions induced by acetic acid in Swiss mice, including the contraction of abdominal muscle and stretching of hind limbs. The inhibition percentage of writhes was reported to be up to 72 percentage (Block *et al.*, 1998).

2.4.5 Antifeedant and antifungal activities

Stem extracts of *Wedelia biflora*, a Thai plant, showed antifeedant activity against the cotton boll weevil, and compounds isolated from the extracts also

exhibited antifeedant and/or antifungal activity (Miles *et al.*, 1990). Other compounds isolated from methylene chloride extract of leaves also exhibited antifeedent activity towards cotton boll weevil and/or antifungal activity (Miles *et al.*, 1993).

2.4.6 Trypanosomicidal effect

Diterpenes isolated from the ethanolic extract of *Wedelia paludosa* showed *in vitro* trypanosomicidal effect. The isolated diterpenes could kill 100% of the parasitic protozoan *Trypanosoma cruzi* at the lowest dose of 0.68 mg/ml. *Trypanosoma cruzi* is a causative agent of Chagas' disease, also named American trypanosomiasis. The transmission vector is insect but recently, the major path of transmission is through infected blood in Brazil (Batista *et al.*, 1999).

2.4.7 Chemical constituents

There are several compounds commonly found in *Wedelia* species. These include lactone from *Wedelia grandiflora* (Bohlmann *et al.*, 1984a), kaurenoic acid (diterpene) and flavone (flavonoid) from *Wedelia paludosa* which exhibit significant inhibition of acetic acid-induced abdominal constriction in mice (Block *et al.*, 1998),

diterpene lactones from *Wedelia regis* (Bohlmann *et al.*, 1984b), ent-kaurenic acids and lactones from *Wedelia hispida*, ent-kauranes from *Wedelia calycina* (Herz and Kulanthaivel, 1984), eudesmanolides, diterpene and sesquiterpene lactones from *Wedelia trilobata* (Bohlmann *et al.*, 1981), eudesmanolide sesquiterpenes from *Wedelia prostrata* (Farag *et al.*, 1996), sesquiterpene lactone, wedelolactone, ($C_{16}H_{10}O_7$) from *Wedelia chinensis* (全國中草藥匯編編寫組, 1983) , and diterpenes and kaurenoic acid from *Wedelia paludosa* (Batista *et al.*, 1999).

2.5 Sesquiterpene lactones

Sesquiterpene lactones are a group of naturally occurring compounds with three isoprene units, 15 carbon atoms, lactone structure, and usually with different degree of unsaturation. The empirical formula of sesquiterpene is $C_{15}H_{24}$. Sesquiterpene lactones can be found in both higher and lower plants, and they are characteristic compounds in compositae plants as secondary metabolites with different structure. They are colorless and bitter. The boiling point of sesquiterpenes is relatively high, usually between 250°C and 280°C (Rodriguez *et al.*, 1976; Ragasa *et al.*, 1993; 肖崇厚, 1996).

Sesquiterpene lactones are classified basically by the carbocyclic skeletons (Rodriguez *et al.*, 1976). Typically occurring sesquiterpene lactones in compositae plants include germacranolides, eudesmanolides, guaianolides, pseudoguaianolides and xanthanolides (Ragasa *et al.*, 1993; Dirsch *et al.*, 2001). Very often, lactones with highest concentration in leaves and flowering heads, the percentage vary with species, and in some species the content also vary with seasons (Rodriguez *et al.*, 1976).

Many sesquiterpene lactones were isolated and identified during the past 50 years. In recent decades, other than the chemical investigation, bioactivities were

also in great interest. Sesquiterpene lactones were documented with diverse biological activities, including antitumor and cytotoxic activity, antimitotic activity, antimicrobial activity, chemoprophylaxis, allergic effect on man, antifeedant acticity on insect, poisoning activity on vertebrate, plant growth regulatory activity, and preventive activities on crop diseases (Rodriguez *et al.*, 1976; Ando *et al.*, 1987).

Many publications related the biological activities of sesquiterpene lactones to the lactone structure, with α-methylene γ-lactones moiety or α,β-unsaturated lactone moiety (Lee *et al.*, 1971; Rodriguez *et al.*, 1976; Ando *et al.*, 1987; 肖崇厚, 1996; Beekman *et al.*, 1998).

Artemisinin (Beekman *et al.*, 1998), helenalin (肖崇厚, 1996; Dirsch *et al.*, 2001), vernolepin (肖崇厚, 1996) and parthenolide (Kang *et al.*, 2002) are examples of sesquiterpene lactone with antitumor activity. Moreover, Arglabin-DMA, the derivative of arglabin, a sesquiterpene lactone from *Artemisia glabella* is registered as a compound with antitumor activity in the Republic of Kazakstan (Shaikenov *et al.*, 2001).

Although cytotoxicity of sesquiterpene lactones is believed to be related to the reaction with sulfhydryl groups via a Michael-type addition, one of the antitumor sesquiterpene lactones that have been widely investigated, Helenalin, was reported to

induce apoptosis in human leukemia Jurkat T cells but not in healthy human peripheral blood mononuclear cells (PBMCs). The apoptosis-inducing mechanism of helenalin was demonstrated to involve the activation of caspase-3, downstream effector and caspase-8, the initiator and cytochrome-c release from mitochondrioa to cytosol. Moreover, the apoptosis-inducing mechanism was independent of CD95 death receptor and antiapoptotic proteins Bcl-x_L and Bcl-2 (Dirsch *et al.*, 2001).

Another example, parthenolide, was reported to be able to uplift the differentiation of human leukemia HL-60 cells into monocytes induced by 1,25-(OH)2D3. And this enhancement was suggested to be the ability of parthenolide to inhibit NF-kappaB binding activity (Kang *et al.*, 2002).

2.6 Cell cycle control and apoptosis

It is very important for multicellular organisms to maintain the balance of cell proliferation and programmed cell death. If the homeostasis of these two events were disrupted, it would be a great problem and may lead to vary diseases. Cancer is recently recognized as a disease caused by both uncontrolled cell proliferation and ineffective cell death. Therefore, cell cycle control and apoptosis have become two major targets for cancer therapy.

2.6.1 Cell cycle and its control in cancer therapy

Each normal cell passes through a tightly controlled cell cycle in order to grow and divide. There are many events associated with the cell cycle, and these events are orderly distributed in different phases of cell cycle, namely M phase, G1 phase, S phase and G2 phase (Figure 2.3). In mammalian cells, generally, the time required for one cycle is about 18-24 hours (Becker *et al.*, 2000).

In **M phase**, also known as mitotic phase, mitosis and cytokinesis take place in series, which means the events of nuclear division and cytoplasmic division take place respectively. After the division of cytoplasm, two daughter cells are produced with identical DNA content. Therefore, the point of actual division of cell occurs in

the M phase.

There are many dramatic events in mitosis, include (1) the condensation of chromatin to produce thick chromosomes which can be seen under microscope; (2) breakage of nuclear envelop; (3) segregation of sister chromatids by mitotic spindle to opposite poles of cell; (4) cytokinesis and formation of new nuclear envelop. However, the time spent in mitotic phase by mammalian cell is relatively short, usually less than an hour although it varies among different cell types (Becker *et al.*, 2000).

Cells spend much more time in the interphase, which is a growth phase, made up of G1, S and G2 phases. During interphase many cellular components are synthesized and so lead to gradual increase in cell mass. In **S phase** DNA is synthesized, and it usually takes six to eight hours in mammalian cell.

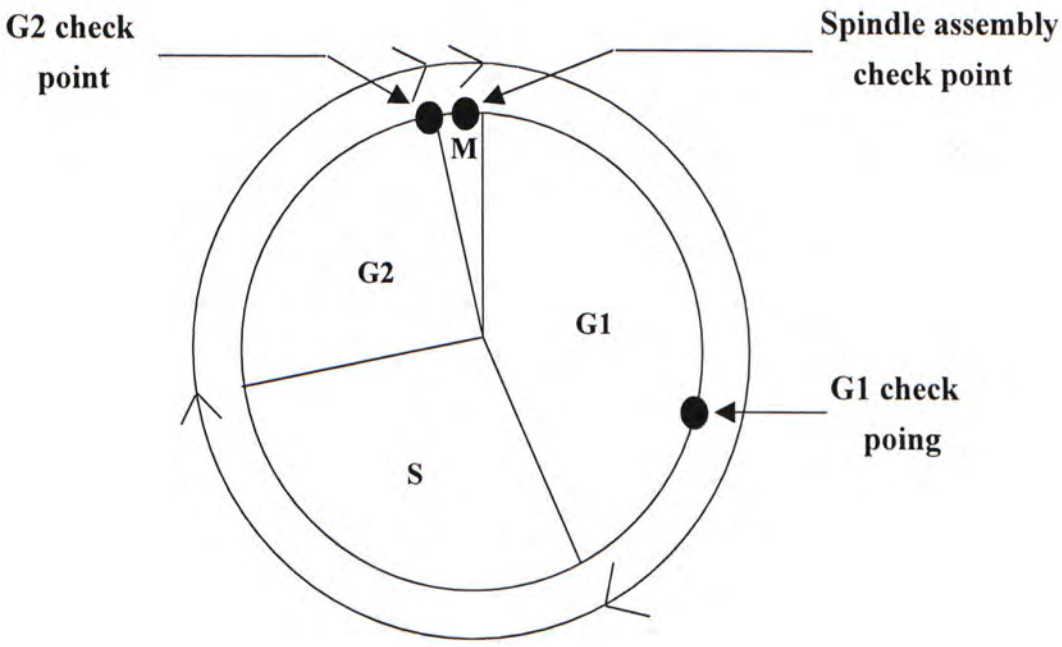
In between M phase and S phase, there are two gaps, the first one is **G1 phase**, which separates M phase to S phase; the second one is **G2 phase**, which separates cells to pass from S phase back to M phase and to undergo division again.

Other than the four phases mentioned above, there are three check points in the cell cycle so as to make sure (1) the corresponding events of each phase undergo in suitable time and correct sequence; (2) the associated events in each phase have

completed before the cell entering the next phase; and (3) allow the cell to respond to external stimuli, for examples, nutrient supply, cell density and growth-signaling molecules. The three check points are G1 check point, G2 check point and spindle assembly checkpoint (Becker *et al.*, 2000; Thomas, 1996).

G1 check point, also known as restriction point in animal cells, is located late in G1 phase, critical check for cells entering S phase. Mammalian cells arrested from division are usually arrested in G1. And cultured cells in exhausted nutrients or overcrowding are also arrested at G1. In this checkpoint, cell size, nutrients, growth factors and DNA damage are checked before allowing cells to pass through. For those, which cannot pass through G1 checkpoint, can be indefinitely retained in G1, or go into a resting state called G0 (Becker *et al.*, 2000; Thomas, 1996).

Figure 2.3 Diagram of cell cycle and the checkpoints.



The second checkpoint is **G2 checkpoint**, located in the G2/M boundary. In some cell types, if cell division is not required, cells are remained at this point indefinitely. Cell size and DNA replication are checked at G2 checkpoint, therefore, in order to pass through G2 phase into M phase, DNA synthesis must be completed (Becker *et al.*, 2000; Thomas, 1996).

The third cell cycle checkpoint is the **spindle assembly checkpoint** located at the boundary between metaphase and anaphase. It checks for the proper attachment of mitotic spindle to chromosome. It is very important because it ensures the newly formed daughter cells to contain identical and complete set of chromosomes. For cells with defects in the spindle attachment, temporary arrestment is found (Becker *et al.*, 2000; Thomas, 1996).

The factors, including completion of associated events in each phase and growth factors, affecting cell behaviour in the checkpoints are mediated by cellular proteins, such as cyclins, cyclin-dependent kinase (Cdk) and retinoblastoma protein (pRb). Cyclins are a group of proteins that activate another group of protein kinase called Cdk, and form the Cdk-cyclin complex. The interaction between Cdk and its corresponding cyclins plays a critical role to regulate cell cycle. The G1 checkpoint is controlled by G1 Cdk-cyclin complex, while the complex is controlled by the

phosphorylation of pRb.

In G1 phase, cyclin D conjugates with Cdk-4 and phosphorylates pRb to release it from and activate E2F, which is a transcription factor to initiate gene transcription of enzymes and proteins required for S phase, so cells can pass through G1 checkpoint into S phase to synthesize DNA. On the other hand, hypophosphorylated pRb binds to E2F, therefore can prevent cells entering into S phase. So, *Rb* gene is also known as tumor suppressor gene, and serves as a brake to inhibit transition from G1 to S phase when no appropriate signal from a growth factor is present. Mutation of *Rb* gene that leads to non-functional pRb and causes uncontrolled cell proliferation has been detected in cancers, for examples, lung, breast, bladder cancers and hereditary retinoblastoma in child. Therefore, antitumor drugs that can inhibit cyclin D, Cdk-4 or induce dephosphorylation of pRb are capable to inhibit the proliferation of cancer cells (Thomas, 1996).

For example, a naturally occurring red pigment isolated from marine sponge, fascaplysin, which arrests U2OS (osteosarcoma) in G1 phase by inhibiting Cdk-4 from phosphorylating pRb, therefore, arrests cell growth. The determination of G1 arrest was done by flow cytometry and decreased Cdk-4 could be examined by western blot and immunodetection (Soni *et al.*, 2000). Another example, Oxindole I

inhibited the proliferation of human mammary carcinoma cell lines MCF-7, ZR-75-1 and BT-549 and Urea II inhibited the proliferation of MCF-7 and BT-75-1. Both Oxindole and Urea II were reported to inhibit cyclin D1-Cdk-4 complex. It demonstrated the importance of inhibiting cyclin D1-Cdk-4 complex to induce phosphorylation of pRb, which in turn, blocked the transition from G1 to S phase in cancer therapy (Kent *et al.*, 1999).

Other than resting in G1 checkpoint, anticancer therapy may be mediated by arresting tumor cells proliferation in the second checkpoint, G2 checkpoint located in G2/M boundary. It was reported that dietary flavone reduces the proliferation of human colon carcinoma cell line HT-29 and the effects are related to G2/M arrest (Wenzel *et al.*, 2000). Besides, analogues of halichondrin B (polyether macrolide found in marine sponges), ER-076349 and ER-086526 inhibited the proliferation of human histiocytic lymphoma cells U937 resulting in G2/M arrest (Towle *et al.*, 2001).

2.6.2 Apoptosis

Cell numbers in multicellular organisms are regulated by the homeostasis of proliferation and programmed cell death. As the regulation of proliferation through

cell cycle regulations has been discussed above, cell death through apoptosis will be discussed in this part.

"Apoptosis is a form of cell death that plays important roles in physiological and pathological phenomena" (Tanuma and Shiokawa, 1996). Cancer can be described as uncontrolled cell growth. Therefore apoptosis also plays an important role against oncogenesis, because it likes a suicide mechanism and serves as a means to remove undesired cells from the body, for examples mutated cells and infected cells.

Although the term programmed cell death (PCD) and apoptosis are widely used interchangeably, but actually, "PCD is an operational definition of a functional relationship in development, whereas apoptosis is an originally morphological, but now biochemical description of a mode of cell death" (Lockshin and Zakeri, 1991). PCD may not accompany with apoptosis although they are often observed together (Gupta, 1996).

When talking about apoptosis, the other form of cell death, necrosis, is usually mentioned. Recently, the term of necrosis was proposed to represent the events after cell death. And the term oncosis should be used instead of necrosis to describe the events that cause the cell death by cell swelling and membrane

disruption and finally lead to inflammation (Lockshin *et al.*, 1998; DeBlois *et al.*, 2000).

Unlike oncosis, morphological characteristics of apoptotic cells include cell shrinkage, condensation of cytoplasm and chromatin, migration of the condensed chromatin to the margin of nuclear envelop, budding of nuclear and plasma membrane to form membrane-bounded apoptotic bodies. The biochemical characteristics include changes in plasma membrane surface, protein synthesis and internucleosome cleavage of DNA (Cameron and Feuer, 2000).

To detect apoptosis, researchers usually use flow cytometry or internucleosomal cleavage detection method. Agarose-gel electrophoresis method, which determines DNA fragmentation qualitatively, is the commonest detection method to detect internucleosomal cleavage. The principle behind this method is that, in apoptotic cells, as the constitutive endonucleases activated by some signal transduction pathway attack the DNA inter-nucleosomes regions, cleave the DNA into non-random oligonucleosomal fragment, with 180-220 base pair and multiples of this unit. Since this ordered fragmentation is a hallmark of apoptotic cells, it is commonly used as a marker of apoptosis. This characteristic can be expressed as DNA ladder (ladder of DNA bands) on agarose electrophoresis gel (Cotter and

Martin, 1996; DeBlois *et al.*, 2000).

As mentioned before, cell proliferation is only one part that contributes to tumor growth, and cell death is the other part. Therefore, for cells that are unable to carry out normal apoptosis due to mutation of genes that execute apoptosis, drugs that activate or induce apoptosis can serve as a new target in cancer therapy, and this is one of the 'heat' topics in this field.

For example, doxorubicin (DOX) is a current drug commonly used to cure hepatocellular carcinoma (HCC). Its effect was reported mainly due to apoptosis. DOX exhibited cytotoxic effect on human HCC cell lines, Hep3B and Huh-7, and human hepatoblastoma cell line HepG2. Apoptotic peak revealed by flow cytometry was observed in all cell lines after treatment of DOX accompanied with DNA fragmentation revealed by agarose gel electrophoresis (Lee *et al.*, 2002).

Other than current drugs, natural products also exhibited the same effect on cancer cells. The aqueous extract of *Salvia miltiorrhiza* (SM), a herb that is used in traditional Chinese medicine to cure liver diseases, showed antitumor activity against HepG2. Aqueous extract of SM inhibited the growth of HepG2 to 37.6% of the control at 2.5 mg/ml. The mechanism behind the antitumor activity was studied to be related to apoptosis, and DNA fragmentation was also observed (Liu *et al.*, 2000).

Magnolo, a hydroxylated biphenyl compound obtained from Chinese medicinal herb *Magnolia officinalis*, inhibit the cell growth of human colon cancer cell line, COLO-205 *in vitro* and *in vivo* and HepG2 *in vitro*. The antitumor effect was reported to be mediated by apoptosis with DNA fragmentation too (Lin *et al.*, 2002).

Apoptosis with DNA fragmentation were also observed in other cell lines, for examples leukaemia cell lines, HL-60 and K-562. Sogawa *et al.* (1998) demonstrated that marine microalgal polysaccharide exhibited strong cytotoxicity to K-562 cells *in vitro* and induced apoptosis. On DNA gel electrophoresis, DNA laddering was also observed. Besides, squamocin, one of the annonaceous acetogenins, is bioactive secondary plant metabolite, with a long alkyl chain and unsaturated r-methyl-r-lactone at the terminal, had potent antiproliferative effect on HL-60 cells *in vitro*. Squamocin was reported to induce apoptosis in HL-60 cells and activate caspase-3, and DNA laddering was also observed in agrose gel electrophoresis (Zhu *et al.*, 2002). Caspase is a family of cystein protease. Now, more then fourteen caspases have been identified and many are involved in apoptosis. Caspase-3 is an effector caspase, like an apoptotic executor, induces apoptosis once activated by different apoptotic signal (Nuñez *et al.*, 1998). Therefore, measuring the activation level of caspase-3 can also reflect the occurrence of apoptosis and commercially

available kit can be used for the measurement of caspase-3 related protease activity. Zhu *et al.* (2002) also concluded that caspase-3 take a critical role in the activation of apoptosis in HL-60 cell by squamocin. In another study, caspase-3 was also reported to be related to the anticancer effect of dietary flavone against human colon carcinoma cells HT-29. Flavone (2-phenyl-4H-1-benzopyran-4-one) was reported to be able to reduce HT-29 cells proliferation, and induce apoptosis. Moreover DNA fragmentation was observed in flavone treated HT-929. Besides, flavone could activate caspase-3, which was reported as the most downstream enzyme to execute apoptosis (Wenzel *et al.*, 2000).

2.7 Four selected Compositae species used in the study

Dendranthema indicum, *Dendranthema morifolium*, *Taraxacum mongolicum* and *Wedelia trilobata* are all Compositae species and were selected to study their antitumor activity in this project.

2.7.1 *Dendranthema indicum* (野菊花)

Dendranthema indicum, (Ye Jiu Hua, wild chrysanthemum flower), is small wild chrysanthemum with yellow flowers, distributed throughout China (Smith, 1911), which are favorite autumn and winter flowers. The flowering seasons are in the autumn and winter and this herb is collected when flowers just bloom.

In traditional description, the properties of *Dendranthema indicum* are bitter, pungent and slightly cold. The flowers are traditionally used to remove toxic-heat, treat inflammation of eyes, boils and carbuncles, relieve red swollen eyes, headache and dizziness caused by high blood pressure. The ethanolic extract of flowers also posses hypotensive effect in rats, cats and dogs. Moreover, it is used to treat retained menses, foment enlarged glands, as a wash in infected and cancerous sores and relieve sore swollen throats. The decoction of flowers has anti-infectious effect against *Shigella dysenteriae* and several other pathogenic bacteria *in vitro*. Besides, it

can increase the phagocytic function of human leukocytes against *Staphylococcus aureus*. Furthermore, the distillate of stem and leaf of the herb is applied in acute bronchitis to relief symptoms of fever, cough and asthma. (Wang, 1983; 中華人民共和國衛生部藥典委員會, 1990; Zhu, 1998; Li, 2002).

2.7.2 *Dendranthema morifolium* (菊花)

Dendranthema morifolium (Jiu Hua), is a perennial herb. The dried anthodium is used as Chinese traditional medicinal herb. It is collected in its flowering seasons from September to November. During this period, the flowers are in full blossom. (Zhu, 1998)

Flowers of *Dendranthema morifolium* are traditionally described to be sweet yet bit and slight cold, used to expel wind-heat, relieve red swollen eyes, headache and dizziness, hypertension, blur vision, as antitoxin, remedy for common cold, for liver claming and heat-clearing, and upper respiratory infection. The decoction of the flower is used to treat coronary diseases and hypertension, effectively relief chest discomfort, palpitation, tachypnea, dizziness, headache and numbness of the extremities. Although a side effect of epigastralgia or diarrhea was reported in few cases, in acute and subacute toxicity tests conducted by Zhu (1998), no strong

toxicity was reported (中華人民共和國衛生部藥典委員會, 1990; Li, 2002).

2.7.3 *Taraxacum mongolicum* (蒲公英)

Taraxacum mongolicum, with common name dandelion, is collected at the flowering seasons, which starts from spring to autumn. The dried herb is used as traditional Chinese medicine with sweet yet bit and cold characteristics, with tonic property, functioning at the liver and stomach. It is used to remove toxic-heat, excess salt and water, induce subsidence of swelling, diuresis, relief dysuria, excreting dampness, relieve breast abscesses, sore throat, swollen red eyes and painful, indigestion, jaundice, and as antispirochetic agent. In clinical application, the herb was reported to increase bile flow and relieve pain in chronic gallbladder spasm. The shoot of *Taraxacum mongolicum* is so tender, can be eaten as a pot-herb. The herb is reported to have low toxicity, induce occasional gastrointertinal reactions, for examples, mild diarrhea and abdominal discomfort. (Smith, 1911; 中華人民共和國衛生部藥典委員會, 1990; Zhu, 1998; Li, 2002).

2.7.4 *Wedelia trilobata* (三裂葉蟛蜞菊)

Wedelia trilobata, with common name yellow wedelia, is an evergreen perennial, scrambling herbaceous plant introduced into Hong Kong. The country of origin is South America. It flowers in summer and can be found in many public gardens as a very attractive decorative ground cover. Stems are round, rooting at the nodes, with three-lobed opposite stalkless leaves. The two lateral lobes found at about the middle of the leaf and the upper surface of leaves is in dark shining green color. *Wedelia trilobata* are widely spread and making a dense ground cover (Thrower, 1984).

Wedelia trilobata belongs to the division Magnoliophyta, class Magnoliopsida, subclass Asteridae, order Asterales, family Asteraceae, subfamily Asteroideae, tribe Heliantheae, subtribe Ecliptinae. Other than decorative purpose, it is used in traditional medicine. The crushed leaves are used as poultice, to relief discomfort symptoms of colds and flu by the tea preparation, and the herb can be used to clear the placenta after birth when used in combination with other herbs. Traditionally, this plant is also used to treat hepatitis, indigestion, and infections. Other than traditional use, in scientific research, the n-hexane extract and ethylacetate extract of *Wedelia trilobata* exhibited antimicrobial activity against both

Gram-positive and Gram-negative bacteria (Taddei and Rosas-Romero, 1999). Although there were several researches studied on the constituents of *Wedelia trilobata*, researches on the bioactivities are relatively less and research on the antitumor effect is even more seldom.

Chapter 3

Materials and Methods

3.1 Extraction

3.1.1 Water extraction

Fifty grams of dried flowers respectively of *Dendranthema indicum* (DI), *Dendranthema morifolium* (DM) and whole plant of Taraxacum *mongolicum* (TM) purchased from Guangzhou supplier were boiled separately with 500 ml distilled water for 1 h for two times. The water extracts were collected after gauze filtration and centrifuged at 22095 g for 30 min by Beckman J2-MI centrifuge. The supernatant collected were then concentrated at 60°C by rotary evaporator (BÜCHI Rotavapor R-200) at reduced pressure and named as DI1, DM1 and TM1 respectively.

Two kilograms of fresh aerial parts of *Wedelia trilobata* (WT) collected on the campus of the Chinese University of Hong Kong (CUHK) during the month April

2001 was washed and boiled at 90 – 100°C with distilled water for 5 min. WT was then homogenized with 6 L distilled water by blender and boiled overnight (16 h) in slow cooker. After gauze filtration, the filtrate was collected. The residue was boiled with another 6 L distilled water overnight (16 h) again and filtrate was collected after filtered by gauze. The two filtrates obtained were pooled together and centrifuged at 22095 g for 30 min. The supernatant was collected and concentrated to 1.2 L at 60°C by rotary evaporator at reduced pressure, named as WT1.

3.1.2 NaOH extraction

Firstly, 1% NaOH was added to the residue obtained after water extraction with stirring and kept overnight (16 h). Secondly, the base extract was centrifuged at 22059 g for 30 min, the supernatant was collected and neutralized with HCl. Thirdly, it was centrifuged at 22059 g for 30 min and concentrated at 60°C by rotary evaporator at reduced pressure. Finally, dialysis extensively against distilled water to remove salts by pore size 12-14 kDa dialysis tubing and lyophilized by freeze drier. WT2 was assigned to represent this extract.

Figure 3.1 (a) *Wedelia trilobata* in the CUHK and (b) *Wedelia trilobata* collected for extraction.

3.1.3 Ethanol precipitation

Firstly, 95% ethanol was slowly added to WT1 with stirring to make up final concentration of 68% ethanol. It was kept overnight (16 h) with stirring. Secondly, it was centrifuged at 22095 g for 30 min, supernatant and precipitate were both collected.

In the supernatant, ethanol was removed by rotary evaporator at 40°C and reduced pressure. Then the supernatant without ethanol was named WT4 and lyophilized by freeze drier.

The precipitate was resuspended in distilled water, named as WT7, and lyophilized by freeze drier.

3.1.4 Bioactivity guided fractionation

3.1.4.1 Macroporous resin column (D_{101})

Two grams of WT4 dissolved in distilled water was loaded into a 3.6 x 40 cm D_{101} macroporous resin column (天津農葯總廠) at flow rate 0.7 ml/min. It was eluted with distilled water, 15% ethanol, 30% ethanol and 60% ethanol one by one, each for four bed volumes, four fractions named WT4-1, WT4-2, WT4-3 and WT4-4 were collected respectively.

The ethanol in WT4-2, WT4-3 and WT4-4 were removed by rotary evaporator at 40°C and reduced pressure. Then all the four fractions were lyophilized by freeze drier.

3.1.4.2 Silica gel 60 column

An amount of 300 g WT4-4 was dissolved in 2 ml methanol, then mixed with 1 g silica gel 60 and dried. The sample was chromatographed on a 30 g silica gel column (Silica gel 60, 0.063 - 0.200mm, Merck), eluted with 100 ml chloroform: ethyl acetate: n-hexane: methanol (3:1:1:0.5) to obtain WT4-4 A, another 100 ml chloroform: ethyl acetate: n-hexane: methanol (3:1:1:0.5) to obtain WT4-4 B, then 100 ml chloroform: ethyl acetate: n-hexane: methanol (2:1:0.5:1) to obtain WT4-4 C and finally 100 ml methanol to obtain WT4-4 D. WT4-4 A was then concentrated by rotary evaporator at 50°C and reduced pressure. The concentrated fraction WT4-4 A was allowed to settle for overnight. The crystals formed were collected.

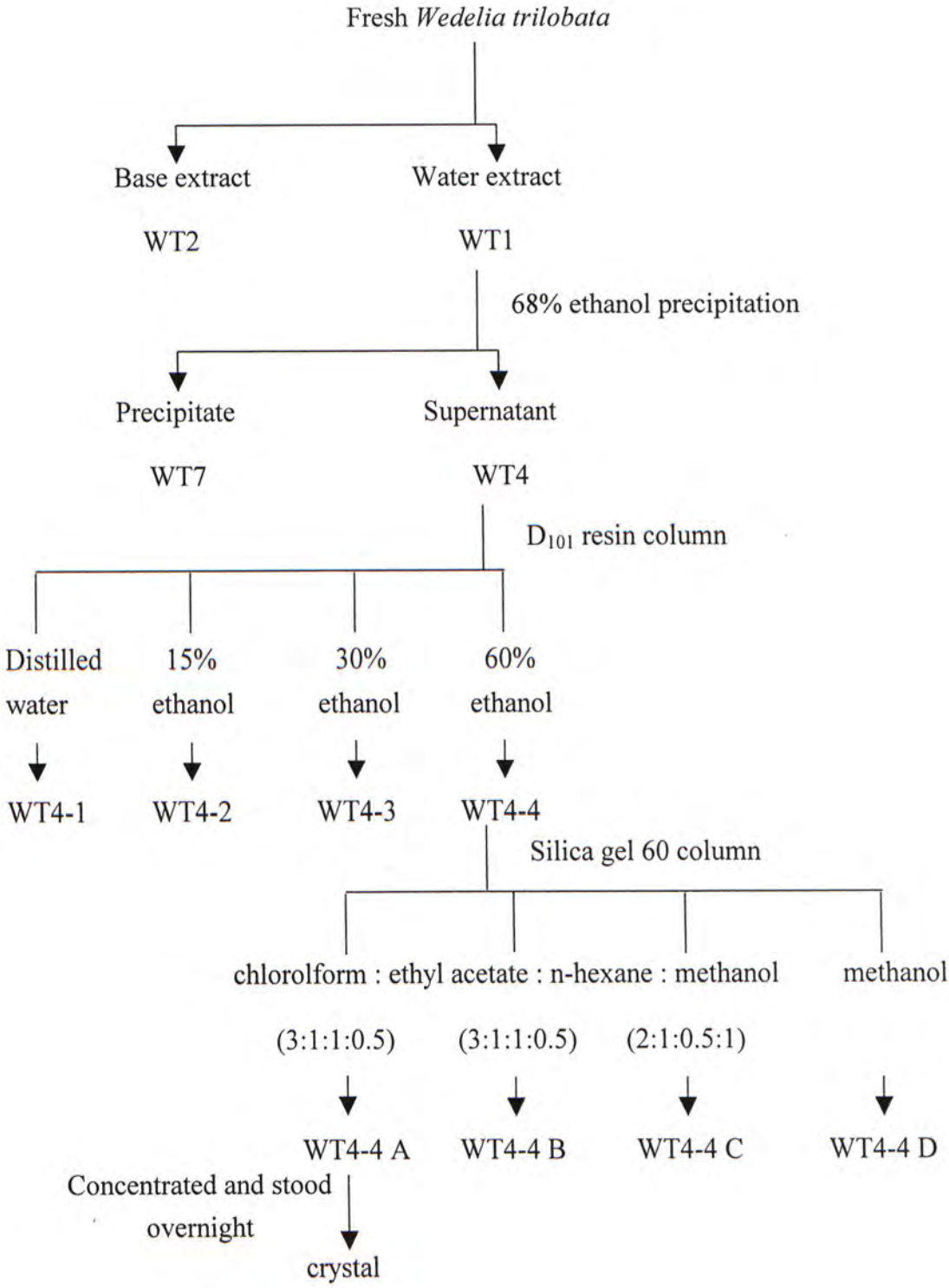


Figure 3.2 Flow chat illustrating the simplified extraction and fractionation of *Wedelia trilobata*.

3.1.5 High Performance Liquid Chromatography (HPLC) analysis

WT4-1, WT4-2, WT4-3, WT4-4, WT4-4 A, WT4-4 B, WT4-4 C, WT4-4 D and the crystal isolated from WT4-4 A were subjected to HPLC analysis (Hewlett Packard series 1100). Reverse-phase HPLC was performed on Bio-Rad Bio-Sil C18 HL 90-5 column (250 x 4.6 mm, particle size: 5 μm) with mobile phase 1% acetic acid and methanol (40:60, v/v), at the flow rate 0.8 ml/min or 0.5 ml/min, column temperature 30°C and the detection wavelength 210 nm.

WT4-1 was dissolved in methanol to water in 40:60 ratio. WT4-2 was dissolved in methanol to water in 80:20 ratio. WT4-4 A, WT4-4 B and the crystal were dissolved in methanol to chloroform in 60:40 ratio. WT4-3, WT4-4, WT4-4 C and WT4-4 D were dissolved in methanol. All the samples were filtered with 0.45 μm filter before HPLC analysis.

3.2 Characterization

3.2.1 Chemical tests

Unless otherwise stated, WT4-4 was dissolved in methanol (1mg/ml) and used in the chemical tests to detect various phytochemical groups. WT4-4 was tested against the presence of alkaloids, lactones and coumarins, flavonoids, sterols and saponins, carbohydrates and polyglucose, terpenoids and anthraquinone.

3.2.1.1 Alkaloids

3.2.1.1.1 Mayer reagent

An amount of 1.36 g $HgCl_2$ and 5 g KI was dissolved in 60 ml and 10 ml distilled water respectively, and then make up to 100 ml. After adding with sample and few drops of HCl, if alkaloids presence, yellowish precipitate would form (Silva *et al.*, 1998).

3.2.1.1.2 Dragendorff reagent

Eight grams of $Bi(NO_3)_3 \cdot H_2O$ in 17 ml 30% HNO_3 and 27.2 g KI in 50 ml distilled water were mixed and kept for 24 h. After that, the solution was filtered and

made up to 100 ml with distilled water. After adding the sample, if alkaloids were present, orange-brown precipitate would form in acidic pH (Silva *et al.*, 1998).

3.2.1.1.3 Wagner reagent

An amount of 1.27 g I_2 and 2 g KI was dissolved in 100 ml distilled water. Few drops of the solution were added to the sample in acidic pH (diluted H_2SO_4). If alkaloids were present, brown precipitate would form (Silva *et al.*, 1998).

3.2.1.2 Lactones and coumarins

3.2.1.2.1 Ferric hydroxamine acid test

Seven grams of $NH_2H_2O{\cdot}HCl$ was dissolved in 100 ml distilled water. 5.6 g potassium hydroxide was dissolved in 100 ml methanol. 1 g ferric chloride was dissolved in 100 ml 1% HCl. The three solutions were mixed together in sequence just before use. A few drops of mixed solution were added to the sample. Red color would indicate the presence of lactones or coumarins (肖崇厚, 1996).

3.2.1.2.2 Emersen reagent

Two grams of ampyrone was dissolved in 100 ml ethanol. 8 g potassium ferricyanide was dissolved in 100 ml distilled water. The two solutions was mixed just before use. A few drops of mixed solution were added to the sample. Red color would indicate the presence of lactones or coumarins (肖崇厚, 1996).

3.2.1.3 Flavonoids

3.2.1.3.1 Shinoda test

Magnesium powder and a few drops of concentrated HCl (37%) were added to the sample. Orange, pink, red to purple colors would indicate the presence of flavones or flavonols (Silva *et al.*, 1998).

3.2.1.3.2 Aluminum chloride reagent

A few drops of 2% aluminum chloride in methanol were added to the sample. Organge to blue fluorescent light at 366 nm would indicate the presence of flavonoids (肖崇厚, 1996).

3.2.1.4 Sterols

2.2.1.4.1 Liebermann-Buchard test

One milliliter anhydrous acetic acid and 1 ml chloroform were mixed and cooled to 0°C. One drop of concentrated sulfuric acid (98%) was added to the solution. Sample dissolved in chloroform was then added to the solution, blue, green, red or orange colors which change with time would represent the presence of sterols (Silva *et al.*, 1998).

3.2.1.4.2 Salkowski reaction

Two milligrams of sample was dissolved in 1 ml chloroform and mixed with 1 ml concentrated sulfuric acid (98%), two phases formed, with red or yellow color would represent the presence of sterols or methylated sterols (Silva *et al.*, 1998).

3.2.1.5 Saponins

Five milligrams of sample was dissolved in 10 ml distilled water and shaked. Foam formed and stable for 15 min or more would represent the presence of saponins (Silva *et al.*, 1998).

3.2.1.6 Carbohydrates

3.2.1.6.1 Molisch reagent

Two to three drops 1% α-naphthol in 80% ethanol and concentrated H_2SO_4 (98%) were added to sample without mixing to form two phases. Purple ring appear in the interface would represent the presence of carbohydrates (Silva *et al.*, 1998).

3.2.1.6.2 Aniline acetate reaction

Sample in solid form was heated on flame. Filter paper impregnated with aniline acetate was place on top of the sample to allow the vapors of sample to react with it. Red color in filter paper would indicate the presence of heterocyclic aldehydes produced by carbohydrate dehydration (Silva *et al.*, 1998).

3.2.1.7 Terpenoids

3.2.1.7.1 Vanillin reagent

An amount of 0.5 g vanillin was dissolved in 80 ml concentrated sulfuric acid (98%) and then made up to 100 ml with ethanol. Few drops of the solution were added to the sample. Red to purple color would indicate the presence of terpenoids (肖崇厚, 1996).

3.2.1.7.2 Carr-price reagent

Five grams of antimonous chloride was dissolved in 25 ml chloroform. Few drops of the solution were added to the sample. Orange color in UV light (366 nm) would represent the presence of terpenoids (肖崇厚, 1996).

3.2.1.8 Anthraquinone

3.2.1.8.1 Borntrager reaction

Five grams of potassium hydroxide was dissolved in 50 ml distilled water. A few drops of the solution were added to the sample. Red color would represent the presence of the anthraquinone (肖崇厚, 1996).

3.2.1.8.2 Magnesium acetate reagent

An amount of 5 g magnesium acetate was dissolved in 50 ml methanol. A few drops of the solution were added to the sample. Orange, red to purple colors would indicate the presence of anthraquinone (肖崇厚, 1996).

3.2.2 X-ray crystallography

The crystals obtained from WT4-4 A was subjected to X-ray crystallographic analysis: $C_{23}H_{32}O_9$, Mr = 452.49, hexagonal, $P6_5$, a = 9.8327(4), c = 42.600(2) Å, V = 3566.8(3) $Å^3$, Z = 6, dx = 1.264 Mg/m^3, F(000)= 1452, μ(Mo-Kα)= 0.097 mm^{-1}. Data collection was performed on a SMART 1000 CCD using graphite monochromated radiation (λ = 0.71073 Å), 4174 unique reflections were collected to θmax = 25.00°, in which 3665 reflections were observed [$F^2 \geq 4\sigma(F^2)$]. The structure was solved by direct method (SHELXTL version 5.1) and refined by full-matrix least-squares on F^2. In the structure refinements, non-hydrogen atoms were refined anisotropically. Hydrogen atoms bonded to carbons were placed on the geometrically ideal positions by the 'ride on' method. Hydrogen atoms bonded to oxygen were located by the difference Fourier method and were included in the calculation of structure factors with isotropic temperature factors. The final R = 0.0550, Rw = 0.0611 and S =1.042.

3.3 *In vitro* Assay

3.3.1 Cell lines

Human acute promyelocytic leukemia HL-60 cells, human chronic myelogenous leukemia K-562 cells, mouse Sarcoma S-180 cells, human hepatoblastoma HepG2 cells and monkey kidney Vero cells were purchased from American Type Culture Collection (ATCC). Human breast cancer MCF-7 cells and human liver L-02 cells were obtained from the Institute of Genetics, Fudan University, Shanghai.

HL-60, K-562 and S-180 are suspension cell lines while HepG2, Vero, MCF-7 and L-02 are adhesive cell lines.

3.3.2 Maintenance of cell lines

All the media used were added with 1% penicillin (10,000 unit/ml)-streptomycin (10,000 μg/ml) (Gibco-BRL) and 0.1% fungizome (amphotericin B 250 μg/ml) (Gibco-BRL).

HL-60, K-562, HepG2 and MCF-7 were maintained in RPMI 1640 medium (Sigma) supplemented with 2 g/L sodium bicarbonate (Sigma), 10% heat-inactivated (56°C for 60 min) fetal bovine serum (Gibco-BRL).

S-180 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL), high glucose, supplemented with 10% heat-inactivated fetal bovine serum and 1.5 g/L sodium bicarbonate.

Vero cell line was maintained in Minimal Essential Medium (MEM) (Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM Hepes, 2 mM glutamine and 0.6% 4 mg/ml gentamycin.

L-02 cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum and 3.024 g/L sodium bicarbonate.

All the cell lines were cultured in 25 cm^2 sterile polystyrene tissue culture flask (Sarstedt) with 10 ml medium for suspension cell lines or 5 ml medium for adhesive cell lines. All the cell lines were maintained at fully humidified atmosphere at 37°C, 5% CO_2 and 95% room air, with three times passage per week.

3.3.3 *In vitro* antitumor assay

The direct cytotoxicity of samples on different cancer cell lines were investigated by direct incubation of cancer cell lines with samples for 72 h. The effects were evaluated by two methods according to the type of cell lines. Trypan blue exclusion

method was used for suspension cell lines and MTT assay method was used for adhesive cell lines.

3.3.3.1 Trypan blue exclusion method

Loss of membrane integrity is one of the morphological characteristics in death cell. Therefore, Trypan blue dye can stain death cells while viable cells can exclude this dye (Cotter and Martin, 1996). The *in vitro* antitumor effect against suspension cancer cell lines HL-60, K-562 and S-180 were revealed by Trypan blue exclusion method.

Water insoluble samples were dissolved in dimethylsulfoxide (DMSO) (Sigma) as stock while water soluble samples were dissolved in phosphate buffered saline (PBS) as stock.

In this assay, stock samples were diluted by corresponding medium to 2-fold higher concentration of the desired final concentration. 50 μl/well of 5 x 10^4 selected tumor cells/ml medium were seeded into a sterile 96-well round bottom tissue culture microplate (IWAKI, Japan) and mixed with 50 μl sample/well. Final concentration of DMSO was kept at 0.5% (v/v) for water insoluble sample in each well. Wells with 50 μl 0.5% DMSO in medium (water insoluble sample) or PBS instead of sample were

used as control. Each concentration was performed in triplicate. The culture plate was incubated at fully humidified atmosphere with 37°C, 5% CO_2 and 95% room air for 72 h.

After 72 h incubation, 100 µl filtered 0.4% Trypan blue (w/v) in PBS was added to each well. After 2-3 min for uptake of dye, aliquot of cells suspension from evenly mixed wells was transferred to hemocytometer (Hausser Scientific, USA) and covered with cover-slip. Viable cells and dead cells in the four 1 mm^2 corners were both counted and recorded separately.

The results of assay were expressed as percentage change in cell density and viability to that of the control. The antiutumor effect was expressed as percentage of inhibition relative to control. Cell density and viability were calculated by the following equations:

$$\text{cell density (cell/ml)} = \frac{\text{cell number}}{4} \times 2 \times 10^4 \text{ cell/ml}$$

$$\text{viability (\%)} = \frac{\text{number of viable cells}}{\text{number of total cells}} \times 100\%$$

Percentage of inhibition (%) was calculated as the following equation:

$$\left(1 - \frac{\text{Average cell density or viability in treatment group}}{\text{Average cell density or viability in control group}}\right) \times 100\%$$

3.3.3.2 MTT assay method

The *in vitro* antitumor effect against adhesive cancer cell lines HepG2 and MCF-7 were revealed by MTT assay method. This method is a quantitative colorimetric method for accessing cell growth and survival. MTT assay method is based on the ability of mitochondrial dehydrogenases in viable cells to convert soluble yellow tetrazolium salt (MTT) to dark blue formazan product. Since the number of viable cells is proportional to the amount of formazan product, cell growth and survival can be determined by measuring the color intensity of formazan product (Cochet *et al.*, 1998).

Stock samples were prepared as in Trypan blue exclusion method and diluted by corresponding medium to 2-fold higher concentration of the desired final concentration. 100 µl/well of 5 x 10^4 selected tumor cells/ml medium were seeded into a sterile 96-well flat bottom tissue microplate (IWAKI, Japan) and mixed with 100 µl sample/well. Final DMSO concentration was kept at 0.5% (v/v) for water insoluble sample in each well. Wells with 100 µl 0.5% DMSO in medium (water insoluble sample) or PBS instead of sample were used as control. Each concentration was performed in triplicate. The culture plate was incubated at fully humidified atmosphere with 37°C, 5% CO_2 and 95% room air for 72 h.

After 72 h, 20 µl freshly prepared MTT (3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide) (Sigma) in 5 mg/ml filtered PBS was added into each well and incubated at 37°C, 5% CO_2 for 5 h. Then the medium was removed and 150 µl of acid isopropanol was added to each well. After pipette vigorously to dissolve the dark blue crystals, the absorbance was determined by microplate reader (SPECTRAmax 250) at 570 nm wavelength (Cochet *et al.*, 1998).

The proliferation of cells was expressed as average value of absorbance. And the antitumor activity was expressed as percentage of inhibition of sample treated group to control group by the following equation:

$$\left(1 - \frac{\text{Average O.D. 570 nm in treatment group}}{\text{Average O.D. 570 nm in control group}}\right) \times 100\%$$

3.3.3.3 Determination of IC_{50}

The IC_{50} was determined. Selected cell lines were incubated with sample in a multiple doses. IC_{50} was determined as the concentration that exerts 50% inhibition on cell density to control after 72 h incubation and estimated by graphical interpolation.

3.3.4 Cytotoxicity assay on normal cell lines

The cytotoxicity of samples on normal cell lines were determined by evaluating the cell density of sample treated Vero and L-02 cell lines. The change in cell density was determined by MTT assay method. In brief, Vero or L-02 cells were incubated with samples at desired final concentration for 72 h. The detailed procedures were same as described in the MTT assay method before.

3.4 *In vivo* Assay

3.4.1 Animals

Seven to eight-week-old male Balb/c mice weighing between 20 and 25 g were obtained from the Laboratory Animal Service Center of CUHK. They were kept in the animal house of the Biology Department of CUHK. The environment was maintained at 21 ± 2°C with 12 h light/12 h dark cycle and mice were fed with normal mouse chow and tape water *ad libitum*. They were acclimatized for at least one week in these environment before used in the maintenance of cell line or for antitumor assay.

3.4.2 Maintenance of Sarcoma 180 cell line

Sarcoma 180 cells were used for the *in vivo* antitumor assay. Sarcoma 180 was maintained in the peritoneal cavity of male Balb/c mice as ascite tumor and weekly passage was performed.

The ascitic fluid with Sarcoma 180 ascite tumor in the abdominal of male Balb/c mouse was collected after sacrificed by cervical dislocation. The cells were washed twice with 0.45% PBS by the aid of centrifuge (514 g for 5 min) (Beckman, Allegra^{TM} 6R centrifuge). The cell pellet was dissolved and diluted to 2.5 x 10^7 living cells/ml in sterile PBS with the aid of 0.4% Trypan blue and hemocytometer. 0.2 ml (5 x 10^6

living cells) cell suspension was intraperitoneally injected into the abdominal cavity of Balb/c mice and kept for seven days. Two mice were injected each time.

3.4.3 Tumor inoculation

2.5×10^7 living cells/ml Sarcoma 180 cell suspension was prepared as in Section 3.3.2. Male Balb/c mice anaesthetized by diethyl ether were subcutaneously inoculated with 0.2 ml cell suspension into the back (Day 0). Three days were allowed for solid tumor formation. At Day 3 (72 h after tumor inoculation), mice were examined carefully by hand, only mice with solid tumor formed were selected. Then they were randomly grouped into 8 per group and caged separately for the use in the experiment.

3.4.4 Preparation of samples

WT4-4 was dissolved in 5% DMSO in sterile PBS at a 2 mg/ml stock solution and dilute to 0.08, 0.16, 0.32, 0.64 mg/ml by 5% DMSO respectively and stored at 4°C until use.

3.4.5 *In vivo* antitumor assay

3.4.5.1 Antitumor effect of WT4-4 on Sarcoma 180 solid tumor

At Day 3, after tumor formation check, WT4-4 at the dose of 0.4, 0.8, 1.6, 3.2 mg/kg body weight were intraperitoneal (i.p.) injected into corresponding groups of mice once per day for ten consecutive days. Injection of 5% DMSO in sterile PBS instead of sample was performed on the control group.

At Day 21 (9 days after the end of i.p. injection of sample dosage), the mice were sacrificed by cervical dislocation and the body weight of each mouse was determined. Then their tumors were excised and weighed.

The average tumor weight of each group was determined and effect of WT4-4 on Sarcoma 180 solid tumor was expressed as percentage of tumor inhibition compared to control. It is calculated by the following equation:

$$\text{Percentage of Tumor inhibition (\%)} = \left[1 - \frac{\text{Average tumor weight of treatment group}}{\text{Average tumor weight of control group}} \right] \times 100\%$$

3.4.5.2 Effect of 5% DMSO in sterile PBS

Since WT4-4 could not be dissolved in PBS, it was dissolved in 5% DMSO in sterile PBS. In order to determine if the solvent had any effect on Sarcoma 180 solid

tumor, tumor size of mice group received i.p. injection of 5% DMSO in sterile PBS was compared to control group received sterile PBS.

3.4.6 Body weight change

In order to investigate the side effect of WT4-4, change in body weight was used as the parameter to access the side effect. Body weight of each mouse before sample injection at Day 3 and before sacrificed at Day 21 was weighed. Average change in body weight was calculated. The change in treatment group was compared to the group without tumor inoculation and treatment.

Table 3.1 Table of schedule for *in vivo* antitumor assay.

Day	**Experiment to carry out**
0	Tumor inoculation
3	Tumor check and random grouping of mice Body weight determination First i.p. injection of WT4-4
4-12	Daily consecutive i.p. injection of WT4-4
21	End of experiment Sacrifice of the mice Body weight determination Tumor excision and weighing

3.5 DNA Agarose Gel Electrophoresis

DNA fragmentation of cells serves as an apoptotic marker and could be accessed by DNA extraction and gel electrophoresis. HL-60, K-562 and S-180 was respectively incubated with WT4-4 for 72 h as described in Section 3.2.3.1 at the corresponding IC_{25}, IC_{50} and IC_{75} concentrations. Cells treated with 1 μM camptothecin (Sigma) for 4 h instead of WT4-4 served as positive control. IC_{25}, IC_{50} and IC_{75} was determined as the concentration of WT4-4 that could exert 25%, 50% and 75% inhibition on cell density to that of control after 72 h incubation.

After 72 h incubation, 2×10^6 cells were harvested with the aid of hemocytometer and washed twice with 5 ml PBS by centrifugation at 800 g for 5 min. The cell pellet was resuspended in 200 μl ice-cold PBS. After that, 5 ml 70% ethanol at -20°C was added and kept at -20°C overnight to fix the cells.

The fixed cells were centrifuged at 800 g for 5 min to remove the ethanol and 1 ml PBS was added to wash the cells. Then it was transferred to 1.5 ml eppendorf tube. The supernatant was removed completely after centrifugation at 800 g for 5 min. Then, the cell pellet was resuspended in 40 μl phosphate-citrate buffer (8 parts 0.1M citric acid with 192 parts 0.2M Na_2HPO_4, pH 7.8) and kept for 60 min at room temperature. After that, it was centrifuged at 1000 g for 5 min, the supernatant was transferred to a new

1.5 ml eppendorf tube and concentrated by SpeedVac concentrator (Savant Speed Vac® SC 110) for 15 min. It was followed by adding 3 µl Nonident NP-40 (0.25%) and 3 µl proteinase K (1 mg/ml) (Amersham), and incubated for 30 min at 37°C (Lee *et al.*, 2002).

DNA content of different samples was measured by BioPhotometer (Eppendorf), and same amount of DNA was used for DNA agarose gel electrophoresis. 1 µl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol) was added to each 5 µl DNA extract, which was loaded into horizontal 0.8% agarose gel (FMC BioProducts) in 0.5X TBE buffer (BCH Medical Supplies Co.). The agarose gel was run at 13 V for 17 h. The gel was stained with 0.625 mg/ml ethidium bromide (1 drop per 50 ml gel) and photographed by UV illuminator (Bio-Rad Gel Doc 1000).

3.6 Statistical Analysis

The results were presented as mean ± standard deviation (S.D.) and analyzed by Student's t-test, only p-value less than 0.05 ($p < 0.05$) were considered as statistically significance. The IC_{25}, IC_{50} and IC_{75} (the concentration of sample that exhibited 25%,

50% and 75% inhibition on cell density in relation to control respectively) in some experiments were estimated by graphical interpolation from individual experiments.

Chapter 4

Results

4.1 Extraction and characterization of *Wedelia trilobata* (WT)

4.1.1 Percentage of yield in extraction and fractionation of WT

After hot water extraction, 190.43 g WT1 was obtained from 2 kg fresh aerial parts of WT and 34.37 g WT4 was obtained after ethanol precipitation. About twenty percent of WT1 was found to be WT4 (Table 4.1).

After fractionation of 20 g WT4 on macroporous resin column, four fractions were collected, WT4-1, WT4-2, WT4-3 and WT4-4, and the total yield was 92.8%. Among this, WT4-1 was the largest fraction contributing nearly 80%, WT4-2 about five percent while WT4-3 and WT4-4 contributing same amount, 4.40% respectively (Table 4.2).

After further fractionation of 300 mg WT4-4 on silica gel column, four

fractions were collected, WT4-4 A, WT4-4 B, WT4-4 C and WT4-4 D. WT4-4 D was the largest fraction, contributing 35% of WT4-4; WT4-4 A was the second large fraction, about 22%; WT4-4 B and WT4-4 C were small fractions, with much lower percentage of yield (Table 4.3 a). 25 mg purified component (crystal) was collected out of 100 mg WT4-4 A (Table 4.3 b).

4.1.2 HPLC chromatograms of WT fractions and the purified component (crystal)

The four fractions obtained from WT4 by macroporous resin column fractionation were analyzed by HPLC and the chromatograms were shown in Figures 4.1 – 4.4. WT4-1 and WT4-2 each showed two peaks while WT4-3 showed three peaks before retention time of 10 min. WT4-4 showed five peaks before retention time of 10 min and two distinct peaks between retention time of 10 to 15 min (Fig. 4.1 – 4.4).

After further fractionation of WT4-4 by silica gel column, the four eluted fractions were also analyzed by HPLC and the chromatograms were shown in Figures 4.5 – 4.9. WT4-4 A showed a distinct major peak at retention time 12.363 min (Fig. 4.6). WT4-4 B showed two peaks (Fig. 4.7), WT4-4 C showed three peaks with largest peak at retention time 4.28 min (Fig. 4.8) and WT4-4 D showed

three broad peaks which close to each other (Fig. 4.9).

The chromatogram of the crystal isolated from WT4-4 A was shown in Figure 4.10. Retention time of the major peak of crystal was corresponding to the major peak in WT4-4 fraction (Fig. 4.11).

4.1.3 Phytochemical groups of WT4-4

WT4-4 showed positive results in chemical tests for lactones and courmarins, flavonoids, and terpenoids but negative results towards alkaloids, sterols and saponins, carbohydrates, and anthraquinone (Table 4.4).

4.1.4 Results on X-ray crystallography of the isolated crystal from WT4-4 A

The very pale yellow crystal isolated from WT4-4 A was found to be sesquiterpene lactone, crystal size 0.68 x 0.50 x 0.38 mm, which had empirical formula of $C_{23}H_{32}O_9$ and formula weight 452.49. Molecular structure of the crystal was shown in Figure 4.12.

Table 4.1 Percentage of yield of WT1 and WT4 from 2 kg fresh aerial parts of WT.

Fraction	**Color**	**Weight (g)**	**Percentage of Yield (%)**
WT1	Brown	190.43	9.25 [a]
WT4	Brown	34.37	1.72 [a]
			18.05 [b]

[a] % yield from fresh aerial parts of WT was calculated by: (weight of the fraction obtained / 2 kg fresh aerial parts of WT) x 100%

[b] % yield of WT4 from WT1 was determined by: (weight of WT4 obtained / 190.43 g WT1) x 100%

Table 4.2 Recovery of WT fractions after application of 20 g WT4 to macroporous resin column.

Fraction	Color	Weight (g)	Percentage of Yield (%)
WT4-1	Brown	15.72	78.60 [a]
WT4-2	Pale yellow	1.08	5.40 [a]
WT4-3	Yellow	0.88	4.40 [a]
WT4-4	Yellow	0.88	4.40 [a]
Total	--	18.56	92.80 [b]

[a] % yield was calculated by: (weight of the fraction obtained / 20 g WT4) x 100%

[b] recovery of total fractions after fractionation was calculated by: (total weight of all fractions / 20 g WT4) x 100%

Table 4.3 a Recovery of WT fractions after application of 300 g WT4-4 to Silica gel column.

Fraction	Color	Weight (mg)	Percentage of Yield (%)
WT4-4 A	Pale yellow	66.0	22.00 [a]
WT4-4 B	Yellow	12.8	4.27 [a]
WT4-4 C	Yellow	19.0	6.33 [a]
WT4-4 D	Brown	106.0	35.33 [a]
Total	--	203.8	67.93 [b]

[a] % yield was determined by: (weight of the fraction obtained / 300 g WT4-4) x 100%

[b] recovery of total fractions after fractionation was determined by : (total weight of all fractions / 300 g WT4-4) x 100%

Table 4.3 b Percentage of yield of the crystal from 100 mg WT4-4 A.

Fraction	Color	Weight (mg)	Percentage of Yield [a] (%)
Crystal	Colorless	25	25

[a] % yield was determined by: (weight of the crystal obtained / 100 g WT4-4 A) x 100%

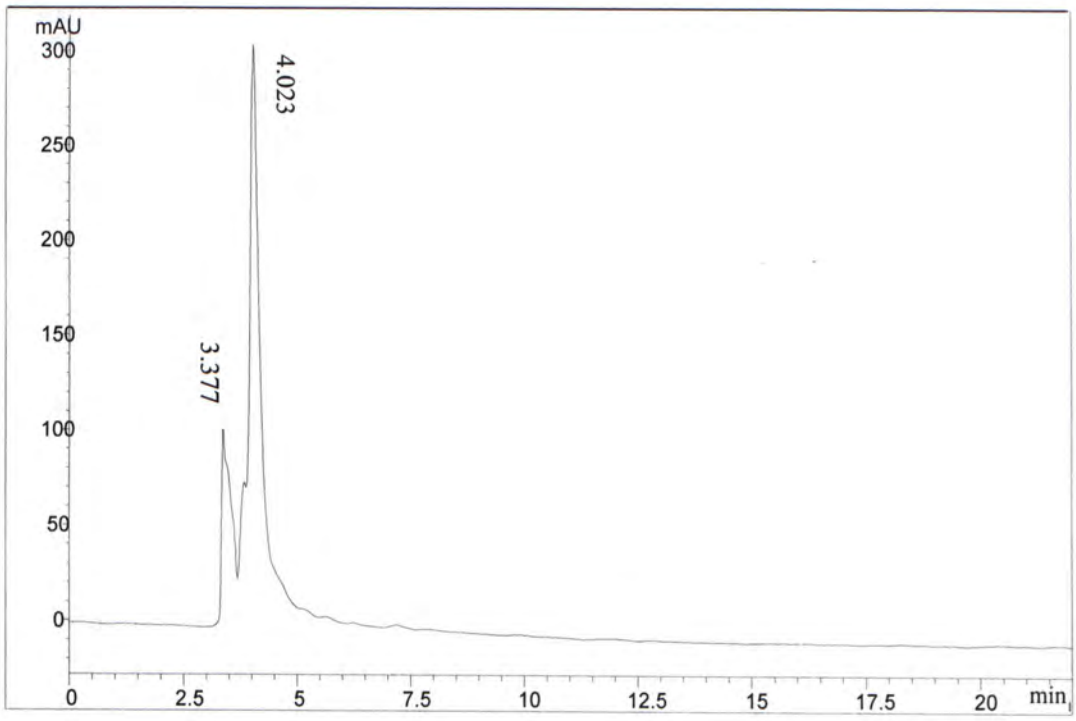


Figure 4.1 The HPLC chromatogram of WT4-1.

WT4-1 was dissolved in methanol:water (40:60, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

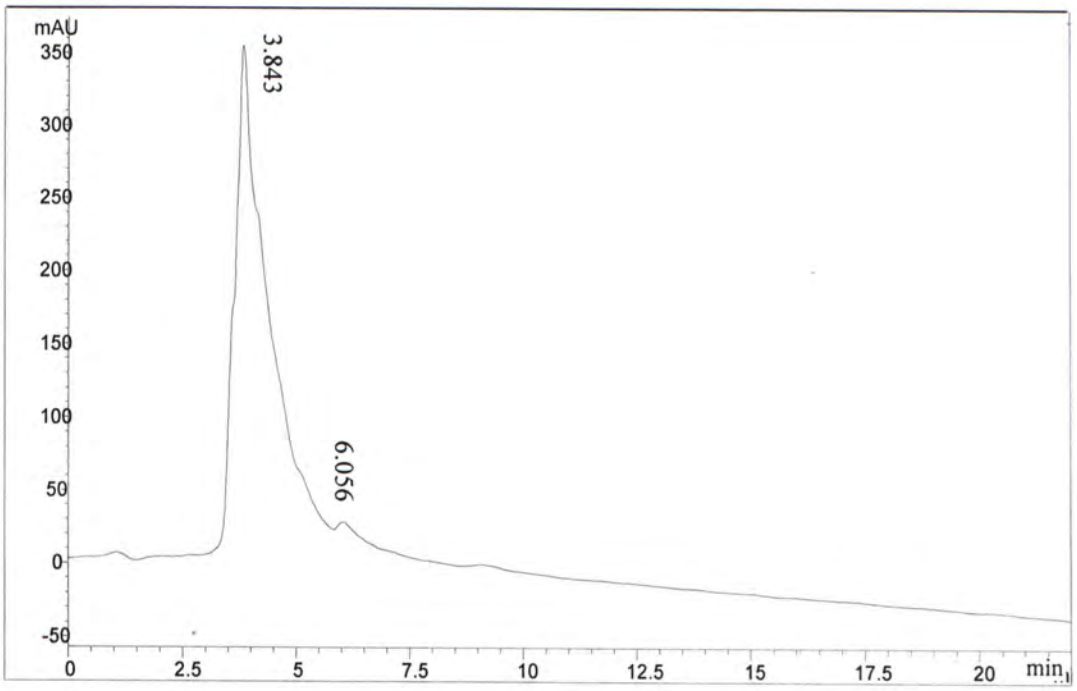


Figure 4.2 The HPLC chromatogram of WT4-2.

WT4-2 was dissolved in methanol:water (80:20, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

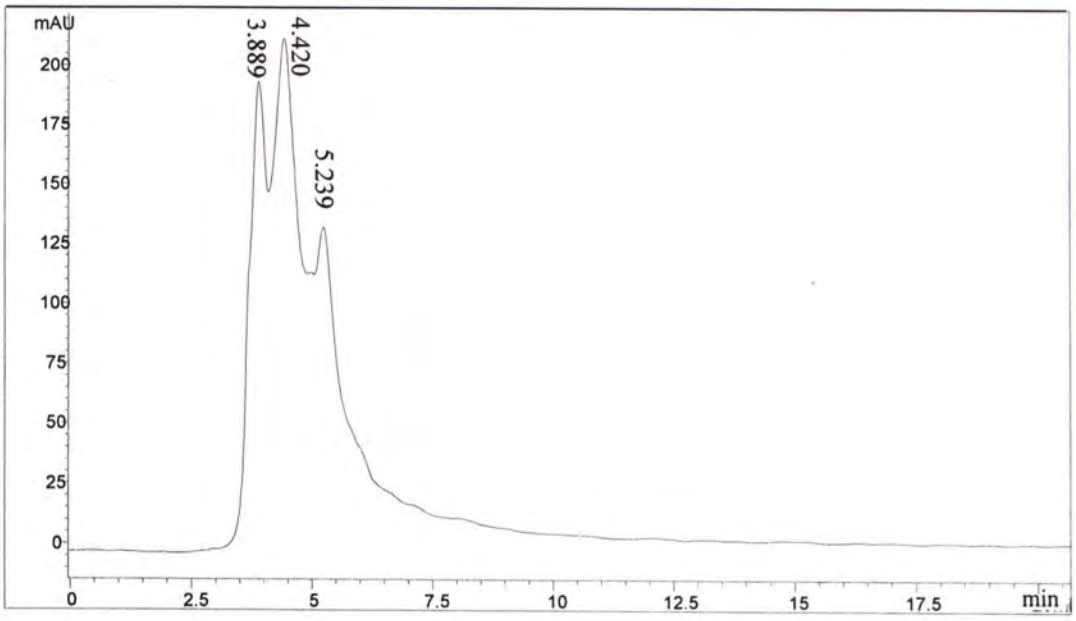


Figure 4.3 The HPLC chromatogram of WT4-3.

WT4-3 was dissolved in methanol, determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

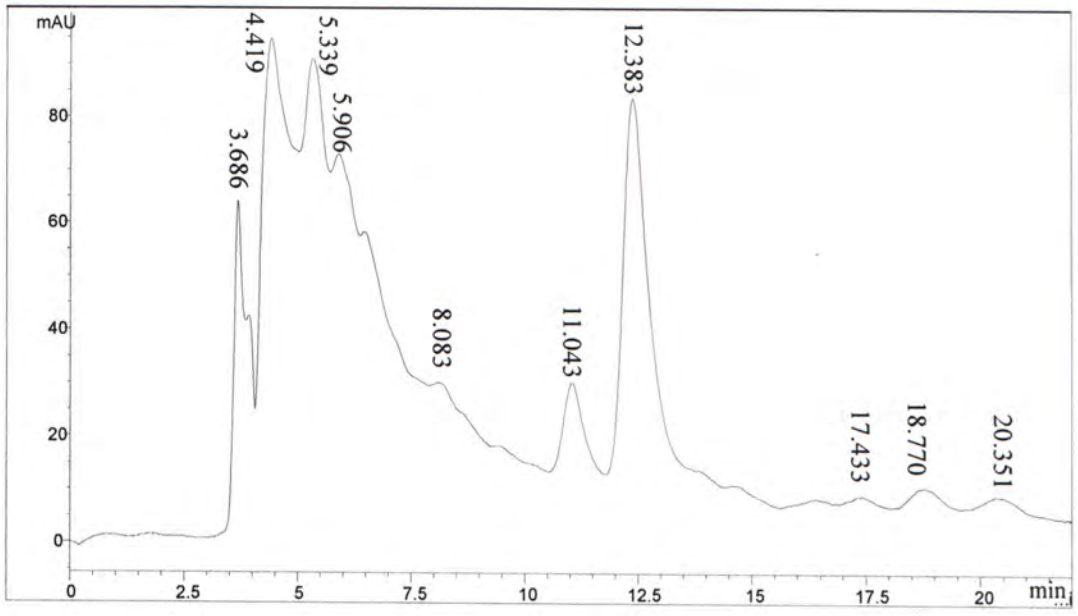


Figure 4.4 The HPLC chromatogram of WT4-4.

WT4-4 was dissolved in methanol, determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

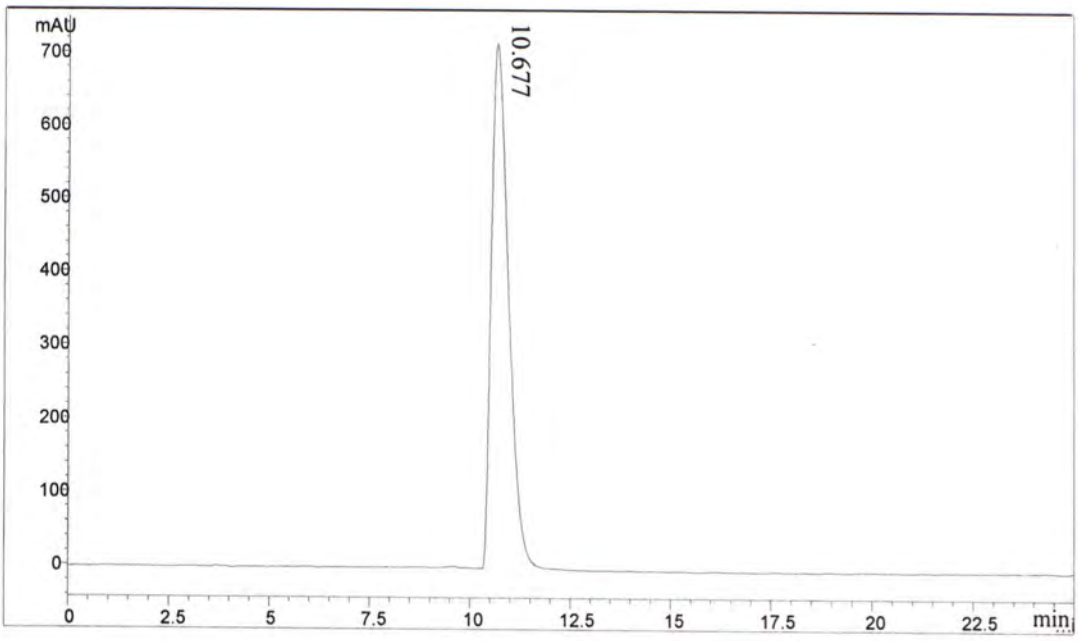


Figure 4.5 The HPLC chromatogram of the solvent methanol:chloroform (60:40, v/v) at flow rate 0.8 ml/min.

Methanol:chloroform (60:40, v/v) was used to dissolve WT4-4 A and WT4-4 B, it's chromatogram was determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

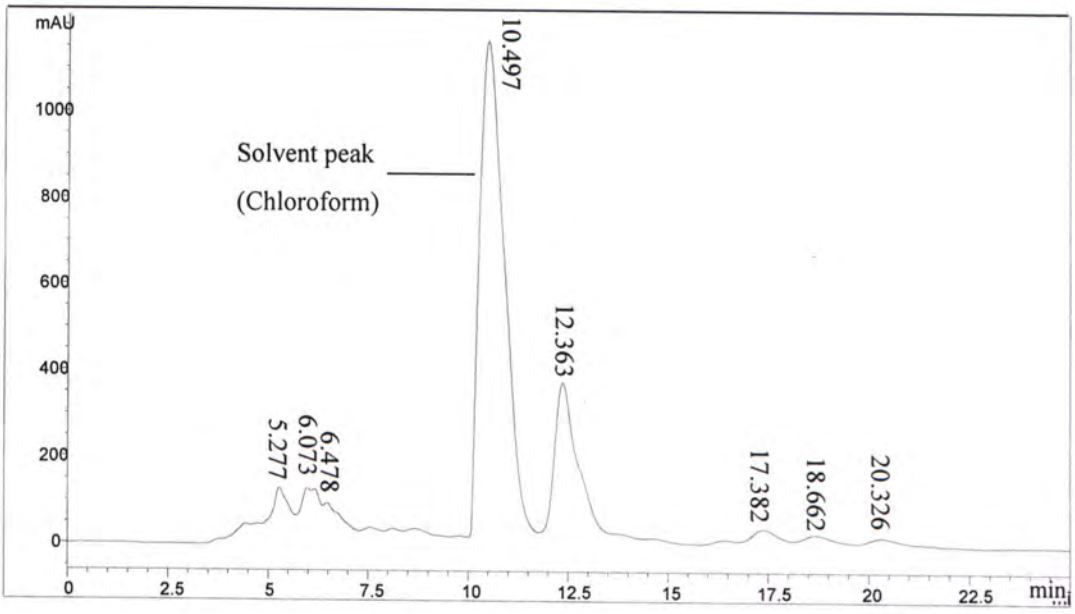


Figure 4.6 The HPLC chromatogram of WT4-4 A at flow rate 0.8 ml/min.

WT4-4 A was dissolved in methanol:chloroform (60:40, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

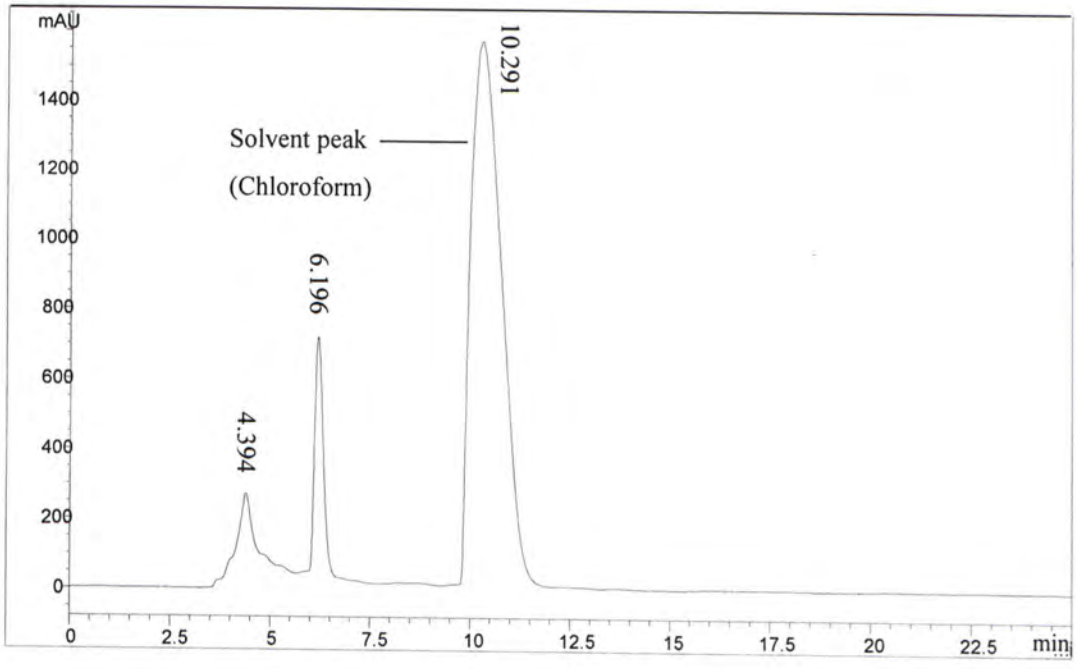


Figure 4.7 The HPLC chromatogram of WT4-4 B at flow rate 0.8 ml/min.

WT4-4 B was dissolved in methanol:chloroform (60:40, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

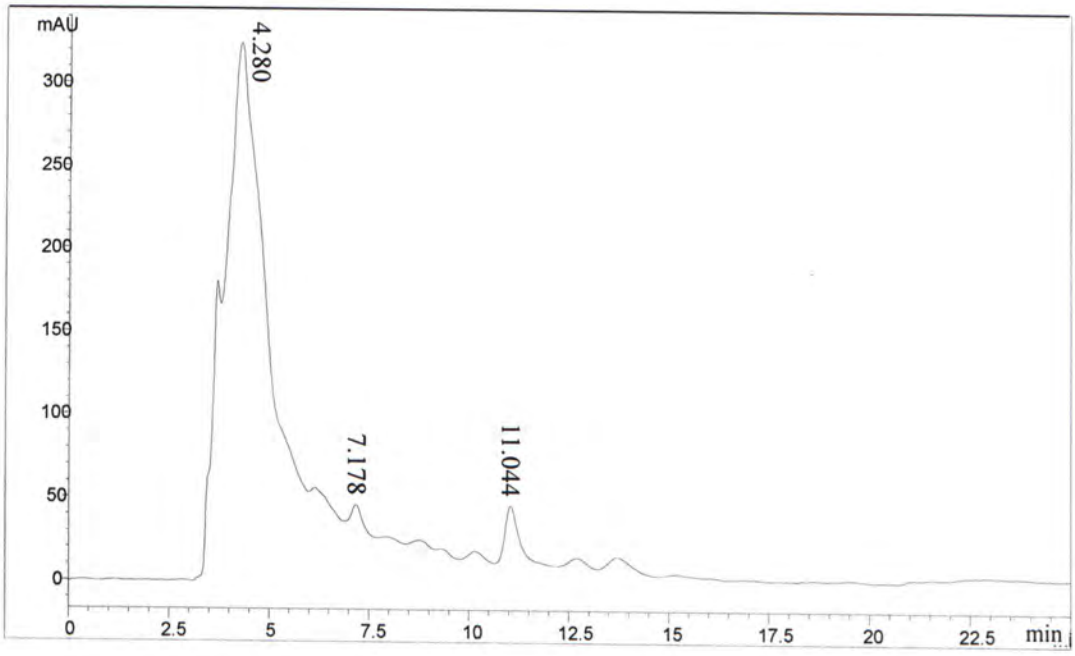


Figure 4.8 The HPLC chromatogram of WT4-4 C at flow rate 0.8 ml/min.

WT4-4 C was dissolved in methanol, determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

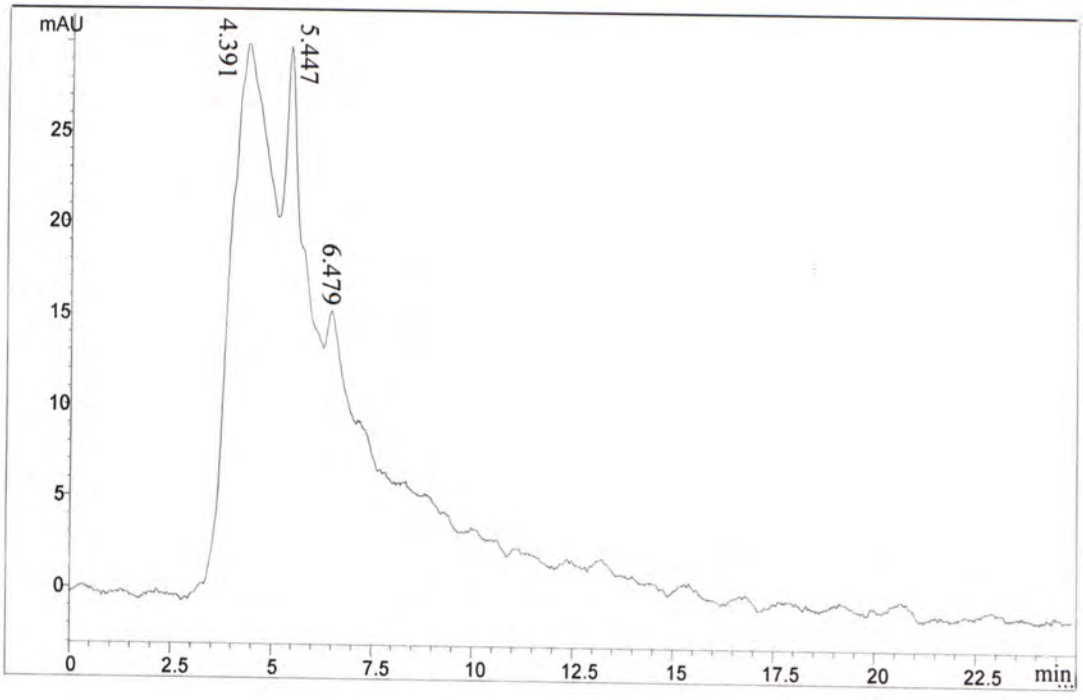
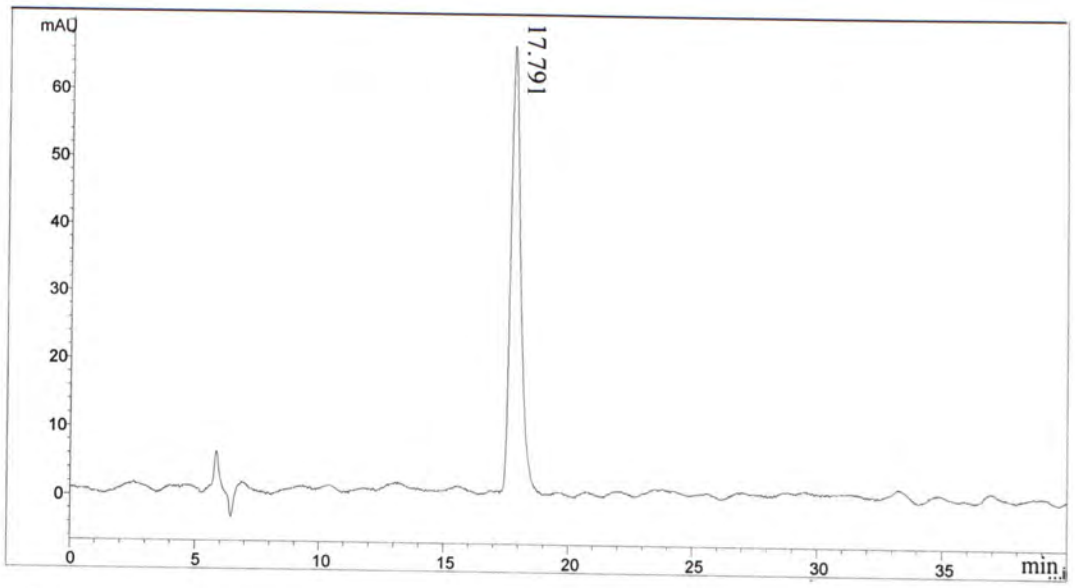


Figure 4.9 The HPLC chromatogram of WT4-4 D at flow rate 0.8 ml/min.

WT4-4 D was dissolved in methanol, determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.8 ml/min.

(a)



(b)

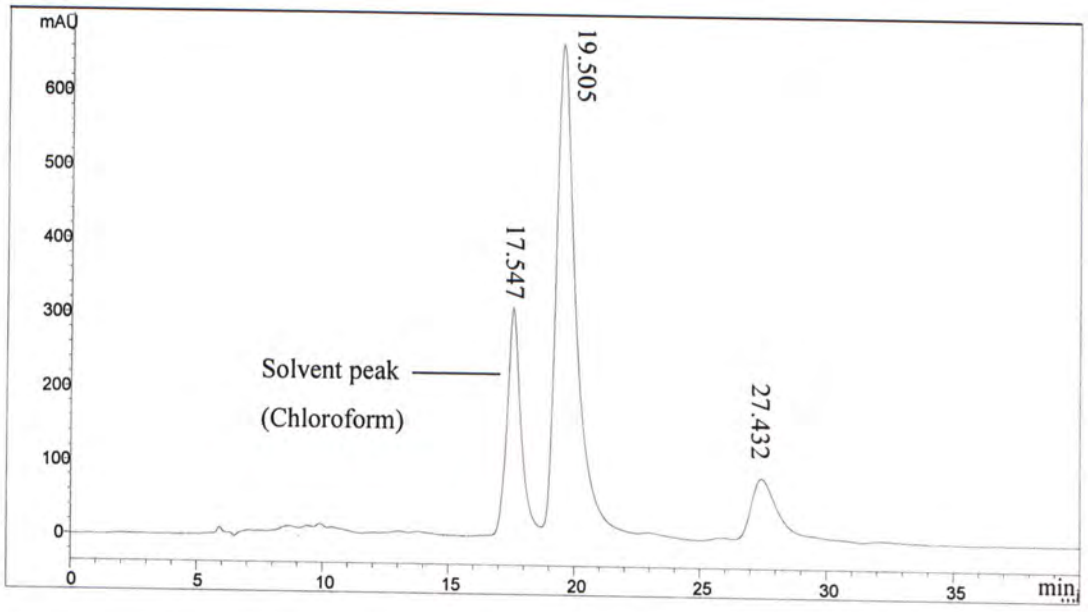


Figure 4.10 (a) The HPLC chromatogram of methanol:chloroform (60:40, v/v) and (b) the chromatogram of the crystal isolated from WT4-4 A at flow rate 0.5 ml/min.

The crystal isolated from WT4-4 A was dissolved in methanol:chloroform (60:40, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.5 ml/min.

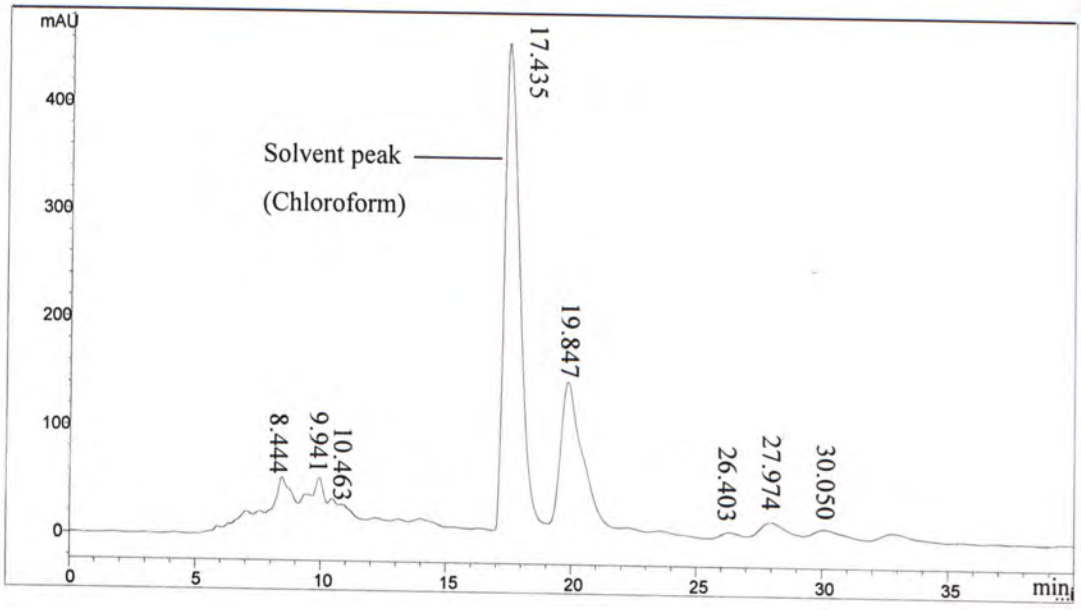


Figure 4.11 The HPLC chromatogram of WT4-4 A at flow rate 0.5 ml/min.

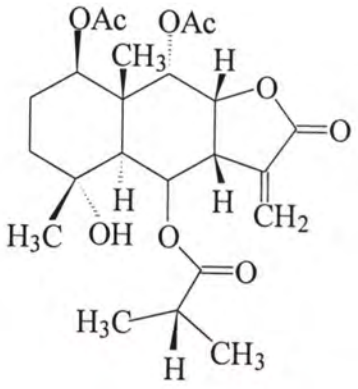
WT4-4 A was dissolved in methanol:chloroform (60:40, v/v), determined by C18 column, detected at 210 nm with mobile phase 0.1% acetic acid:methanol (40:60, v/v), temperature at 30°C and flow rate was set at 0.5 ml/min.

Table 4.4 Results of chemical tests on phytochemical groups of WT4-4.

Tested phytochemical groups	Tests	Results [a]
Alkaloids	Mayer reagent	-
	Dragendorff reagent	-
	Wagner reagent	-
Lactones and coumarins	Ferric hydroxamie acid reaction	+
	Emersen reagent	+
Flavonoids	Shinoda test	+
	Aluminum chloride reagent	+
Sterols	Liebermann-Buchard test	-
	Salkowski reaction	-
Saponins	Foam test	-
Carbohydrates	Molisch reagent	-
	Aniline acetate reaction	-
Terpenoids	Vanillin reagent	+
	Carr-price reagent	+
Anthraquinone	Borntrager reaction	-
	Magnesium acetate reagent	-

[a] Results were presented as '+' for positive results and '-' for negative results towards the corresponding tests.

(a)



(b)

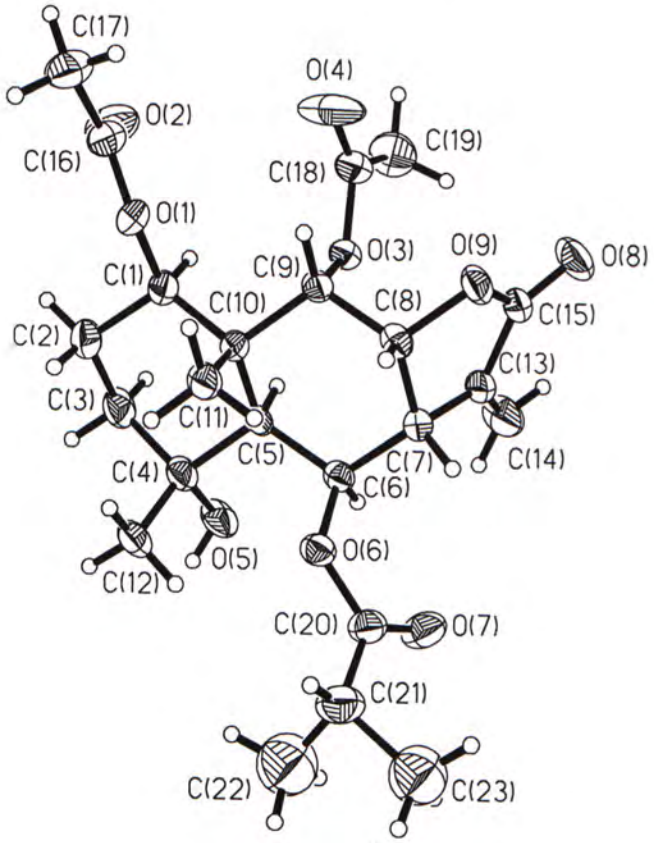


Figure 4.12 (a) Chemical structure of the crystal $C_{23}H_{32}O_9$ (sesquiterpene lactone) isolated from WT4-4 A and (b) the molecular structure showing the atom labeling scheme. The C and O atoms were drawn as 30% thermal ellipsoids.

4.2 *In vitro* Antitumor Assay

4.2.1 Effects of DI1, DM1, TM1 and WT1 on suspension cancer cell line

The *in vitro* effects of crude water extracts from DI, DM, TM and WT on HL-60 and K-562 were evaluated by Trypan blue exclusion method. All the four samples inhibited the proliferation of HL-60 and K-562 in a dose-dependent manner but only WT1, DI1 and TM1 showed statistically significant inhibition. WT1 gave the most promising results in both cell lines, which significantly inhibited the proliferation of HL-60 and K-562 by 97.3% and 80.3% at 200 μg/ml respectively. WT1 reduced the viability of HL-60 by 93.2% at 200 μg/ml but no significant effect on viability of K-562 was observed. Although both DI1 and TM1 significantly inhibited the proliferation of HL-60, only TM1 significantly inhibited the viability at 200 μg/ml by 10.6% (Fig. 4.13 and 4.14).

4.2.2 Effects of WT fractions and the purified component (crystal) on suspension cell lines

The effects were investigated by Trypan blue exclusion method. According to Figures 4.15 and 4.16, the water extract of WT (WT1) exhibited much stronger inhibition on proliferation and viability on both HL-60 and K-562 than the base (1% NaOH) extract (WT2), but both fractions did not significantly affect the

viability of K-562. WT2 did not show any significant effect on proliferation or viability of K-562.

The ethanol supernatant fraction, WT4, exhibited much stronger inhibitory effect on proliferation and viability of HL-60 than the ethanol precipitated fraction WT7. At 200 μg/ml WT4 could significantly inhibit the proliferation of HL-60 by 95.4% but the inhibition rate was only 21.4% for WT7. WT4 could significantly inhibit the viability of HL-60 by 83.8% but WT7 could not show any significant change (Fig. 4.17). As shown in Figure 4.18 WT4 significantly inhibited the proliferation of K-562 by 90.7% at 200 μg/ml but no significant change in cell viability was observed. WT7 could neither inhibit the proliferation nor viability of K-562 (Fig. 4.18).

The effects of the four fractions, WT4-1, WT4-2, WT4-3 and WT4-4, obtained from WT4 after macroporous resin column fractionation on HL-60, K-562 and S-180 were demonstrated in Figures 4.19 – 4.21. WT4-4 was the most effective fraction to inhibit the proliferation and viability of all the three cell lines. It significantly inhibited the proliferation of HL-60, K-562 and S-180 by 96.1%, 96.5% and 96.3% respectively at 100 μg/ml and the viability by 100% for all cell lines at 100 μg/ml. WT4-3 was the second most effective fraction, followed by

WT4-2. At 200 μg/ml WT4-3 and WT4-2 significantly inhibited the proliferation of HL-60 by 74.5% and 48.6%; K-562 by 65.2% and 38.1%; and S-180 by 23.2% and 27.4% respectively. WT4-1 could only significantly inhibit the proliferation of S-180 at 200 μg/ml by 17.3%. WT4-1, WT4-2 and WT4-3 could not affect the viability of all the cell lines. The IC_{50} of WT4-4 on proliferation on HL-60, K-562 and S-180 were determined to be 4 μg/ml, 6.5 μg/ml and 8 μg/ml respectively (Fig. 4.22 – 4.24).

The effects of the four fractions, WT4-4 A, WT4-4 B, WT4-4 C and WT4-4 D, fractionated from WT4-4 after silica gel column chromatography on HL-60, K-562 and S-180 were investigated. At the tested doses, WT4-4 A was the only antitumor fraction effective on all the cell lines. All the other fractions could not inhibit the proliferation or viability on all the tested cell lines. WT4-4 A could significantly inhibit the proliferation of all three cell lines even at the lowest tested dose 2 μg/ml. It could significantly inhibit the proliferation of HL-60, K-562 and S-180 by 94.3%, 96.2% and 96.7%; and the viability by 97%, 22.2% and 29% respectively at 8 μg/ml. The IC_{50} on proliferation of HL-60, K-562 and S-180 were determined to be 3 μg/ml, 3.4 μg/ml and 2.8 μg/ml respectively (Fig. 4.25 – 4.30).

The effects of the crystal isolated from WT4-4 A on HL-60 were investigated.

The lowest tested concentration of WT4-4 A that exhibited significant inhibition on proliferation of HL-60 was determined to be 1.56 μg/ml with 28.9% inhibition. The viability of HL-60 was significantly inhibited by 100% at 6.25 μg/ml (Fig. 4.31). The IC_{50} on proliferation of HL-60 was determined to be 2.2 μg/ml (Fig. 4.32).

4.2.3 Effects of WT fractions on adhesive cancer cell lines

The effects of the four fractions, WT4-1, WT4-2, WT4-3 and WT4-4, obtained from WT4 after macroporous resin column fractionation on MCF-7 and HepG2 were determined by MTT assay method. Only WT4-4 could significantly inhibit the proliferation of MCF-7 in the experiment. It significantly inhibited the proliferation by 77.4% at 50 μg/ml (Fig. 4.33) with the IC_{50} equal to 36 μg/ml (Fig. 4.34). Besides, WT4-4 significantly inhibited the proliferation of HepG2 by 51.4% at 25 μg/ml and up to 100% at 100 μg/ml (Fig. 4.35), with the IC_{50} equal to 24 μg/ml (Fig. 4.36).

4.2.4 Effects of WT4-4 on normal cell lines

The effects of WT4-4 on Vero and L-02 were investigated by MTT assay

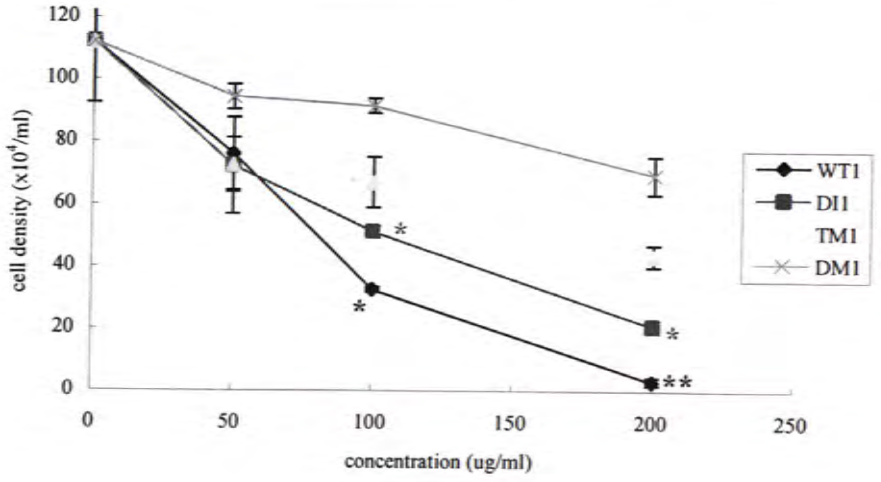
method. WT4-4 did not show significant cytotoxicity on L-02 up to 50 μg/ml (Fig. 4.37) and on Vero up to 6.25 μg/ml (Fig. 4.38). However, WT4-4 at the concentration of 12.5 μg/ml or higher exhibited significant cytotoxicity on Vero cells (Fig. 4.38).

(a) †

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-32.0	-71.0	-97.3
DI1	-35.4	-54.3	-81.4
TM1	-35.1	-40.2	-61.2
DM1	-15.6	-18.2	-37.9

(b) ††

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-1.5	-5.7	-93.2
DI1	-2.4	-5.3	-22.7
TM1	-1.0	-1.0	-10.6
DM1	-2.6	-3.3	-1.7



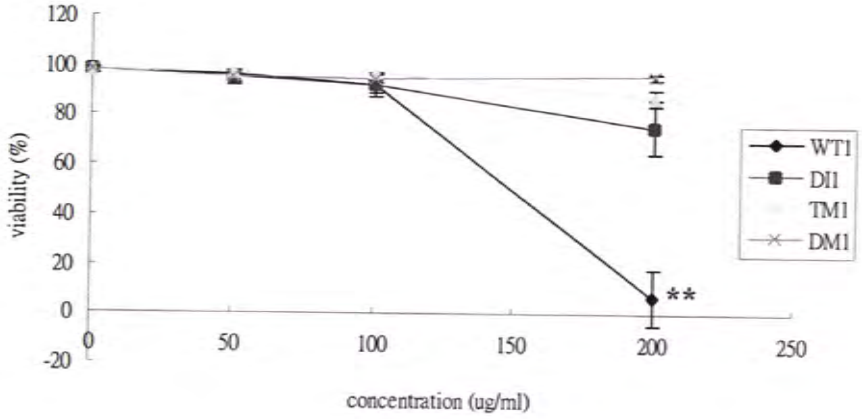


Figure 4.13 Effects of different Compositae water extracts on proliferation and viability of HL-60 cells.

The effects on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 μg/ml, 100μg/ml and 200μg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of HL-60 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-21.5	-24.1	-80.3
DI1	-9.7	-11.3	-26.9
TM1	-18.7	-11.3	-21.7
DM1	-6.6	-10.7	-13.6

(b) ††

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-0.6	-0.8	-3.4
DI1	1.3	1.1	1.6
TM1	-1.1	-4.1	-0.1
DM1	1.2	2.0	1.3

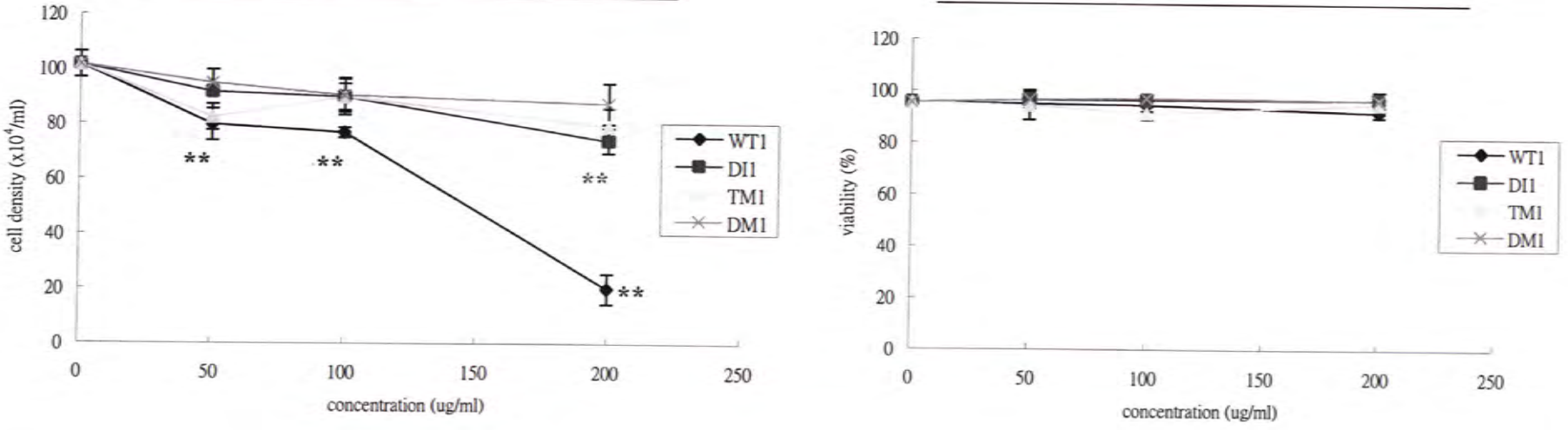


Figure 4.14 Effects of different Compositae water extracts on proliferation and viability of K-562 cells.

The effects on K-562 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 μg/ml, 100μg/ml and 200μg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of K-562 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-18.6	-44.6	-94.9
WT2	-0.2	-0.6	-13.3

(b) ††

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-0.5	-0.5	-87.9
WT2	-1.4	0.2	-2.0

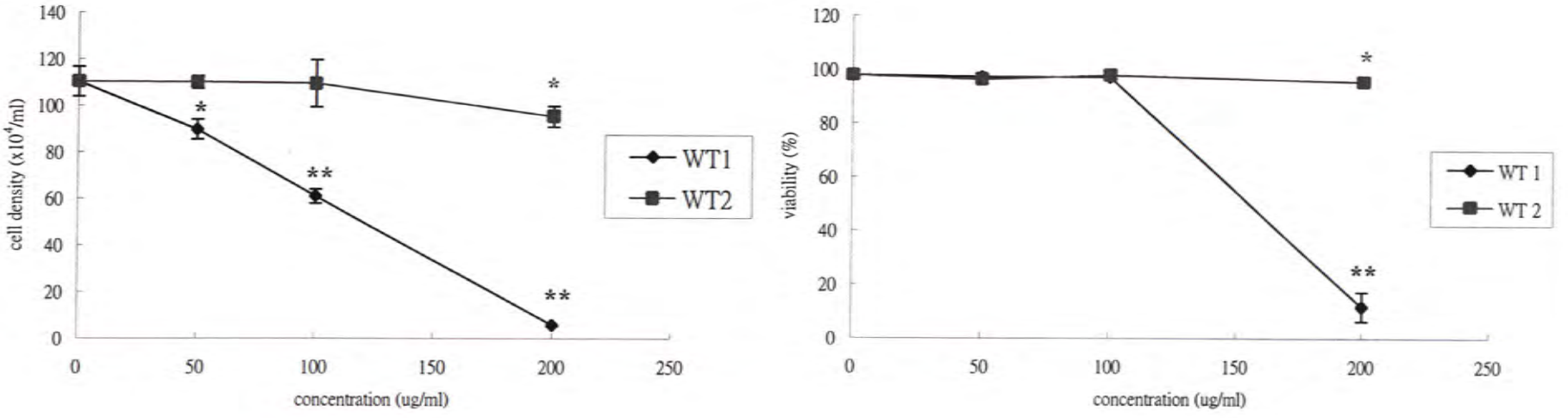


Figure 4.15 Effects of WT1 and WT2 on proliferation and viability of HL-60 cells.

The effects on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 μg/ml, 100μg/ml and 200μg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of HL-60 respectively.

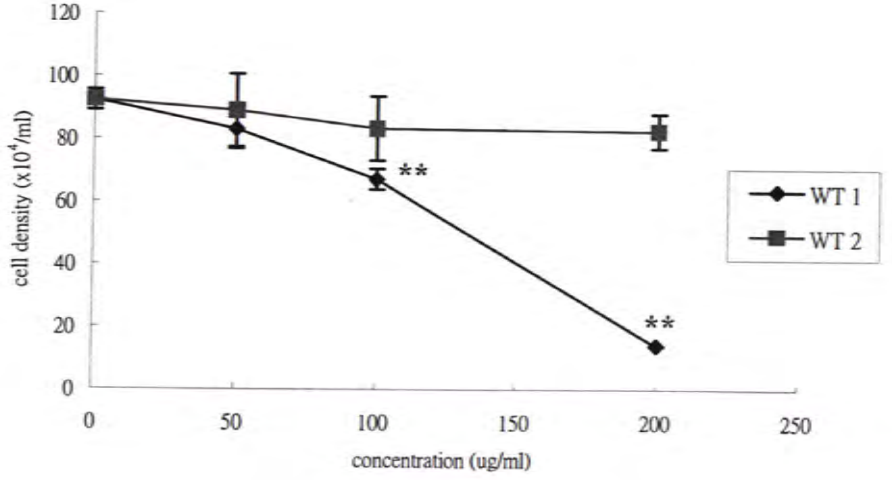
* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-10.1	-27.3	-84.8
WT2	-3.6	-9.7	-10.5

(b) ††

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT1	-3.4	-2.4	-11.2
WT2	-2.6	-4.7	-6.7



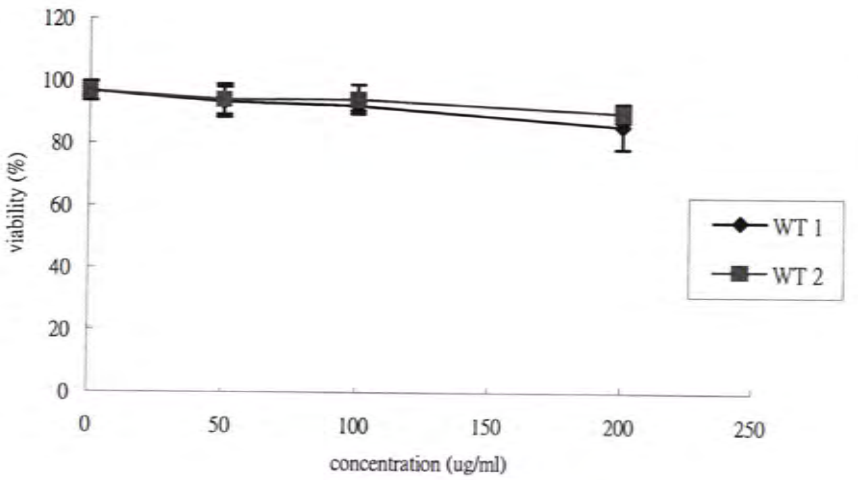


Figure 4.16 Effects of WT1 and WT2 on proliferation and viability of K-562 cells.

The effects on K-562 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 μg/ml, 100μg/ml and 200μg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of K-562 respectively.

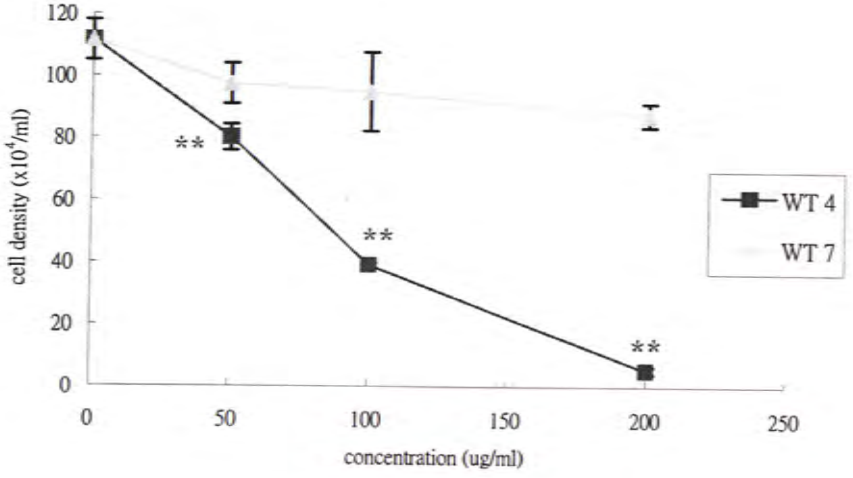
* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations		
	50 µg/ml	100 µg/ml	200 µg/ml
WT4	-28.1	-65.0	-95.4
WT7	-12.6	-14.8	-21.4

(b) ††

sample	% of inhibition at different concentrations		
	50 µg/ml	100 µg/ml	200 µg/ml
WT4	-2.3	-5.1	-83.8
WT7	-4.7	-5.4	-5.4



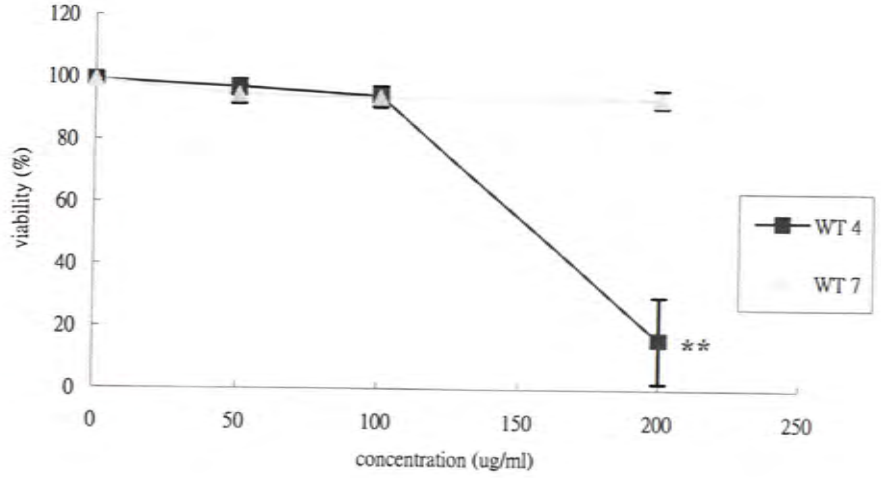


Figure 4.17 Effects of WT4 and WT7 on proliferation and viability of HL-60 cells.

The effects on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 µg/ml, 100µg/ml and 200µg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of HL-60 respectively.

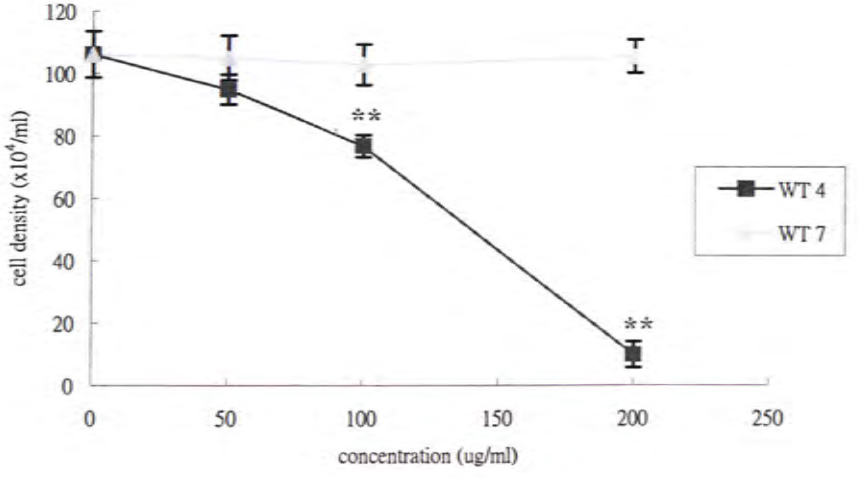
* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT4	-10.5	-27.7	-90.7
WT7	-0.9	-3.0	-0.5

(b) ††

sample	% of inhibition at different concentrations		
	50 μg/ml	100 μg/ml	200 μg/ml
WT4	-0.1	-1.4	-5.8
WT7	0.7	-2.8	-0.2



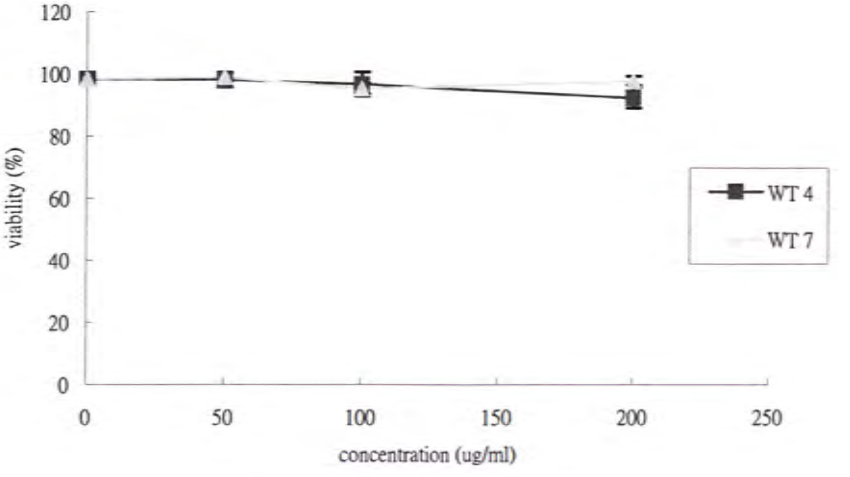


Figure 4.18 Effects of WT4 and WT7 on proliferation and viability of K-562 cells.

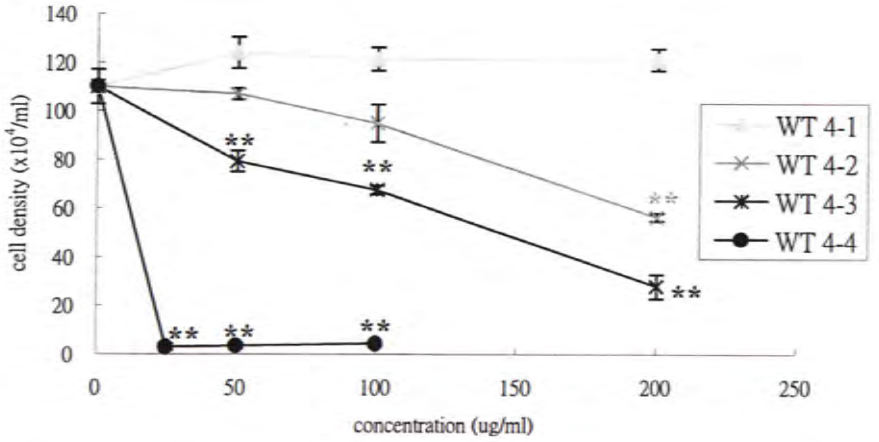
The effects on K-562 were evaluated by Trypan blue exclusion method after 72 h incubation with samples at 50 μg/ml, 100μg/ml and 200μg/ml. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of K-562 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	12.7	10.5	10.3
WT4-2	--	-2.7	-13.6	-48.6
WT4-3	--	-27.9	-38.6	-74.5
WT4-4	-97.3	-96.8	-96.1	--



(b) ††

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	0.0	0.4	0.7
WT4-2	--	0.6	0.8	-0.6
WT4-3	--	-2.5	0.3	-6.5
WT4-4	-100	-100	-100	--

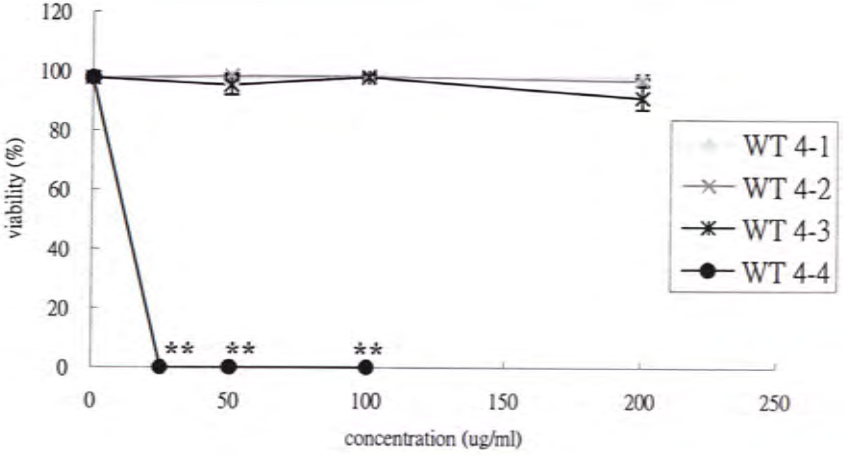


Figure 4.19 Effects of WT4 fractions on proliferation and viability of HL-60 cells.

The effects on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation with samples. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of HL-60 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	-11.9	-16.1	-6.1
WT4-2	--	-8.9	-20.2	-38.1
WT4-3	--	-16.9	-38.5	-65.2
WT4-4	-95.4	-97.0	-96.5	--

(b) ††

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	-6.0	-4.4	-3.8
WT4-2	--	-3.9	-5.7	-8.2
WT4-3	--	-5.9	-8.3	-15.4
WT4-4	-10.8	-100	-100	--

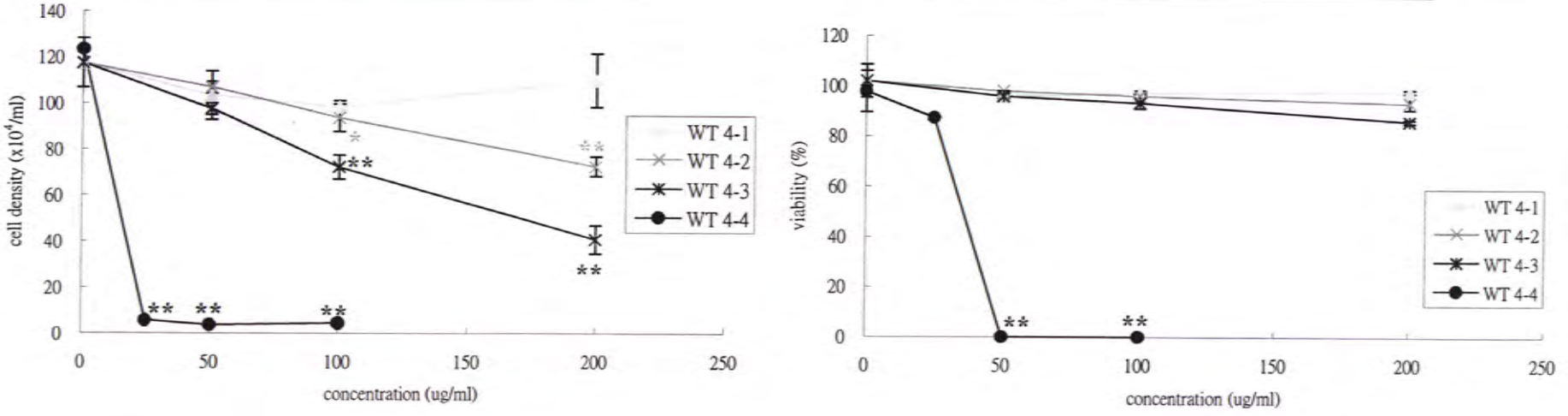


Figure 4.20 Effects of WT4 fractions on proliferation and viability of K-562 cells.

The effects on K-562 were evaluated by Trypan blue exclusion method after 72 h incubation with samples. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of K-562 respectively.

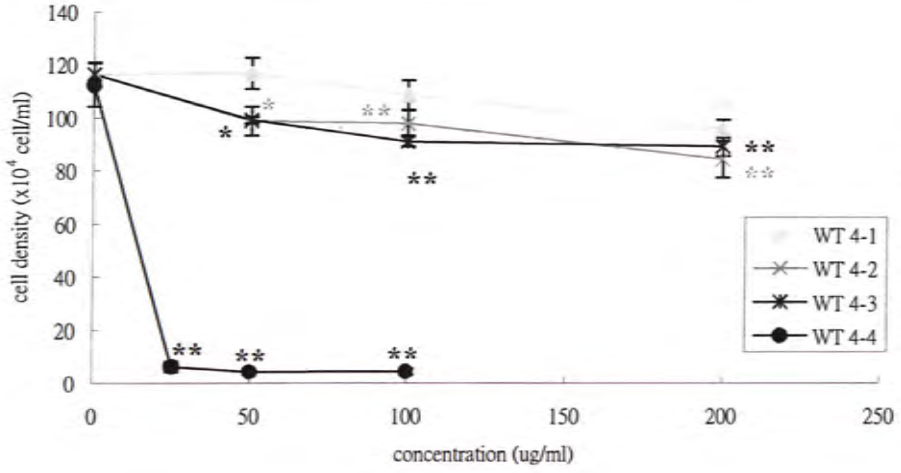
* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	0.4	-6.6	-17.3
WT4-2	--	-15.2	-15.8	-27.4
WT4-3	--	-14.8	-21.8	-23.2
WT4-4	-94.5	-96.4	-96.3	--

(b) ††

sample	% of inhibition at different concentrations			
	25 μg/ml	50 μg/ml	100 μg/ml	200 μg/ml
WT4-1	--	2.2	1.2	0.3
WT4-2	--	2.3	1.4	0.9
WT4-3	--	1.6	1.3	1.6
WT4-4	-2.8	-100	-100	--



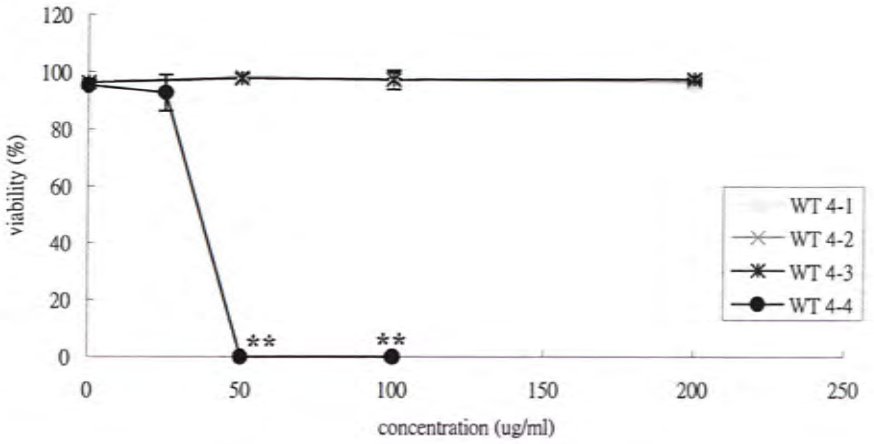
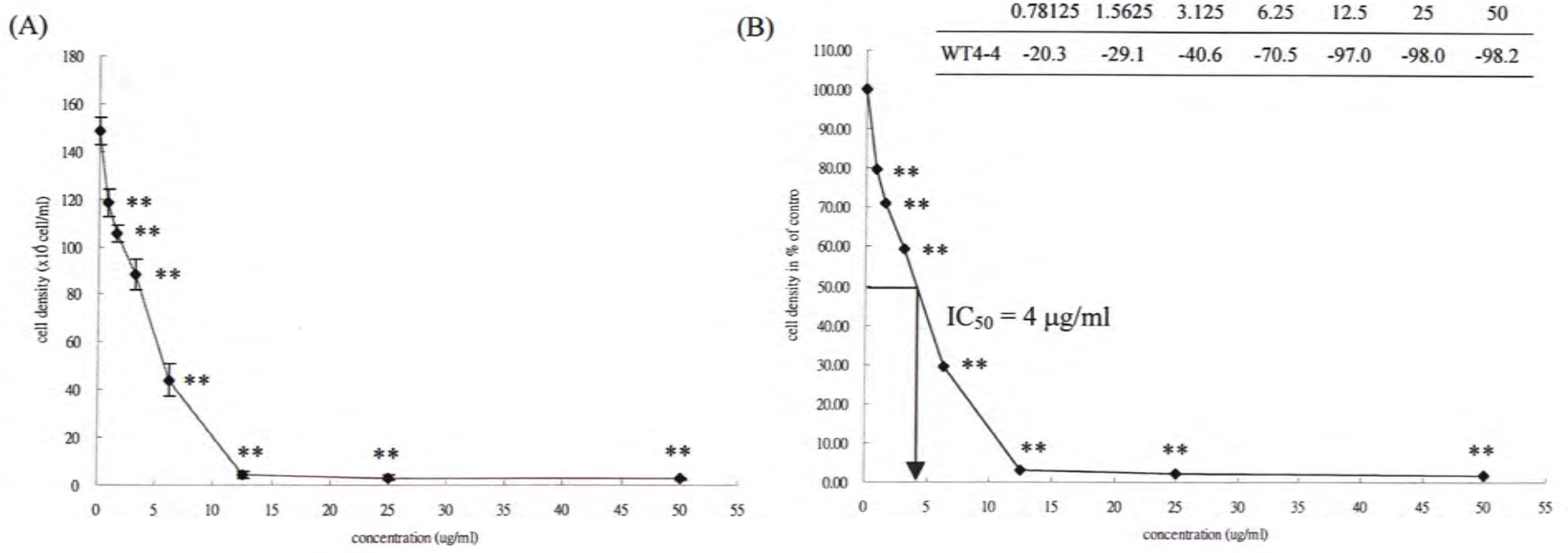


Figure 4.21 Effects of WT4 fractions on proliferation and viability of S-180 cells.

The effects on S-180 were evaluated by Trypan blue exclusion method after 72 h incubation with samples. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of S-180 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).



†

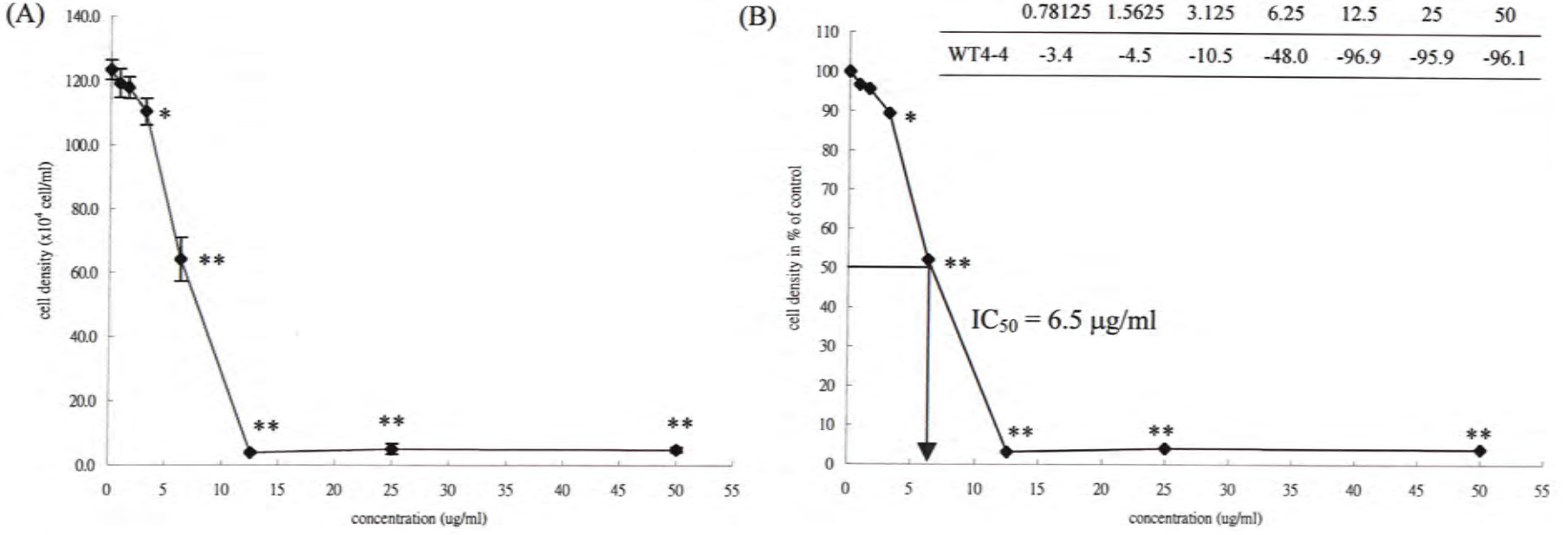
sample	% of inhibition at different concentrations (µg/ml)						
	0.78125	1.5625	3.125	6.25	12.5	25	50
WT4-4	-20.3	-29.1	-40.6	-70.5	-97.0	-98.0	-98.2

Figure 4.22 IC_{50} of WT4-4 on HL-60 cells

(A) The effect of WT4-4 on proliferation of HL-60 cells at 0.78125 µg/ml, 1.5625 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml respectively after 72 h incubation. The effect was evaluated by Trypan blue exclusion method. (B) The results of same set of data but expressed as cell density in % of control to determine the IC_{50} of WT4-4 on HL-60 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

† showed the % of inhibition on proliferation of HL-60.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).



†

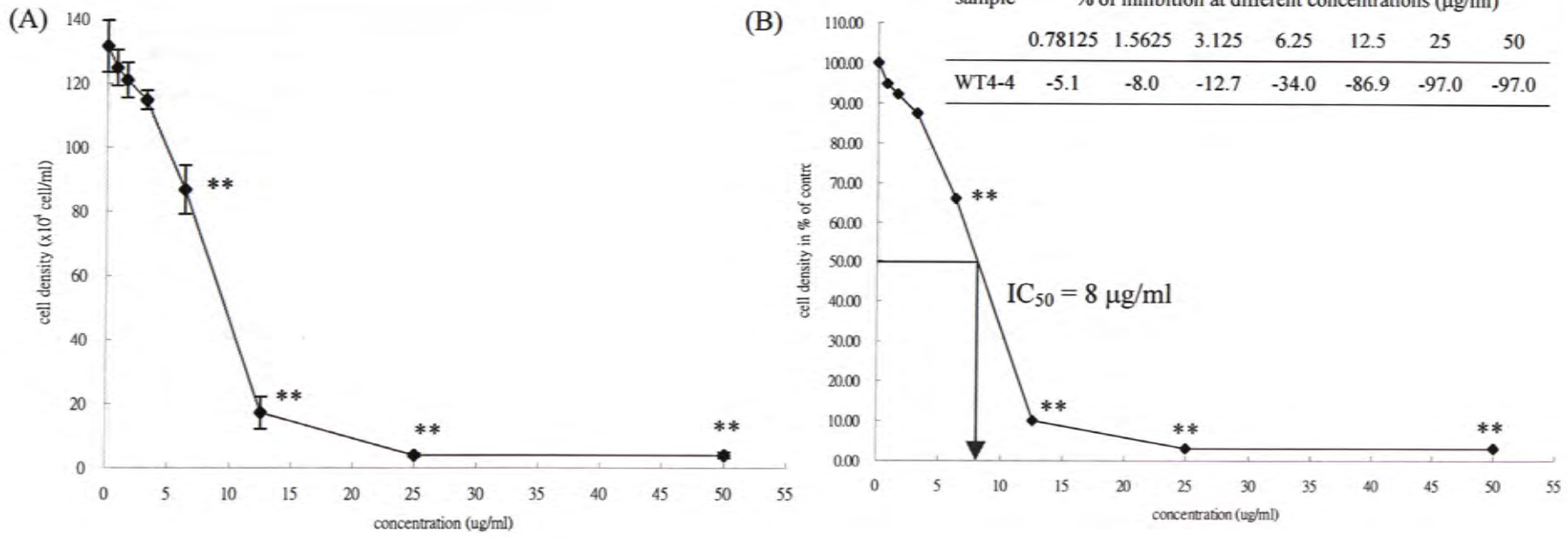
sample	% of inhibition at different concentrations (µg/ml)						
	0.78125	1.5625	3.125	6.25	12.5	25	50
WT4-4	-3.4	-4.5	-10.5	-48.0	-96.9	-95.9	-96.1

Figure 4.23 IC_{50} of WT4-4 on K-562 cells

(A) The effect of WT4-4 on proliferation of K-562 cells at 0.78125 µg/ml, 1.5625 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml respectively after 72 h incubation. The effect was evaluated by Trypan blue exclusion method. (B) The results of same set of data but expressed as cell density in % of control to determine the IC_{50} of WT4-4 on K-562 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

† showed the % of inhibition on proliferation of HL-60.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).



†

sample	% of inhibition at different concentrations (µg/ml)						
	0.78125	1.5625	3.125	6.25	12.5	25	50
WT4-4	-5.1	-8.0	-12.7	-34.0	-86.9	-97.0	-97.0

Figure 4.24 IC_{50} of WT4-4 on S-180 cells

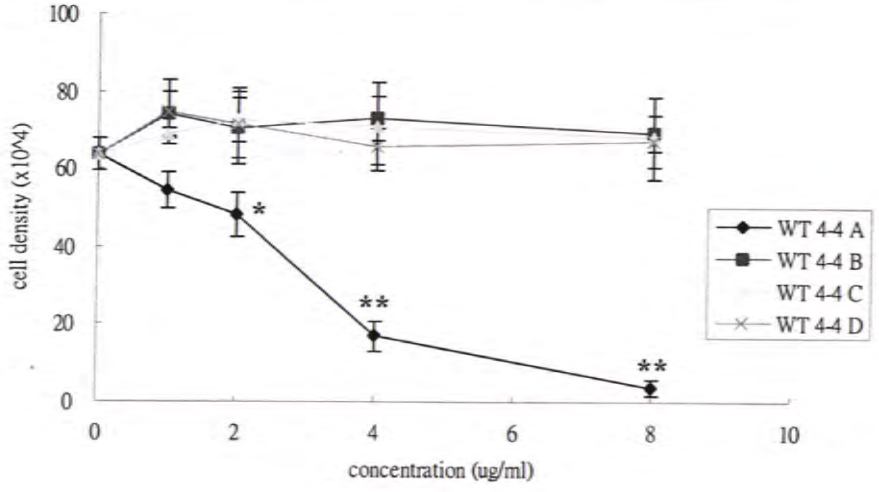
(A) The effect of WT4-4 on proliferation of S-180 cells at 0.78125 µg/ml, 1.5625 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml and 50 µg/ml respectively after 72 h incubation. The effect was evaluated by Trypan blue exclusion method. (B) The results of same set of data but expressed as cell density in % of control to determine the IC_{50} of WT4-4 on S-180 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

† showed the % of inhibition on proliferation of HL-60.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-15.1	-24.5	-73.4	-94.3
WT4-4 B	15.9	10.4	14.4	8.9
WT4-4 C	8.1	13.8	11.5	6.8
WT4-4 D	17.0	12.3	3.1	5.5



(b) ††

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-1.6	-4.9	-21.0	-97.0
WT4-4 B	5.2	5.6	5.6	5.5
WT4-4 C	5.5	5.1	5.5	5.4
WT4-4 D	5.5	5.8	6.4	4.5

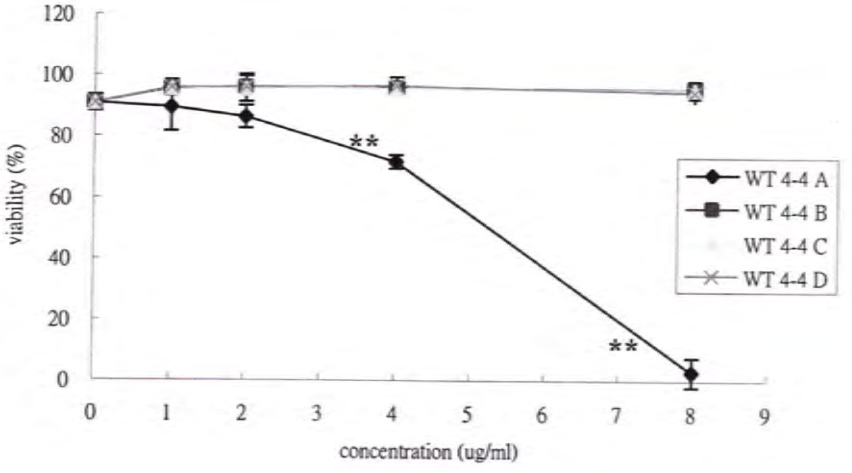


Figure 4.25 Effects of the fractions from WT4-4 on proliferation and viability of HL-60 cells.

The effects of the four fractions obtained after silica gel column on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of HL-60 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

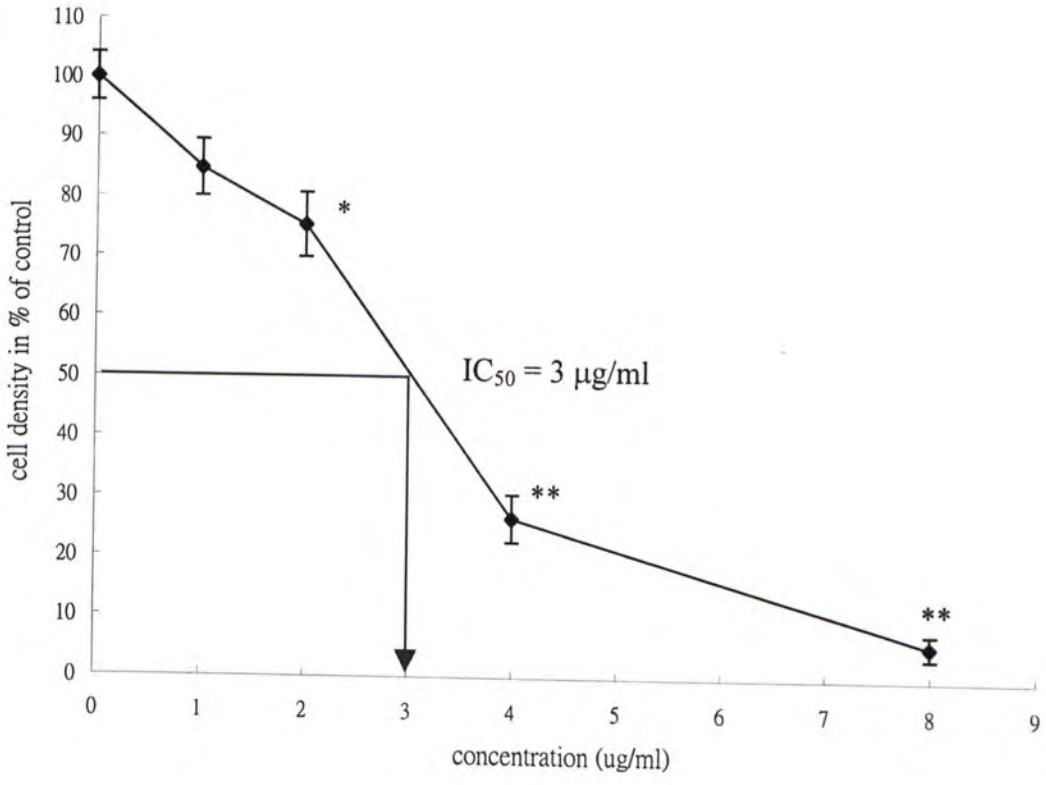


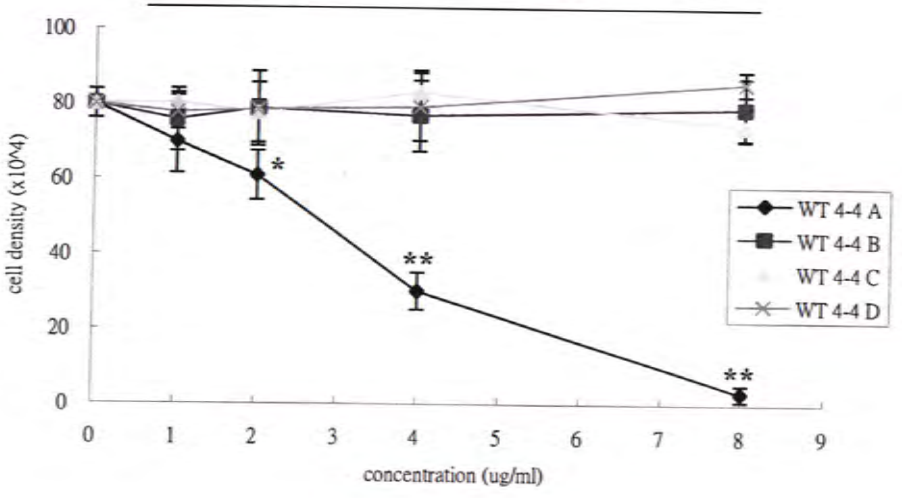
Figure 4.26 IC_{50} of WT4-4 A on HL-60 cells

The effect of WT4-4 A on HL-60 was evaluated by Trypan blue exclusion method after 72 h incubation. The result was expressed as cell density in % of control to determine the IC_{50} of proliferation on HL-60 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-12.5	-23.8	-62.2	-96.2
WT4-4 B	-5.2	-1.5	-3.5	-0.8
WT4-4 C	0.6	-2.7	4.4	-6.7
WT4-4 D	-2.7	-1.7	-0.6	7.5



(b) ††

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-3.5	-5.6	-15.7	-22.2
WT4-4 B	-2.6	-0.4	-3.4	-2.7
WT4-4 C	1.4	2.4	0.0	-3.6
WT4-4 D	-1.1	1.1	0.6	-3.8

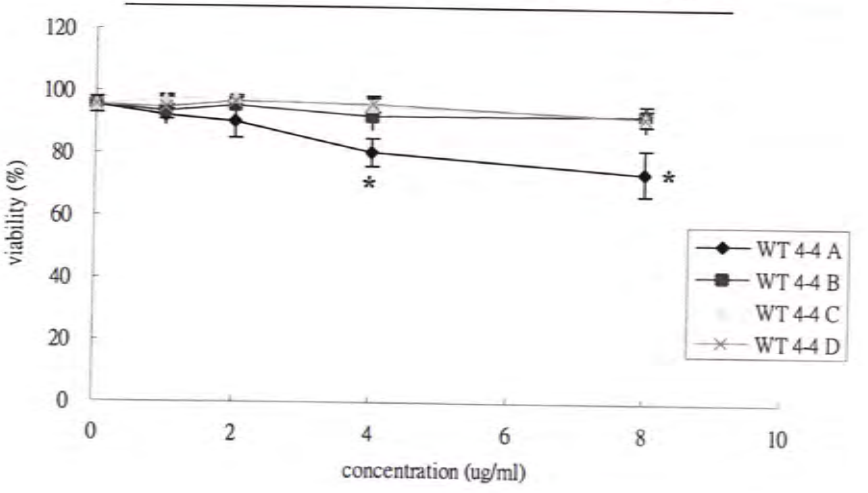


Figure 4.27 Effects of the fractions from WT4-4 on proliferation and viability of K-562 cells.

The effects of the four fractions obtained after silica gel column on K-562 were evaluated by Trypan blue exclusion method after 72 h incubation. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of K-562 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

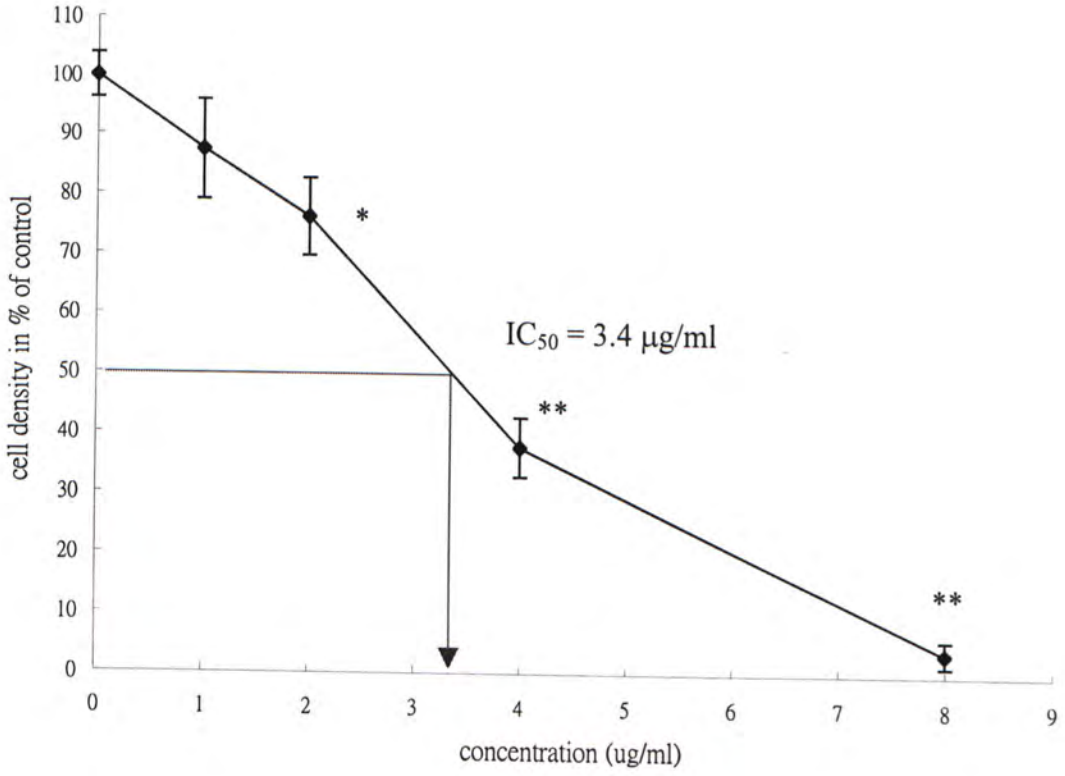


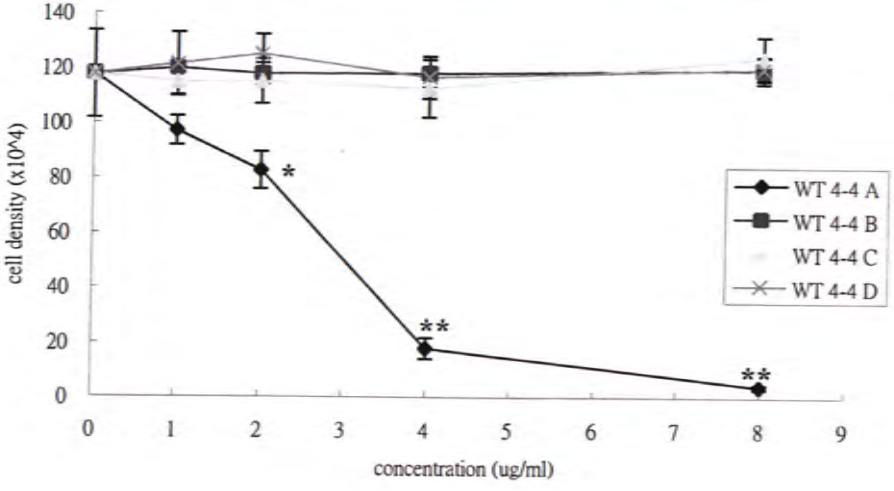
Figure 4.28 IC_{50} of WT4-4 A on K-562 cells

The effect of WT4-4 A on K-562 was evaluated by Trypan blue exclusion method after 72 h incubation. The result was expressed as cell density in % of control to determine the IC_{50} of WT4-4 A on K-562 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(a) †

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-17.4	-29.7	-84.8	-96.7
WT4-4 B	1.8	0.1	0.6	2.0
WT4-4 C	-2.3	-2.1	-4.1	5.7
WT4-4 D	3.3	6.4	-0.7	2.4



(b) ††

sample	% of inhibition at different concentrations			
	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml
WT4-4 A	-2.4	-3.1	-14.7	-29.0
WT4-4 B	-1.0	-1.7	-1.8	-3.8
WT4-4 C	-5.1	-0.4	-2.5	-5.3
WT4-4 D	-2.1	-6.3	-3.8	-4.1

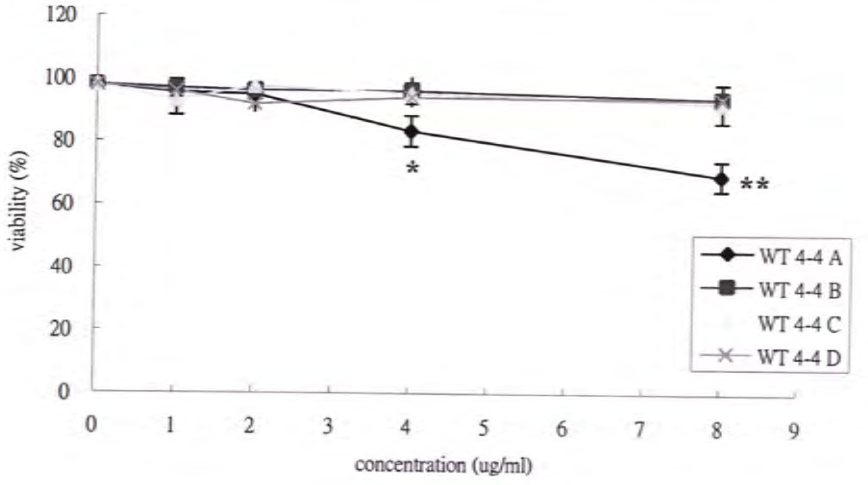


Figure 4.29 Effects of the fractions from WT4-4 on proliferation and viability of S-180 cells.

The effects of the four fractions obtained after silica gel column on S-180 were evaluated by Trypan blue exclusion method after 72 h incubation. The results were expressed as mean of (a) cell density and (b) viability ± S.D. of three replicates.

† and †† showed the % of inhibition on proliferation and viability of S-180 respectively.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

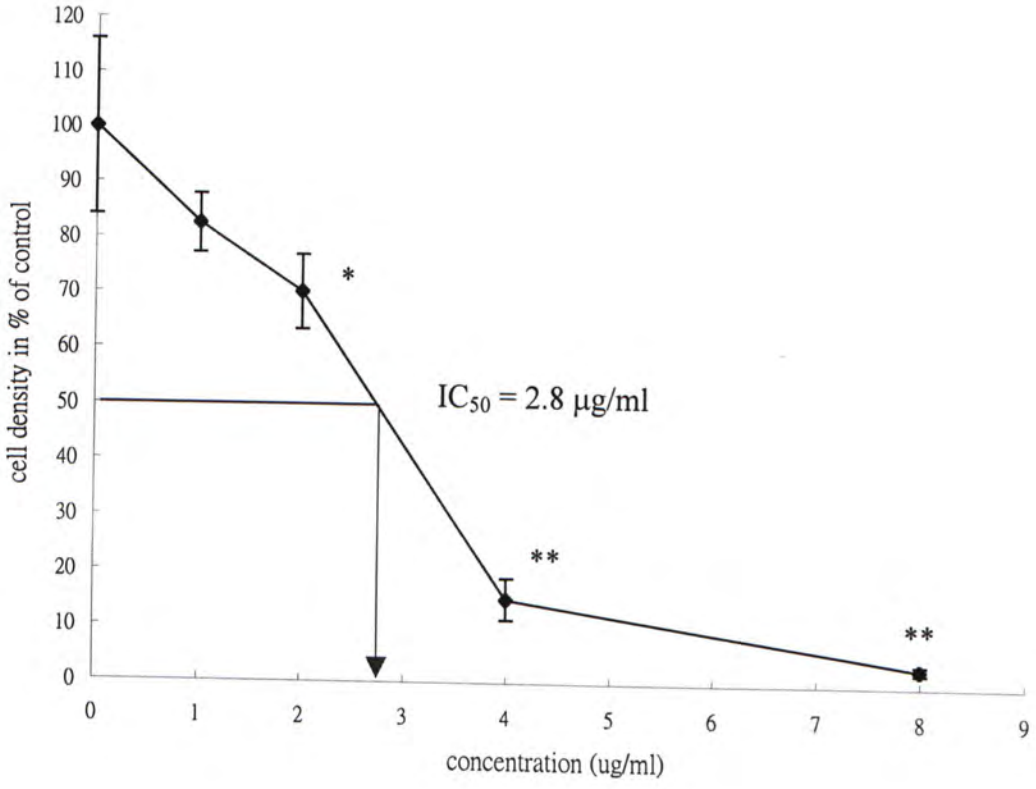


Figure 4.30 IC_{50} of WT4-4 A on S-180 cells

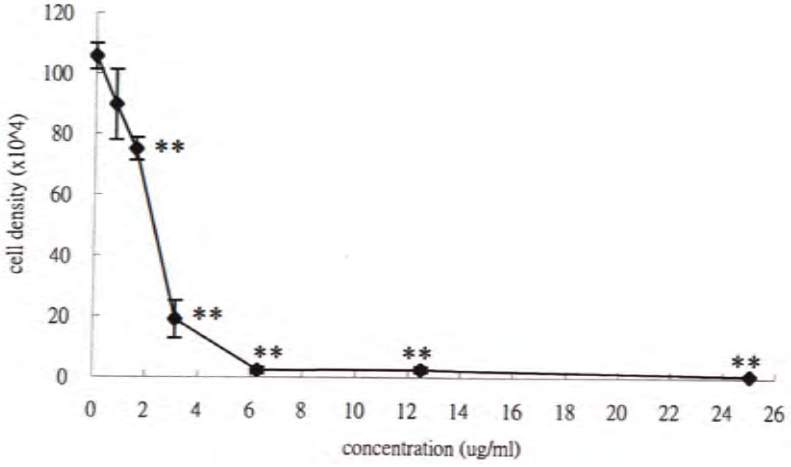
The effect of WT4-4 A on S-180 was evaluated by Trypan blue exclusion method after 72 h incubation. The result was expressed as cell density in % of control to determine the IC_{50} of WT4-4 A on S-180 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

(A)

†

sample	% of inhibition at different concentrations (μg/ml)					
	0.78	1.56	3.13	6.25	12.5	25
Crystal	-15.0	-28.9	-82.0	-97.8	-97.6	-99.4



(B)

††

sample	% of inhibition at different concentrations (μg/ml)					
	0.78	1.56	3.13	6.25	12.5	25
Crystal	1.3	1.6	-9.1	-100	-95.1	-100

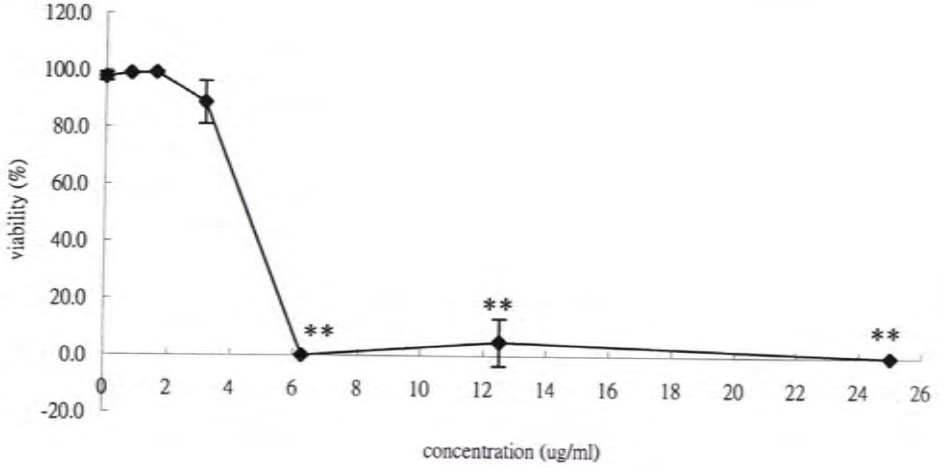


Figure 4.31 Effects of the crystal isolated fromWT4-4 A on HL-60 cells

The effects on HL-60 were evaluated by Trypan blue exclusion method after 72 h incubation with the crystal at 0.78 μg/ml, 1.56 μg/ml, 3.13 μg/ml, 6.25 μg/ml, 12.5 μg/ml and 25 μg/ml respectively. The results were expressed as (A) cell density and (B) viability ± S. D. of three replicates.

† and †† showed the % of inhibition on proliferation of HL-60.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

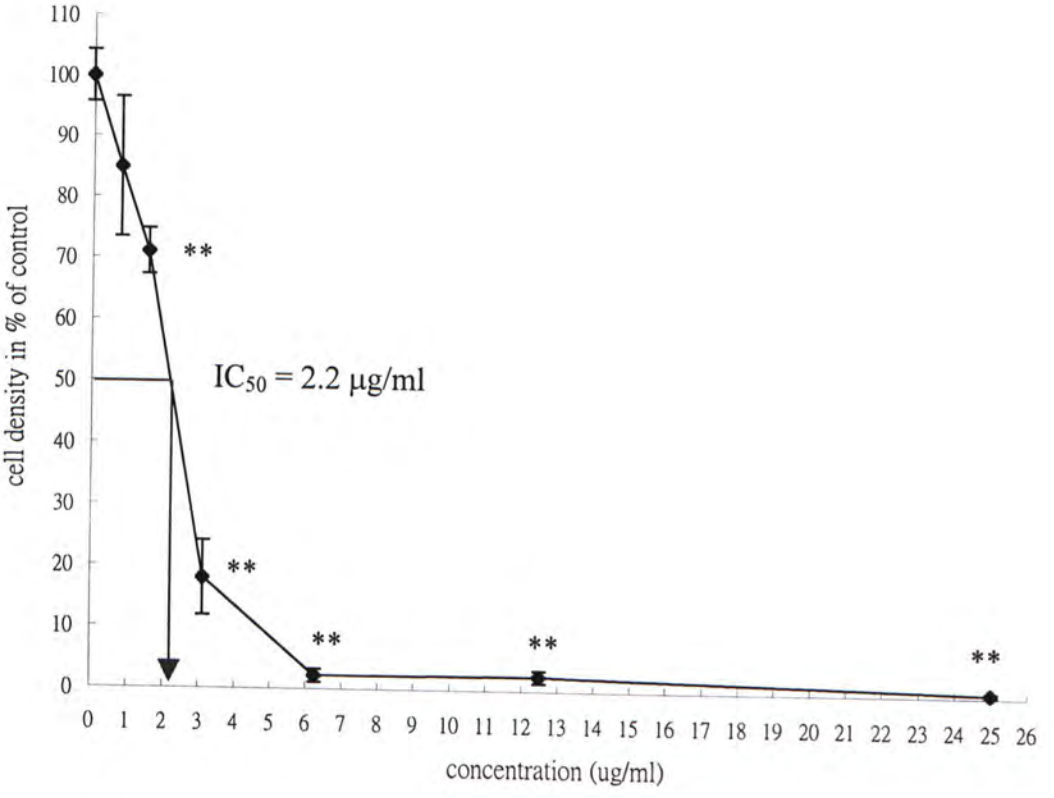


Figure 4.32 IC_{50} of the crystal isolated from WT4-4 A on HL-60 cells

The effect of WT4-4 A on HL-60 was evaluated by Trypan blue exclusion method after 72 h incubation. The result was expressed as cell density in % of control to determine the IC_{50} of the crystal isolated from WT4-4 A on HL-60 cells. Cell density in % of control was calculated by: (average cell density of treatment group / average cell density of control group) x 100%.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

†

sample	% of inhibition at different concentrations (μg/ml)								
	0.78	1.56	3.13	6.25	12.5	25	50	100	200
WT4-1	--	--	--	--	--	--	-2.1	-1.5	5.1
WT4-2	--	--	--	--	--	--	21.6	20.6	23.5
WT4-3	--	--	--	--	--	--	9.8	1.0	-17.2
WT4-4	-5.2	-5.5	-7.5	-8.7	-20.5	-29.3	-77.4	--	--

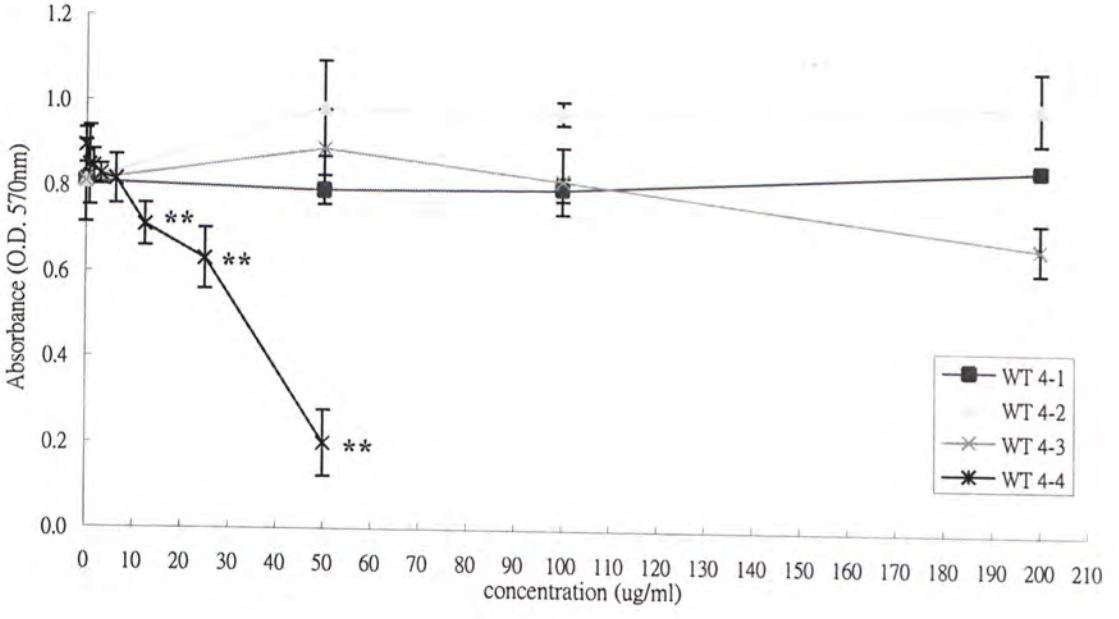


Figure 4.33 Effect of the four fractions from WT4 on proliferation of MCF-7 cells.

The effects of the four fractions obtained after macroporous resin column fractionation on MCF-7 were evaluated by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm ± S.D. of three replicates.

† showed the % of inhibition on proliferation of MCF-7.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

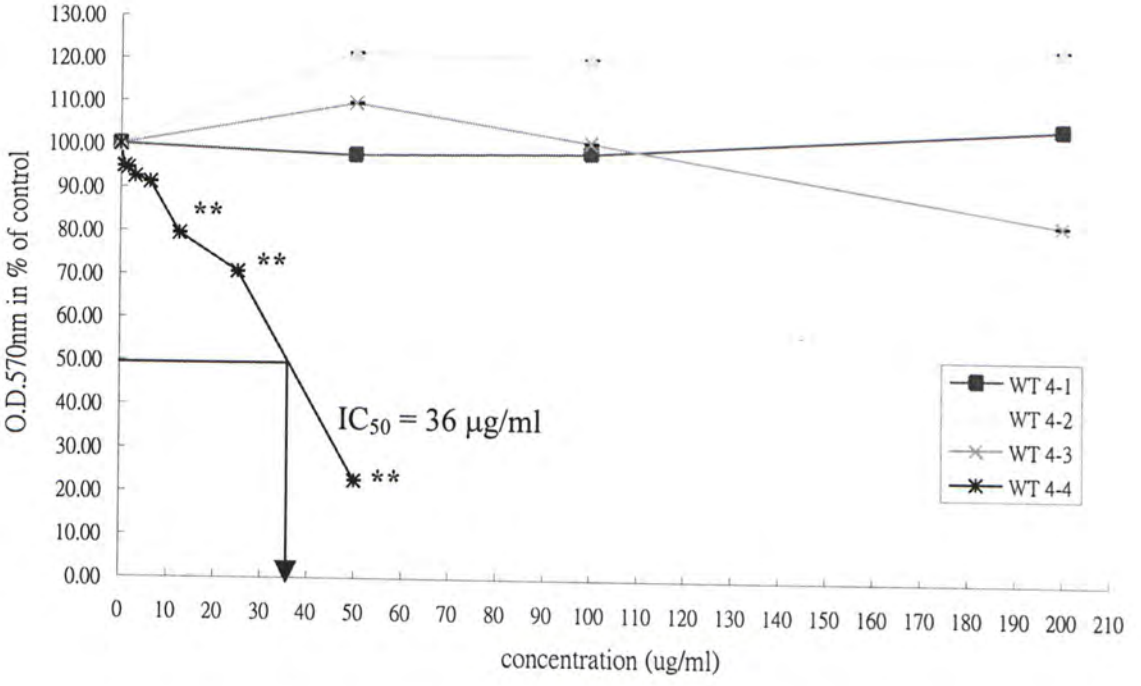


Figure 4.34 IC_{50} of the WT4-4 on proliferation of MCF-7 cells.

The effects of the four fractions obtained after macroporous resin column fractionation on MCF-7 were evaluated by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm in % of control of three replicates. The O.D. 570 in % of control was calculated by: (average absorbance of treatment group / average absorbance of control group) x 100%

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

†

sample	% of inhibition at different concentrations (μg/ml)								
	0.78	1.56	3.13	6.25	12.5	25	50	100	200
WT4-1	--	--	--	--	--	--	-3.6	0.8	-10.0
WT4-2	--	--	--	--	--	--	-25.9	-35.5	-45.2
WT4-3	--	--	--	--	--	--	-24.8	-38.4	-50.4
WT4-4	28.8	24.1	24.1	14.7	-17.5	-51.4	-79.5	-100	--

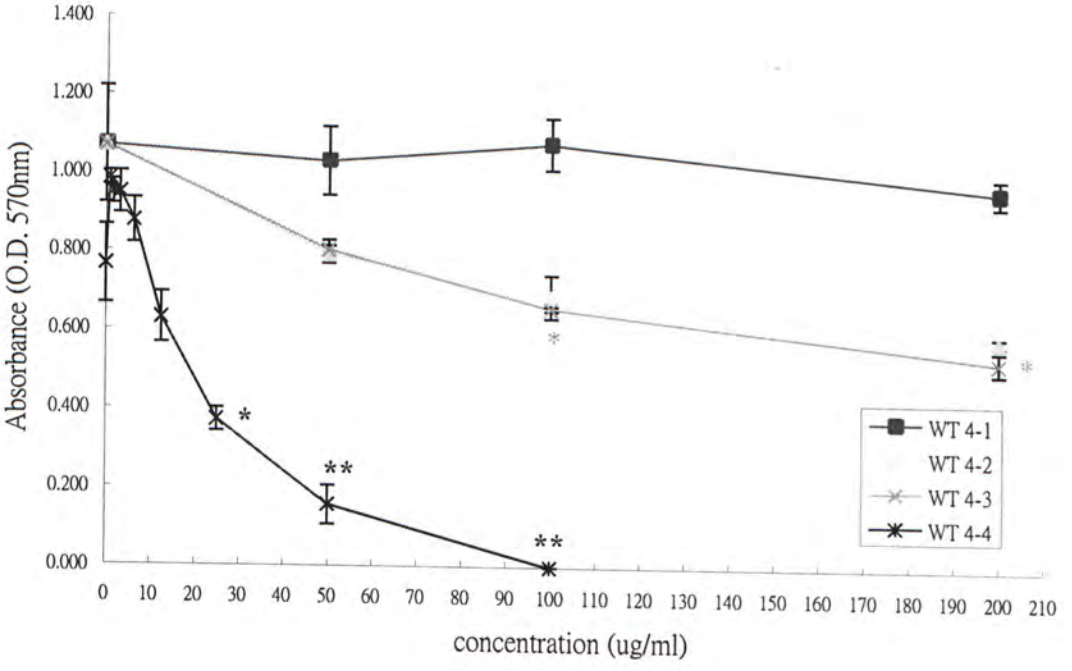


Figure 4.35 Effect of the four fractions from WT4 on proliferation of HepG2 cells.

The effects of the four fractions obtained after macroporous resin column fractionation on HepG2 were evaluated by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm ± S.D. of three replicates.

† showed the % of inhibition on proliferation of HepG2.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

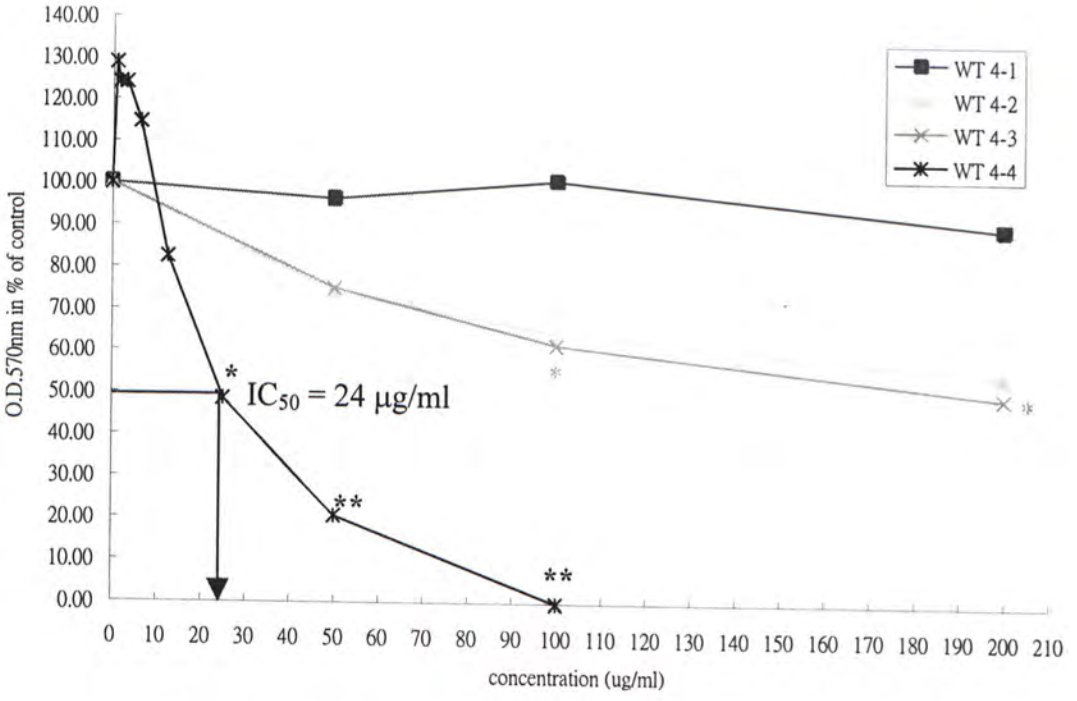


Figure 4.36 IC_{50} of the WT4-4 on proliferation of HepG2 cells.

The effects of the four fractions obtained after macroporous resin column fractionation on HepG2 were evaluated by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm in % of control of three replicates.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test).

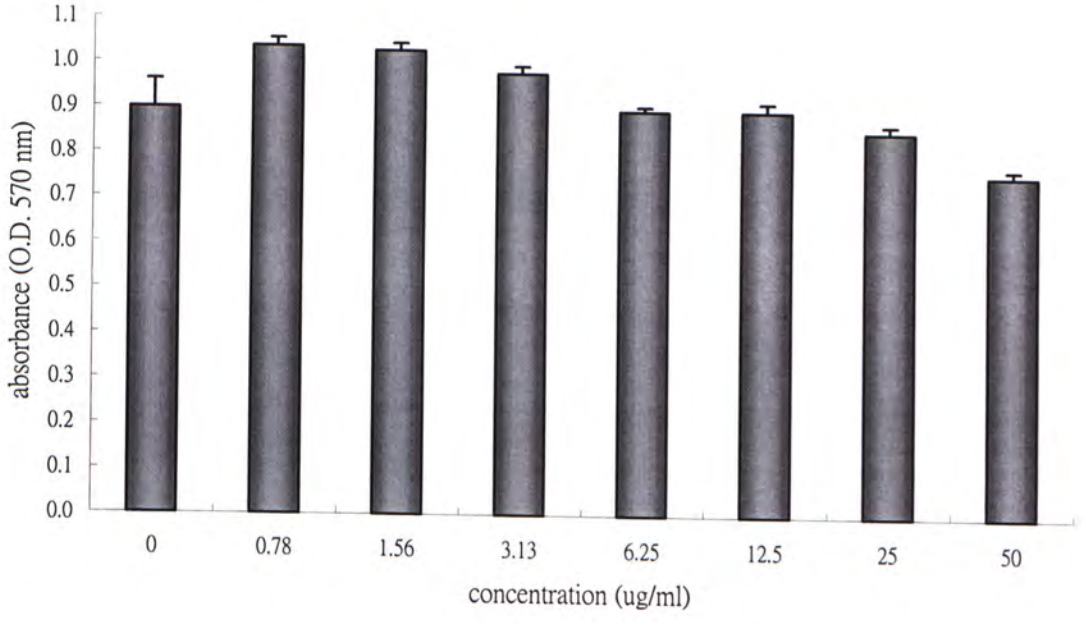


Figure 4.37 Effect of WT4-4 on proliferation of L-02 cells.

The cytotoxicity effect of WT4-4 on normal cell line L-02 cells was determined by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm ± S.D. of three replicates.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test)

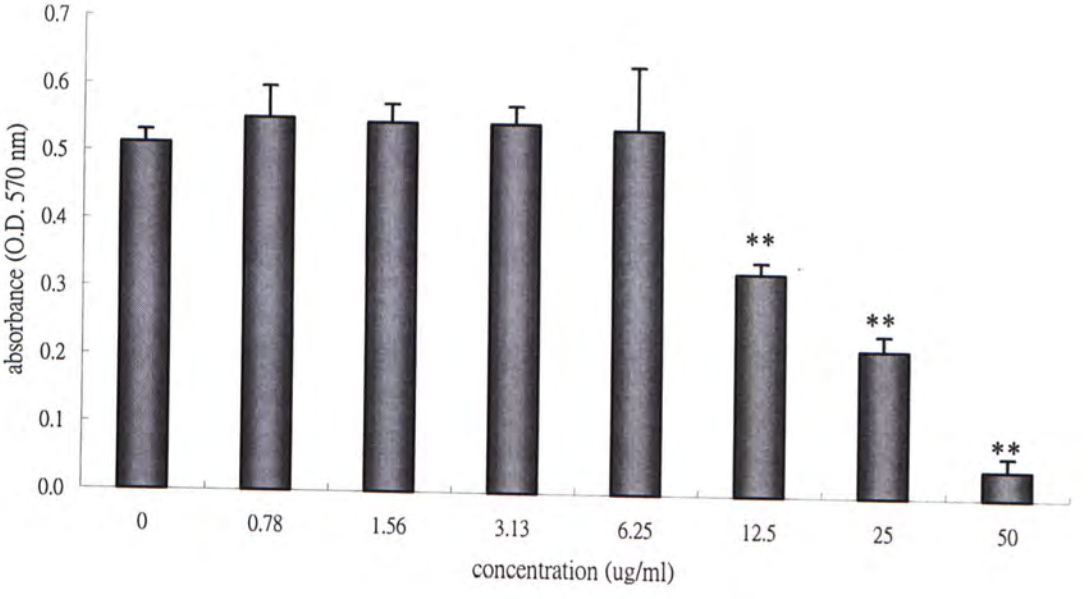


Figure 4.38 Effect of WT4-4 on proliferation of Vero cells.

The cytotoxicity effect of WT4-4 on normal cell line Vero cells was determined by MTT assay method after 72 h incubation. The results were expressed as mean of absorbance at O.D. 570 nm ± S.D. of three replicates.

* $p<0.05$ (t-test); ** $p<0.01$ (t-test)

4.3 *In vivo* Antitumor Effect of WT4-4

WT4-4 was dissolved in 5% DMSO in the *in vivo* assay, and the effect of this solvent on Sarcoma 180 growth in Balb/c mice was not significantly different from that of PBS (Fig. 4.39).

WT4-4 could significantly inhibit the growth of Sarcoma-180 tumor in Balb/c mice from 0.8 mg/kg by i.p. injection in a dose-dependent manner. It inhibited the growth of Sarcoma 180 by 62.79% at the dose of 3.2 mg/kg (Fig. 4.40).

WT4-4 at the dose of 0.4 mg/kg, 0.8 mg/kg and 1.6 mg/kg did not significantly affect the body weight in Sarcoma 180-bearing mice from control group. At 3.2 mg/kg, WT4-4 significantly increased the body weight by 5.26% when compared to control group but there was no significant different when compared to weight control group (Table 4.5).

†

Treatment	Average tumor weight (g) ± S.D. [a]	% change in tumor weight [b]
PBS	0.45 ± 0.16	--
5% DMSO	0.50 ± 0.13	11.22

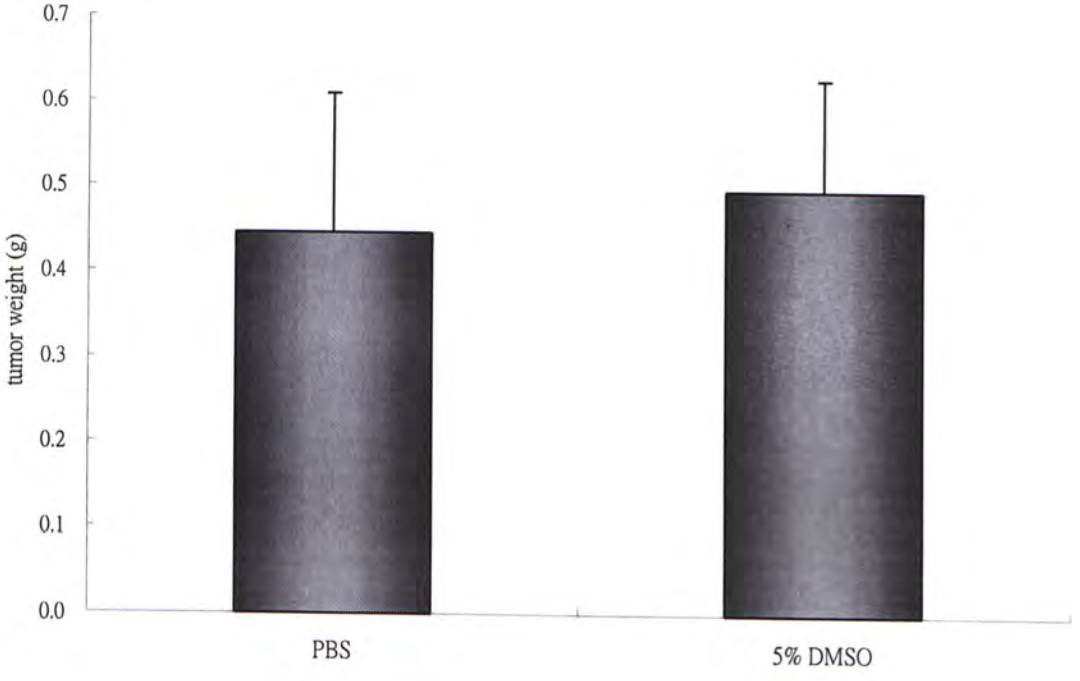


Figure 4.39 Effects of PBS and 5% DMSO on Sarcoma 180 solid tumor.

5 x 10^6 Sarcoma 180 cells were subcutaneously injected into the back of male Balb/c mice. Three days later, PBS or 5% DMSO were daily i.p. injected into the tumor-bearing mice for 10 consecutive days.

The effects of PBS and 5% DMSO on Sarcoma 180 solid tumor in male Balb/c mice were expressed as average tumor weight (g) ± S.D. (n = 7). Statistically significant value was marked with * and ** for $p < 0.05$ and $p < 0.01$ respectively.

† showed the average tumor weight of PBS or 5% DMSO treated tumor-bearing mice and the % change of 5% DMSO treatment to PBS treatment.

[a] The tumor was excised and weighed 9 days after the 10 consecutive days injection.

[b] % change was calculated by: (average tumor weight of treatment group – control group) / control group x 100%

†

Treatment	Concentration	Tumor weight (g) ± S.D. [a]	% of inhibition [b]
DMSO (Control)	5%	0.86 ± 0.31	--
WT4-4	0.4 mg/kg	0.60 ± 0.25	-30.23
	0.8 mg/kg	0.57 ± 0.24	-33.72 *
	1.6 mg/kg	0.48 ± 0.19	-44.19 **
	3.2 mg/kg	0.32 ± 0.16	-62.79 **

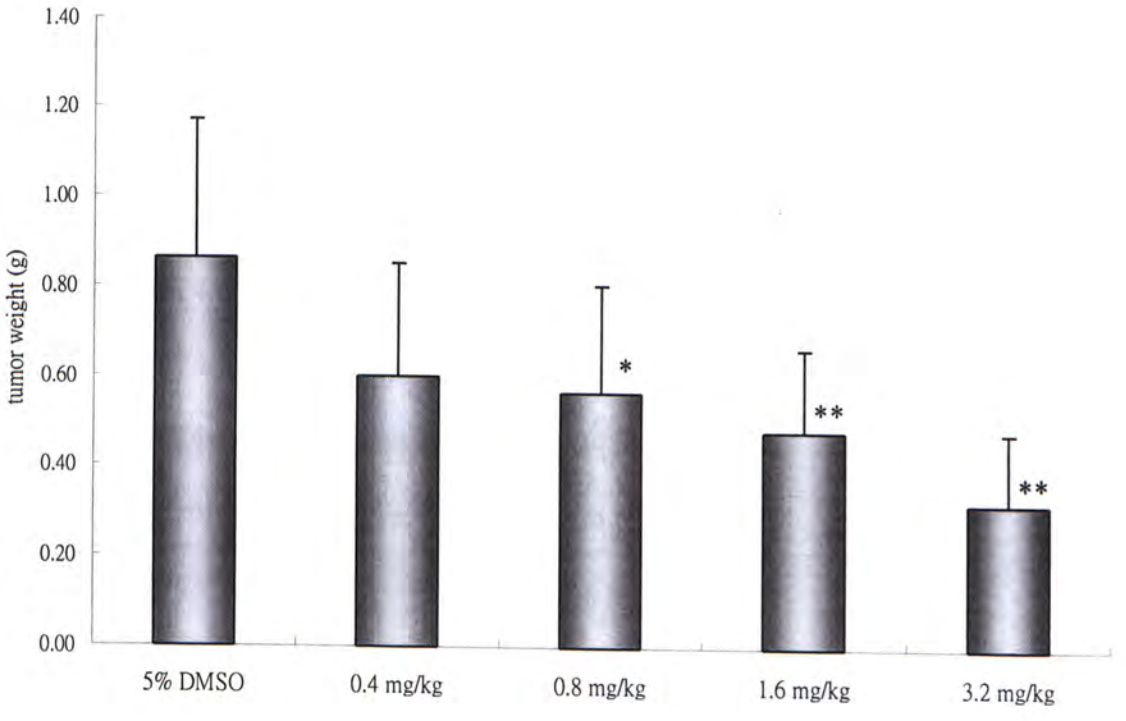


Figure 4.40 Effects of WT4-4 on Sarcoma 180 solid tumor.

5 x 10^6 Sarcoma 180 cells in 0.2 ml were subcutaneously injected into the back of male Balb/c mice. Three days later, WT4-4 in different concentrations was daily i.p. injected into the tumor-bearing mice for 10 consecutive days.

The effects on Sarcoma 180 solid tumor in male Balb/c mice were expressed as average tumor weight (g) ± S.D. (n = 8). Statistically significant values were marked with * and ** for $p < 0.05$ and $p < 0.01$ respectively.

† showed the average tumor weight of WT4-4 treated groups and the % change of treatment groups to control groups (5% DMSO).

[a] The tumor was excised and weighed 9 days after the 10 consecutive days injection.

[b] % of inhibition was calculated by: (average tumor weight of treatment group – control group) / control group x 100%

Table 4.5 Effect of WT4-4 on body weight change of Sarcoma 180 bearing mice.

Group	Average body weight (g) ± S.D.		% change in body weight [a]
	Initial (I)	Final (F)	
Control (5% DMSO)	23.42 ± 1.79	24.86 ± 1.54	+2.48
0.4 mg/kg WT4-4	23.18 ± 1.10	24.77 ± 1.03	+4.27
0.8 mg/kg WT4-4	22.96 ± 1.53	24.27 ± 1.69	+3.22
1.6 mg/kg WT4-4	22.40 ± 0.68	23.84 ± 0.71	+4.29
3.2 mg/kg WT4-4	23.01 ± 1.80	24.54 ± 1.78	+5.26 *
Weight control	24.55 ± 1.64	25.63 ± 1.18	+4.40

5 x 10^6 Sarcoma 180 cells in 0.2 ml were subcutaneously injected into the back of male Balb/c mice. Three days later, WT4-4 in at the dose of 0.4 mg/kg, 0.8 mg/kg, 1.6 mg/kg, 3.2 mg/kg or 5% DMSO were daily i.p. injected into tumor-bearing mice for 10 consecutive days. Mice without tumor inoculation or treatment were used as weight control group. Initial weight (I) of mice was weighed before the experiment and final weight (F) of mice was weighed at the end of the experiment before tumor excision.

The effect of WT4-4 on Sarcoma 180 tumor bearing mice was expressed as average body weight (g) ± S.D. (n=8).

[a] % change in body weight was calculated by: (F – tumor weight – I) / I x 100% Statistically significant difference in % change in body weight of treatment group to control (5% DMSO) group was marked with * for $p < 0.05$.

Statistically significant difference in % change in body weight of treatment group to weight control group was marked with # for $p < 0.05$.

4.4 Results of DNA Agarose Gel Electrophoresis

DNA extracted from WT4-4-treated HL-60, K-562 and S-180 respectively after 72 h incubation were run on agarose gel electrophoresis. The results were presented in Figure 4.41. When compared to the positive control, WT4-4 did not cause DNA laddering on gel electrophoresis in HL-60, K-562 or S-180 at the corresponding IC_{25}, IC_{50} and IC_{75}.

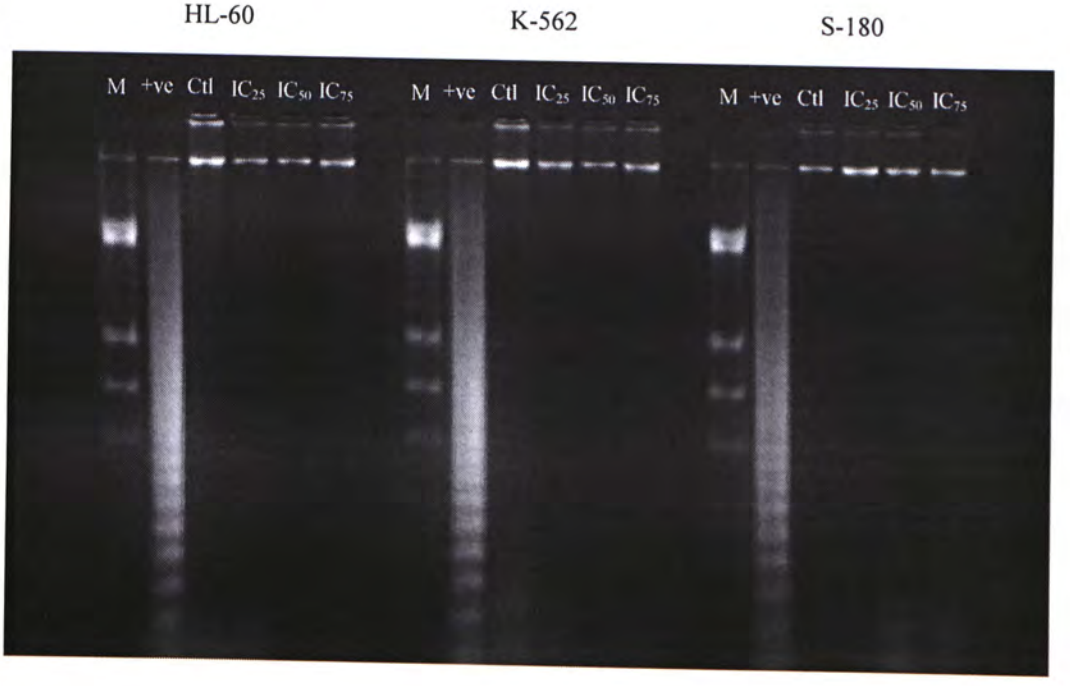


Figure 4.41 DNA agarose gel photo of WT4-4 treated HL-60, K-562 and S-180.

HL-60, K-562 and S-180 were incubated with WT at the corresponding IC_{25}, IC_{50} and IC_{75} for three days. Their DNA were extracted and subjected to DNA agarose gel electrophoresis on 0.8% gel. Positive control (+ve) was DNA from cells treated with 1μM camptothecin for 4 h. Control (Ctl) was DNA from cells that without any treatment. IC_{25}, IC_{50} and IC_{75} were the concentrations of WT4-4 that induced 25%, 50% and 75% inhibition on cell proliferation respectively on corresponding cell lines.

Chapter 5

Discussion

5.1 Characterization of Antitumor Fraction and Compound of *Wedelia trilobata* (WT)

Active fractions from WT were extracted by hot water extraction, ethanol precipitation and further fractionated by bioactivity-guided purification. According to Figures 4.17 – 4.18, the active fraction from WT water extract (WT1) was found to be the small molecular fraction in the supernatant (WT4), which exhibited antitumor effect on both HL-60 and K-562 leukemia cells.

Since WT4 was derived from water extraction at the beginning, in order to purify the active fraction, macroporous resin column that is usually used to separate samples with stronger polarity was employed to briefly fractionate WT4. Four solutions with decreasing polarity, i.e. water, 15% ethanol, 30% ethanol and 60%

ethanol, were used in sequence to fractionate WT4, which then gave rise to WT4-1, WT4-2, WT4-3 and WT4-4 fractions respectively. WT4-4, the less polar fraction eluted by 60% ethanol, was the most active fraction against the proliferation of HL-60, K-562, S-180, MCF-7 and HepG2 cell lines (Fig. 4.19 – 4.21, Fig. 4.33, Fig. 4.35). According to the HPLC chromatograms (Fig. 4.1 – 4.4), WT4-4 contained a distinct large peak at retention time of 12.383 min (17%) that was not found in the other fractions at flow rate 0.8 ml/min. The chemical tests of phytochemical groups on WT4-4 (Table 4.4) showed that there were lactones and coumarins, flavonoids and terpenoids, but no alkaloids, sterols, saponins, carbohydrates, polyglucose nor anthraquinone. This implied that the active ingredients of WT4-4 might be lactones, coumarins, flavonoids or terpenoids. These compounds can also be found in other Compositae plants. Some showed bioactivities, for example, sesquiterpene lactones from *Ratibida mexicana* exhibited antitumor activity against solid tumor cell lines (Calera *et al.*, 1995); diterpene lactones were found in *Wedelia regis* (Bohlmann *et al.*, 1984b); coumarin with antitumor activity was found in *Saussurea eopygmaea* (Zong *et al.*, 1994); a flavonoid, silymarin, from milk thistle exhibited antihepatotoxic effect, antioxidative activity and against skin tumor promotion (Chatterjee *et al.*, 1999); diterpenoid, triterpene and flavonoids were found in

Wedelia asperrima (Calanasan and Macleod, 1997); sesquiterpenoids from *Achillea millefolium* exhibited *in vivo* antitumor activity against mouse P-388 leukemia cells (Tozyo *et al.*, 1994); and terpenoids were found in *Wedelia chinensis* (Huang *et al.*, 1997).

Although WT4-4 contained a distinct peak in HPLC at retention time 12.383 min, it also contained some broad and overlapping peaks before retention time of 10 min. WT4-4 was thus further purified by silica gel column in order to find out the active ingredients contributed to the antitumor effect. Among the four eluted fractions, the first one (WT4-4 A) with lower polarity, obtained by elution of first 100 ml chloroform : ethyl acetate : n-hexane : methanol (3 :1 : 1 : 0.5) was found to be the active fraction (Fig. 4.25, 4.27, 4.29). The HPLC chromatogram of this fraction (Fig. 4.6) showed a major distinct peak at retention time of 12.363 min (39%) that was not found in the other three less active fractions. Besides, the retention time of this peak was consistent with the unique peak in WT4-4 with retention time (12.383 min). Therefore this compound was believed to be a marker peak of the antitumor effect in WT. Although there were still some minor peaks with retention time before 10 min in WT4-4 A, the amount was much smaller than that in WT4-4, that means WT4-4 A was more homogenous fraction and proportion of the marker

peak was higher.

After the fraction WT4-4 A was concentrated and stood overnight, crystals were formed. This compound could exhibit antitumor effect on HL-60 (Fig. 4.31). From the HPLC chromatogram of the crystal at flow rate 0.5 ml/min (Fig. 4.10), there was only one major peak at retention time 19.505 min (83%) and a small peak at retention time of 27.432 min. The retention time of the major peak of the crystal was consistent with the major maker peak in WT4-4 A but in higher proportion (Fig. 4.11) at flow rate 0.5 ml/min (retention time at 19.847 min). Since the major peak contributed over 80% so the antitumor activity was properly due to this compound.

According to the X-ray crystallography results of the crystal (Fig. 4.12), the empirical formula was determined to be $C_{23}H_{32}O_9$, with one lactone ring and the chemical structure showed this antitumor compound was sesquiterpene lactone. The lactone and terpenoid structures of this compound were consistent with the chemical tests for phytochemical groups of WT4-4. Isolation of sesquiterpene lactones from other Compositae species had also been reported. For examples, several sesquiterpene lactones have been isolated from *Artemisia annua* (Beekman, *et al.*, 1998), *Podachaenium eminens* (Castro *et al.*, 2000), *Arnica montana*, *Arnica chamissonis* (Woerdenbag *et al.*, 1994) and *Neurolaena lobata* (Francois *et al.*, 1996).

Many of these sesquiterpene lactones possessed antitumor activities.

Sesquiterpene lactones with same empirical formula of the crystal obtained in this project were also reported in other publications, and these compounds were found in *Wedelia* species, including the aerial parts of *Wedelia trilobata* (Bohlmann *et al.*, 1981), the aerial parts of *Wedelia prostrata* (Ragasa and Padolina, 1993) and also leaves of *Wedelia prostrata* (Farag *et al.*, 1996). However, only the sesquiterpene lactone reported by Farag *et al.* (1996) was same as the crystal in this project. The other two sesquiterpene lactones reported by Bohlmann *et al.* (1981) and Ragasa and Padolina (1993) were different from the sesquiterpene lactone isolated in this project in stereochemistry.

5.2 Antitumor Effects of WT Fractions and Purified Component (Crystal)

5.2.1 *In vitro* assay

In the screening of most prominent antitumor agents in Compositae among *Dendranthema indicum* (DI), *Dendranthema morifolium* (DM), *Taraxacum mongolicum* (TM) and *Wedelia trilobata* (WT), WT1 was found to be the most effective water extract on both HL-60 and K-562 leukemia cells while DM1 did not affect the proliferation nor viability of both cell lines (Fig. 4.13, Fig. 4.14). For all the four selected Compositae species, HL-60 was more sensitive than K-562. Moreover, none of the water extracts could inhibit the viability of K-562.

When comparing WT1 and WT2, the antitumor activity of WT1 was much stronger in both HL-60 and K-562 (Fig. 4.15, Fig. 4.16). WT1 could exhibit significant inhibition (18.6%) on proliferation of HL-60 at the lowest dose of 50 μg/ml and 27.3% on K-562 at 100 μg/ml. At 200 μg/ml, it could inhibit the proliferation of HL-60 and K-562 by 94.9% and 84.8% respectively. However, WT2 could only inhibit the proliferation of HL-60 by 13.3% at 200 μg/ml. These implied that the antitumor effect of water extract of WT was more effective than that of the base extract on HL-60 and K-562. Therefore, water extracts was used in further

study.

The ethanol supernatant (WT4) from WT1 was found to be a stronger antitumor fraction than the ethanol precipitate (WT7) on both HL-60 and K-562 (Fig. 4.17, Fig. 4.18). The lowest effective dose for both cell lines were same as WT1, but the percentage of inhibition on proliferation was higher, especially in HL-60 cells, increased from 18.6% to 28.1% at 50 μg/ml.

It was not surprised that the more pure fraction after resin column fractionation would have stronger inhibitory effect on the cancer lines. Among the four eluted fractions, WT4-1 was inactive to all the tested cell lines, except on S-180, with mild inhibitiory effect on proliferation at high dose. Although all the other three fractions exhibited antitumor effect on the suspension cell lines HL-60, K-562 and S-180 in a dose-dependent manner, WT4-4 was the most prominent fraction and the percentage of inhibition on HL-60 and K-562 was much higher than that of WT4, which was obtained before column fractionation. The percentage of inhibition on proliferation of HL-60, K-562 and S-180 was 96.8%, 97% and 96.4% at 50 μg/ml (Fig. 4.19 – 4.21). The antiproliferative effect of WT4-4 increased by 244% on HL-60 cells and 824% on K-562 cells when compared to WT4 at the dose of 50 μg/ml. The viability of WT4-4 on all these suspension cell lines reached 100% inhibition. The effect was

also much stronger in WT4-4 than that in WT4.

When the tested concentration shifted to lower range in multiple from 0.78 μg/ml to 50 μg/ml, the effect also showed in a dose dependent manner, the lowest statistically significant inhibition on proliferation of HL-60, K-562 and S-180 was found to be 0.78 μg/ml with 20.3% inhibition, 3.13 μg/ml with 10.5% inhibition and 6.25 μg/ml with 34% inhibition respectively. The IC_{50} on HL-60, K-562 and S-180 was determined to be 4 μg/ml, 6.5 μg/ml and 8 μg/ml respectively (Fig. 4.22 – 4.24). These data further indicated the more purified fraction WT4-4 with higher percentage of the active ingredient and HL-60 was the most sensitive suspension cell line.

The effects of the four fractions obtained from resin column fractionation on adhesive cell lines were similar to that in suspension cell line, WT4-1 was inactive to both MCF-7 and HepG2 while WT4-4 was the most effective antitumor fraction, but the effects were not as strong as in the suspension cell lines. The lowest effective dose was lower in MCF-7 (12.5 μg/ml) than HepG2 (25 μg/ml). But the IC_{50} of HepG2 (24 μg/ml) was lower than MCF-7 (36 μg/ml). This difference may due to the difference in sensitivity of different cell lines to the active ingredient. WT4-2 and WT4-3 could inhibit the proliferation of HepG2 in a dose dependent manner but in a

much lower percentage than WT4-4, both with the lowest significant dose at 100 μg/ml (Fig. 4.33 – 4.36).

Other than the antitumor effects, cytotoxicity on normal cells of antiutumor substance are also important, therefore the *in vitro* cytotoxicity of WT4-4 on L-02 and Vero were determined. There was no cytotoxicity effect on normal human liver cell line, L-02, up to the highest tested dose, 50 μg/ml; and on normal monkey kidney cell line, Vero, up to 6.25 μg/ml (Fig. 4.37, Fig. 4.38). This data indicated that WT4-4 did not has cytotoxic effect on L-02 up to 64 times, 16 times, 8 times, 4 times and 2 times of the lowest significant antiproliferative dose of HL-60, K-562, S-180, MCF-7 and HepG2 respectively. When IC_{50} was considered, WT4-4 did not has cytotoxic effect on L-02 up to 12.5 times, 7.7 times, 6.25 times, 1.4 times and 2.1 times of the IC_{50} of HL-60, K-562, S-180, MCF-7 and HepG2 respectively. When compared the antitumor effect of WT4-4 to the cytotoxic effect on Vero, WT4-4 did not has cytotoxic effect on Vero up to 8 times and 2 times of the lowest significant antiproliferative dose of HL-60 and K-562 respectively, and no cytotoxic effect was observed at the lowest significant antiproliferative dose of S-180. However, for MCF-7 and HepG2, there was cytotoxic effect on Vero cells at the statistically significant antiproliferative dose. The cytotoxicity results on normal cell lines

indicated that L-02 was more resistance to WT4-4 but Vero was more sensitive, this may due to the fact that difference cells with different sensitivity or due to the two cell lines come from different species, one from human (L-02) and the other from monkey (Vero). Besides, the effective doses of WT4-4 on all cell lines were not cytotoxic to the normal human cell line, L-02.

After further purification of WT4-4 by silica gel, WT4-4 A was found to be the only antitumor fraction which exhibited antiproliferative effects on HL-60, K-562 and S-180, the effects were in dose dependent manner. As expected, this more purified fraction exhibited stronger antitumor effect on the three cell lines than WT4-4. The IC_{50} on proliferation of HL-60, K-562 and S-180 were 3 μg/ml, 3.4 μg/ml and 2.8 μg/ml (Fig. 4.25 – 4.30), decreased by 25%, 48% and 65% respectively when compared to WT4-4. The inhibition on viability was also increased. These results indicated that the active ingredient on WT4-4 had been further purified and increased in WT4-4 A by the silica gel column purification, the inactive ingredients were separated form WT4-4 A to the other three fractions.

The crystal obtained from WT4-4 A was a more purified compound, and through X-ray crystallography, it was found to be an antitumor sesquiterpene lactone, with IC_{50} equal to 2.2 μg/ml on HL-60 cell proliferation (Fig. 4.32) which was 27%

lower than that of WT4-4 A. The proportion of the major peak of this crystal increased as purification proceeded from WT4-4 to the crystal finally obtained, and the antitumor effect also became stronger and stronger. Therefore, this sesquiterpene lactone ($C_{23}H_{32}O_9$) is considered as the major antitumor compound account for the antitumor effect.

Sesquiterpene lactones with same empirical formula with the crystal of this project but different stereochemistry were reported in the aerial parts of *Wedelia trilobata* (Bohlmann *et al.*, 1981) and in the leaves of *Wedelia prostata* (Ragasa and Padolina, 1993). However, none of these publications mentioned about the antitumor activity. Sesquiterpene lactone same as the crystal of this project was obtained from the aerial parts of *Wedelia prostata* (Farag *et al.*, 1996) was reported to have no inhibitory effect on the growth of cervical cancer cell line, HeLa S_3 cells.

It is difficult to compare the antitumor activities of this sesquiterpene lactone to other antitumor sesquiterpene lactones because the cancer cell lines used may be different, the incubation time may also different, the assay method may be different, moreover, the percentage of purity of the sesquiterpene lactone in this project are not exactly known. All these factors would affect the antitumor activities when comparing different compounds. However, some datum can be used for reference.

Helenalin exhibited antitumor activity with IC_{50} 0.1 μg/ml on colon carcinoma HT-29, helenine, with IC_{50} 7 μg/ml, the assay method was cell colony formation (Powis *et al.*, 1994). On human leukemia Jurkat T cells, by MTT assay method, helenalin with an IC_{50} 6 μM (Dirsch *et al.*, 2001). On lymphocytic leukemia P388, cell growth assayed by Coulter counter, (11S)-3-Oxoeudesmano-13,6α-lactone exhibited 16% cell growth inhibitory ratio at 10 μg/ml and 2α-Bromo-3-oxoeudesm-11(12)-eno-13,6α-lactone exhibited 106% cell growth inhibitory ratio at 1 μg/ml (Ando *et al.*, 1987).

5.2.2 DNA agarose gel electrophoresis

Since the viability of WT4-4 treated HL-60, K-562 and S-180 respectively decreased, they were subjected to DNA agarose gel electrophoresis to see whether the antitumor effect was mediated by apoptosis accompanying with DNA fragmentation. However, all the three WT4-4 treated cell lines at IC_{25}, IC_{50} and IC_{75} did not show the DNA laddering pattern, which is a marker of apoptosis. These results indicated that the WT4-4 did not cause DNA fragmentation and was not likely to induce apoptosis *in vitro*. Nevertheless there was exception, although DNA

fragmentation is a hallmark of apoptosis but not all apoptotic cells were accompanied with DNA fragmentation. For examples, dietary polyunsatureated fatty acids, arachidonic acid and eicosapentaenoic acid, which induce apoptosis in HL-60 cells but DNA fragmentation was not induced (Chiu *et al.*, 2000).

5.2.3 *In vivo* assay

Since WT4-4 exhibited antitumor effect in S-180 solid tumor cell lines, the *in vivo* antitumor effect on Sarcoma 180 was investigated. According to the results, WT4-4 exhibited *in vivo* antitumor effect against Sarcoma 180 in Balb/c mice from the lowest dose of 0.8 mg/kg to 3.2 mg/kg in a dose dependent manner with 33.72% and 62.79% respectively (Fig. 4.40). This positive result was consist with another *Wedelia* species, *Wedelia chineses*, the water extracts of this whole plant also exhibited *in vivo* antitumor effect in mice (江蘇新醫學院, 1977).

The *in vivo* side effect of WT4-4 was expressed as change in body weight. According to the results (Table 4.5), WT4-4 did not affect the body weight of tumor-bearing mice when compared to normal mice without tumor inoculation nor any treatment at all the tested doses. The body weight of tumor-bearing mice

received WT4-4 at 3.2 mg/kg even having significant increase in body weight when compared to the control tumor-bearing mice group which received 5% DMSO. These data indicated that WT4-4 did not confer *in vivo* side effect in body weight at those effective doses.

According to the results from *in vitro* assay and *in vivo* assay, the sesquiterpene lactone isolated in this project exhibited promising antitumor activities, but for further development, specificity of antitumor activity should be investigated in more detailed because many but not all sesquiterpene lactones were reported with different degree of cytotoxicity or allergic effect in humans (Rodriguez *et al.*, 1976). On the other hand, the solubility and effect of sesquiterpene lactones could be increased by chemical modification. A good example is arglabin-DMA, deriviative of sesquiterpene lactone arglabin with stonger antitumor activity and higher solubility, is registered as a compound with antitumor activity in the Republic of Kazakstan (Shaikenov *et al.*, 2001). The sesquiterpene lactone isolated in this project is worthy for further development.

5.3 Further Study

The antitumor effects of WT *in vitro* and *in vivo* have been studied in a great extent, but according to the results of DNA agarose gel electrophoresis, it only showed that WT4-4 did not cause DNA fragmentation on HL-60, K-562 and S-180, and the mechanism of the antitumor activity have not been fully illustrated. Therefore further study on the mechanism is needed in order to find out the pathway for the antitumor effect, for example, the change in cell cycle can be observed by DNA-PI flow cytometry, which can reveal whether the cells are arrested in any particular phase of cell cycle. Besides, bivariate annexin V/PI flow cytometry, which utilizes the characteristic of apoptotic cell other than DNA fragmentation, can reveal the change in membrane property of cells. In which, early apoptotic cells will be stained by annexin V (green) in the membrane due to the presence of phosphatidylserine (PS) but resist the penetration of propidium iodide (PI) (red). On the other hand, necrotic cells with ruptured cell membranes will be stained in both green and red.

Moreover, increase in the level of Caspase-3 can also be observed in apoptotic cells. Caspase-3 is an effector caspase, which acts like an apoptotic executor, and

induces apoptosis once is activated by different apoptotic signal (Nuñez *et al.*, 1998). Therefore, the occurrence of apoptosis can also be reflected by measuring the activation level of caspase-3, as commercially available kit can be used for the measurement of caspase-3 related protease activity.

Furthermore, western blot and immunodetection of cellular proteins, like pRb and cyclin D can also be used to investigate any changes in these protein levels in the treated cells. In which, *Rb* is known as tumor supressor gene and cyclin D is related to pRb in controlling the passage through G1 phase.

Other than hand, the cytotoxiciy of the isolated sesquiterpene lactone should also be investigated in more detail for further development.

Chapter 6

Conclusion

In this project, antitumor fractions and compound from *Wedelia trilobata* were extracted and isolated by bioactivity-guided fractionation. Firstly, by hot water extraction (WT1) and the active fraction was found to be the small molecules in supernatant fraction after ethanol precipitation (WT4). Further purification was carried out by macroporous resin column D_{101}. The fourth fraction WT4-4 with weaker polarity, was found to be the most effective one among the four eluted fractions. Lactones, coumarins, flavonoids and terpenoids were found in WT4-4 by phytochemical tests. After silica gel column fractionation of WT4-4, the most effective fraction was found to be the first fraction, WT4-4 A, with weaker polarity. After that, the more purified component sesquiterpene lactone with empirical formula $C_{23}H_{32}O_9$ was isolated form WT4-4 A. The antitumor effects of the active

fractions were in dose-dependent manner.

The *in vitro* antitumor effects increased as purification proceed. WT1 could inhibit the proliferation of HL-60 by 18.6% at the lowest dose of 50 μg/ml and K-562 by 27.3% at 100 μg/ml. WT4 could inhibit the proliferation of HL-60 by 28.1% at the lowest dose of 50 μg/ml and K-562 by 27.7% at 100 μg/ml. WT4 could inhibit the viability of HL-60 by 83.8 % at 200 μg/ml but no effect on K-562. WT4-4 could inhibit the proliferation of HL-60, K-562 and S-180 by 96.8%, 97% and 96.4% at 50 μg/ml. The lowest significant inhibition on proliferation of HL-60, K-562 and S-180 were 0.78 μg/ml with 20.3% inhibition, 3.13 μg/ml with 10.5% inhibition and 6.25 μg/ml with 34% inhibition. The IC_{50} was 4 μg/ml, 6.5 μg/ml and 8 μg/ml respectively. The inhibition on viability of the three cell lines was 100% at 50 μg/ml. The lowest effective dose in MCF-7 was 12.5 μg/ml and HepG2 was 25 μg/ml. The IC_{50} of MCF-7 was 36 μg/ml and HepG2 was 24 μg/ml. WT4-4 did not show cytotoxicity on normal human liver cell line, L-02, up to the highest tested dose, 50 μg/ml; and on normal monkey kidney cell line, Vero, up to 6.25 μg/ml. The IC_{50} of WT4-4 A on proliferation of HL-60, K-562 and S-180 were 3 μg/ml, 3.4 μg/ml and 2.8 μg/ml respectively. The isolated crystal had antitumor activity with the IC_{50} on HL-60 equal to 2.2 μg/ml.

WT4-4 also exhibited *in vivo* antitumor activity on Sarcoma 180 solid tumor. The significant doses ranging from the lowest concentration 0.8 mg/kg to 3.2 mg/kg in a dose-dependent manner with 33.72% and 62.79% respectively.

In DNA agarose gel electrophoresis of WT4-4 treated HL-60, K-562 and S-180, no DNA laddering pattern was observed, indicating that the *in vitro* antitumor activity of WT4-4 was not accompanied with DNA fragmentation in these cell lines and so apoptosis was less likely to be induced by WT4-4.

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