Study on the *In Vitro* Anti-tumor Effect of *Acanthopanax senticosus*

WAN, Chung Tin

A Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of
Master of Philosophy
in
Biochemistry

September 2008

I declare that the assignment here submitted is original except for source material explicitly acknowledged, and that the same or related material has not been previously submitted for another course. I also acknowledge that I am aware of University policy and regulations on honesty in academic work, and of the disciplinary guidelines and procedures applicable to breaches of such policy and regulations, as contained in the website.

[signature]	17-9-2008
Signature	Date
WAN CHUNG TIN	06947370
Name	Student ID
BCH 8062	Thesis Research
Course code	Course title

Thesis / Assessment Committee

Professor Kwok Tim Tak (Chair)
Professor Fung Kwok Pui (Thesis Supervisor)
Professor Kong Siu Kai (Thesis Supervisor)
Professor Leung Kwok Nam (Thesis Supervisor)
Professor Tsang Suk Ying, Faye (Committee Member)
Professor Wong Ngok Shun, Ricky (External Examiner)

Acknowledgement

I would like to express my gratitude to my supervisors, Prof. K. P. Fung, Prof. S. K. Kong and Prof. K. N. Leung for their guidance and constructive advice throughout the course of my study. I sincerely appreciate the opportunity to learn from them. And I am deeply indebted to Prof. Fung for providing all the research facilities in my pursuit of Master degree.

I would also like to give my heartfelt thanks to Dr. Grace Yue and Dr. Patrick Tang for their valuable suggestions and indispensable contributions to the project. The generous assistance they offered has helped a lot from the very beginning of the project. Nonetheless, their encouragement and support also means a lot to me.

I must also thank Franky Choi and Dr. Hon for their guidance and support in course of extraction and purification. I am grateful for their help and suggestions in the course of purification.

Besides, I would like to thank Dr. Johnny Koon, Dr. Erik Ko, Dr. David Lau, Julia Lee, Virginia Lau and Ben for their patience, caring and encouragement.

Last but not least, I have to thank for the financial support from Ming Lai Foundation and the International Association of Lions Clubs District 303 – Hong Kong and Macau Tam Wah Ching Chinese Medicine Resource Centre

Abstract

Cancer is one of the leading causes of death in modern world. Though plenty of drugs have been developed for chemotherapy, most of them pose severe side effects on the patients which make the patients even more vulnerable. Undoubtedly, there is a need to search for more effective and safe anti-cancer drugs. Traditional herbal medicine has gained attention for its long history and mild action on the body, therefore providing us with a rich source of relatively safe phytochemicals for anti-cancer drug screening.

The anti-tumor activities of *Acanthopanax* were reported previously, for instance, it can inhibit the proliferation of leukemia cell line HL-60 and stomach cancer cell line KATO III. However, the mechanism behind this anti-tumor effect remains unclear. Therefore, the anti-tumor effect of *Acanthopanax* and its mechanism of action were studied in this project.

Acanthopanax was initially extracted through the process of boiling under reflux with water for 2 hours. Its *in vitro* anti-proliferative activities on different human cancer cell lines (MCF-7, MDA-MB-231 and Hep G2) were examined by the colorimetric MTT assay. The aqueous extract of *Acanthopanax* could inhibit the growth of tested cancer cell lines in a dose-dependent manner and the IC_{50} was around 400 μg/ml. To further investigate the anti-tumor activities, the methanol extract of

Acanthopanax was partitioned and column chromatography was performed on the ethyl-acetate fraction. It was found that one of the sub-fractions (Fc) generated from column chromatography was able to induce apoptosis in the breast cancer cells, with an IC_{50} of about 42 μg/ml and 65 μg/ml towards MCF-7 and MDA-MD-231 cells respectively. Apoptotic features such as mitochondrial membrane depolarization, phosphatidyl-serine externalization and DNA fragmentation could be observed in the MDA-MB-231 cells after 48 hour treatment with Fc. By Western blotting, it was revealed that Fc could induce the intrinsic apoptotic pathway by reducing expression of Bcl-2, procaspase-9 and procaspase-3 and PARP in MDA-MB-231 cells. On the other hand, G1 phase cell cycle arrest in the MCF-7 cells was induced by Fc.

摘要

癌症乃當今致命率最高的疾病之一，縱然有多種的抗癌藥物已被廣泛應用在化療當中，惟現今大部份的抗癌藥物均會對病人造成各種嚴重的副作用，因此有需要研發更爲有效以及安全的抗癌藥物。傳統草藥擁有悠久的歷史，已經逐漸被現代科學所認同及重視，而同時成爲抗癌藥物研究的新方向，提供了許多相對安全的化合物作爲研究的目標。

近年對於刺五加的抗癌功效的研究已是略有所成，例如發現刺五加對白血病細胞 HL 60 及胃癌細胞 KATO III 的生長具有抑制作用，可是針對刺五加的抗癌作用的機理研究依然是十分有限。因此，本研讀對刺五加的抗癌功效及其背後的作用基理作出較爲深入的探討。

我們發現以回流加熱法所得的刺五加水提取物對於乳癌細胞(MCF-7 和 MDA-MB-231)及肝癌細胞(Hep G2)的生長均顯示出一定的抑制作用，其 IC_{50} 大約爲 400 μg/ml。爲了作進一步的研究，我們以萃取法從刺五加甲醇提取物中取得具有對抗癌細胞生長作用的乙酸乙酯提取物；其後又以管柱層析法從乙酸乙酯提取物中分離出有效成分 Fc。Fc 對於乳癌細胞 MCF-7 及 MDA-MB-231 的 IC_{50} 分別爲 42 μg/ml 及 65 μg/ml。其中 Fc 導致 MDA-MB-231 出現細胞凋亡現象，使其顯示出磷脂酰絲氨酸外翻、去氧核醣核酸段裂等等的細胞凋亡特徵。其後發現 Fc 能誘發內在性細胞凋亡，減低了細胞的 Bcl-2、procaspase-9、procaspase-3 和 PARP 表達。此外, Fc 亦對 MCF-7 造成細胞週期阻滯, 可見 Fc 對乳癌細胞生長顯

示出一定的抑制作用。

List of Abbreviations

AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor 1
APC	Antigen presenting cell
APS	Ammonium persulphate
AV	Annexin-V
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAD	Caspase activated deoxyribonuclease
CARD	Caspase recruitment domain
Cdk	Cyclin dependent kinase
ConA	Concanavalin A
COX	Cyclooxygenase
DcR1	Decoy death receptor 1
DD	Death domain
DED	Death receptor domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DM	Diabetes Mellitus
DMSO	Dimethylsulfoxide
EAT	Ehrlich Ascites Tumor
endoG	Endonuclease G
ER	Estrogen receptor
FADD	Fas-associating protein with death domain
FBS	Fetal bovine serum
FLIP	FLICE inhibitory protein
HCC	Hepatocellular carcinoma
ICAD	Inhibitor of caspase activated deoxyribonuclease
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
LPS	Lipopolysaccharide
MMC	Mitomycin-C
MTT	3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide
NK cells	Natural killer cells

NO	Nitric oxide
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PHA	Phytohemagglutinin
PMS	Phenazine methosulfate
PI	Propidium iodide
PS	Penicillin-Streptomycin
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
RIP	Receptor-interacting protein
Smac	Second mitochondria-derived activator of caspases
TEMED	Tetramethylethylenediamine
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TLC	Thin layer chromatography
TRAIL	TNFR apoptosis-inducing ligand
XIAP	X-chromosome linked inhibitor of apoptosis
XTT	Sodium 3′-[1- (phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate

List of Tables

List of Figures

Contents

Chapter 1 Introduction

1.1. Overview of cancer

Cancer is one the leading causes of death in the world. Despite the medical advances, the mortality of cancer remains at a high level. Cancer accounts for 13 % of all deaths and caused the death of 7.6 million people in 2005. Cancers of lung, stomach, liver, colon and breast were listed as the top five cancers in causing mortality. Though the number of deaths caused is frightening enough, there are 10.9 million of new cancer cases and 24.6 million people are still living with cancer according to figures in 2002. More importantly is that the number of deaths from cancer is projected to grow in the future. From 2007 to 2030, an estimated growth as high as 45 % is predicted in the number of global cancer deaths, meanwhile new cases of cancer are estimated to increase from 11.3 million in 2007 to 15.5 million in 2030 (http://www.who.int/cancer). Since about 40 % of cancer deaths could be prevented, cancer prevention has become one of the major concerns of cancer development. However, to deal with such a large number of cancer patients, the urge to search for effective drugs in treating cancer is highly desirable.

1.1.1 Breast cancer

Breast cancer is a leading cause of mortality in women, especially in developed countries. In the United States, an estimated number of 46,000 women will die from

breast cancer every year with over 2,000,000 new cases adding to the number of breast cancer patients (Perera and Gui, 2003). The treatment of women in estrogen-dependent breast cancer has been improved, owing to the recent advances in diagnosis and therapy. Endocrine therapy has shown to be effective in treating estrogen-dependent breast cancer through deprivation of estrogen stimulation to breast tumor cells by either reducing the local and systemic estrogen concentration or blocking the estrogen receptor (Howell and Wardley, 2005). In contrast, the treatment for estrogen-independent tumors is not satisfactory and responses of breast cancer to anti-tumor agents are not very good (Bergh et al., 2001). Combination chemotherapies gave a better survival rate compared with monotherapies, and has become a standard treatment to metastatic hormone-refractory breast cancer. However, severe side effects and the development of resistance have hindered the effectiveness of such practices (Rock and DeMichele, 2003;Hsieh et al., 2005).

1.1.2 Hepatocellular carcinoma (HCC)

HCC is listed at the third place in causing mortality among different kinds of cancers. HCC is notorious for its high lethality, with a yearly fatality ratio near to 1, implying that most patients cannot survive for more than a year (Yoshida et al., 2005). Viral infections with hepatitis B or hepatitis C is the leading cause of hepatocellular carcinoma, therefore, the control of viral infection is an important measure in

preventing HCC prevalence (Omata and Yoshida, 2004).

Surgical resection could possibly remove the entire HCC nodules. However, the option of surgery is only available to less than 20 % of HCC patients (Johnson, 2002). Even if surgical resection is possible, *de novo* carcinogenesis may still take place, probably due to the fact that the remaining liver is often cirrhotic. Therefore, the recurrence rate does not fall with time (Sakon et al., 2000).

Treatment of HCC highly depends on chemotherapy. Unfortunately, the efficacy of chemotherapy is limited by the multi-drug resistance which is common in liver cancer, and over-expression of P-glycoprotein could be found in 50 – 70 % of the patients (Grude et al., 2002). Since liver is the organ responsible for detoxification in our body, liver malignancy would impair such function and as a result of this, the side effects of chemotherapy becomes even more prominent in HCC patients (Nowak et al., 2004).

1.2. Role of natural products in the fight against cancer

Plant is a great source of natural products and provides lots of metabolites and most of them do not have any specific function in the plant and therefore termed as secondary metabolites. Throughout history, plant-based formulations have been used as medicine in treating various human and animal ailments (Singh et al., 2003) that have supplied a rich source of compounds that were applied in the field of medicine,

pharmacology as well as biology, and still playing a vital role in modern drug development. As many as 70 % of therapeutic drugs used nowadays were developed from plants (Sreenivasan et al., 2006). Many new natural compounds isolated from plants have been used as prototypes for later structural modification and give rise to compounds with practically useful pharmacological activities (Mukherjee et al., 2001;Lee, 1999;Gordaliza, 2007). Successful examples include Camptothecin and Taxol. Camptothecin was first isolated from *Camptotheca acuminata*, and was discovered to exert its anti-tumor effect by inhibiting the topoisomerase I. Two analogues of camptothecin are now applied in the treatment of ovarian, colorectal, and small cell lung cancers, and there are newer analogues still under testing in clinical trials. Taxol isolated from *Taxus brevifolia* was found to inhibit cancer cell proliferation by stabilizing the microtubules. Taxol is currently used in the treatment of breast and non-small cell lung cancers and Kaposi's sarcoma (Oberlies and Kroll, 2004). With a long history of more than a thousand years, the experience from traditional Chinese medicine can surely afford valuable information in the search for new antineoplastic drugs (Han, 1988). In the present study, we are interested in three herbs (Astragalus, Acanthopanax and Maitake) in particular.

1.2.1 Introduction of *Astragalus membranaceus*

As a tonic herb, the immunoregulatory effect of Astragalus is well-noted and

there are lots of reports regarding the action of Astragalus extract on the immune system. Astragalus extract could enhance the proliferation of murine splenocytes and human cord blood lymphocytes, and the effect is even more profound when it is co-treated with mitogens such as Lipopolysaccharide (LPS), Concanavalin A (ConA) or Phytohemagglutinin (PHA). The IL-2 receptor expression level of mouse splenocytes was also elevated after the *in vitro* treatment of Astragalus extract (Cho and Leung, 2007a). In addition, Astragalus administration also modulated other aspects of immunity as well, for instance, increasing superoxide anion production and promoting phagocytic activity of macrophages of mice, stimulating the NK cell activity of human peripheral blood lymphocytes and restoring the NK cell activity inhibited by steroid intake (2003). Astragalus could also stimulate the activity of cytotoxic T-lymphocytes and promote the TNF and IL-6 release from macrophages (Yoshida et al., 1997).

It was proposed that the polysaccharide in Astragalus was responsible for the immunostimulatory effect. Astragalus polysaccharide exhibited mitogenic effect on splenocytes and splenic B cells, and stimulated the cytokine secretion including IL-1β and TNF-α from macrophages. It was shown that the Astragalus polysaccharide would bind to the surface of macrophages and B cells and the membrane immunoglobulin may act as the binding receptor in B cell activation (Shao et al.,

2004). Another study showed that Astragalus polysaccharide could stimulate the production of NO in both *in vitro* and *in vivo* conditions. The inducible NO synthase transcription was also increased through the activation of the NF-κB/Rel (Lee and Jeon, 2005).

A vast number of studies about the effect of Astragalus on tumor cells were carried out and the results usually coincide to the same conclusion. Astragalus could modify the immune system in our body and help defending against cancer. In contrast, there were much fewer reports about the direct cytotoxicity of Astragalus towards cancer cells. A study tested the cytotoxicity of Astragalus extracts on a number of cancer cell lines including macrophage-like tumor J774 A.1, PU5-1.8, $P388D_1$, myeloid tumors HL-60, Ml and WEHI-3, T-cell lymphoma MBL-2 and YAC-1, mineral oil-induced plasmacytoma NS-1, fibroblast-like tumor L929 and WEHI-164 and Ehrlich ascites tumor EAT (Cho and Leung, 2007b). However, none of them shown significant decrease in cell viability after treating with Astragalus extracts although cytostatic effect could be observed, especially for macrophage-like tumor and myeloid tumor cells. On the other hand, Astragalus extract demonstrated anti-tumor activities when it comes to an *in vivo* environment. A significant reduction in number of viable tumor cells recovered from the peritoneal cavity of the mice after Astragalus administration was noted. Astragalus administration also inhibited the

growth of EAT in tumor-bearing mice (Cho and Leung, 2007b). Immunopotentiating effect could also be found in the same set of experiments. Administration of Astragalus extracts could result in the influx of macrophages into the peritoneal cavity and activating the phagocytic activity of peritoneal exudate cells *in vivo* while the *in vitro* treatment of macrophages with Astragalus extract led to increased cytostatic activity towards MBL-2 tumor. Injection of Astragalus extract together with LPS led to a much profound increase in the level of TNF-α in tumor-bearing mice when compared with that of LPS-injection alone (Cho and Leung, 2007b). Therefore, it is very likely that the anti-tumor effect *in vivo* of Astragalus was mediated through its immunoregulatory effect.

Nevertheless, a recent report proposed that the total saponins from Astragalus could induce apoptosis and G2/M phase cell cycle arrest in human colon cancer cells HT-29 and demonstrated significant *in vivo* inhibition of tumor growth in nude mice xenograft, without lethal effect and did not cause significant drop in the body weight of the mice (Tin et al., 2007). In addition, it was reported that Astragalus extract could induce apoptosis in erythroleukemia HEL cells through stimulating apoptosome formation and activating caspase-3 (Cheng et al., 2004). Therefore, the anti-tumor effect of Astragalus may be partly contributed by its ability to induce apoptosis of malignant cells and it seems that whether Astragalus extract would induce apoptosis

in tumor cells depends on the cell line tested and the extraction method employed.

Some reports have suggested the possibility of using Astragalus as an adjunct therapy, for example, a ten-fold potentiation of cytotoxicity of recombinant IL-2 generated lymphokine-activated killer cells against murine renal carcinoma could be induced by Astragalus extract (Wang et al., 1992). Intravenous injection of Astragalus to tumor-bearing mice could restore the blastogenic response of splenic lymphocytes. On the other hand, intraperitoneal (i.p.) injection of Astragalus in mice could also reduce the immunosuppressive effect caused by cyclophosphamide administration (Cho and Leung, 2007a). A clinical study involving 120 tumor patients in China demonstrated that the immuno-stimulating effect of Astragalus is still valid in patients undergoing chemotherapy. The IgG and IgM levels were increased in Astragalus-treated patients. More importantly, the side effects caused by chemotherapy such as diarrhea, vomiting and proteinuria were lowered after Astragalus treatment when compared with that of the cancer patients with chemotherapy only (Duan and Wang, 2002). This result shows that Astragalus may help protecting our body during chemotherapy. In another randomized controlled clinical trial, Astragalus i.p. injection in combination with platinum-based chemotherapy has been shown to significantly reduce the risk of death at 12 months, compared with platinum-based chemotherapy alone (Zou and Liu, 2003). This result

shows that Astragalus may increase the effectiveness of treatment when used in combination with chemotherapy (McCulloch et al., 2006).

In addition to the activities mentioned before, other biological activities of Astragalus were also reported. For instance, it was found that the saponins in Astragalus could inhibit the formation of lipid peroxides in the myocardium and decrease blood coagulation at the same time (2003). It was shown that Astragalus had an antioxidant effect in 43 patients suffering from acute myocardial infarction. The red blood cell superoxide dismutase activity was elevated and the lipid peroxidation content in the plasma was reduced as a result of Astragalus oral consumption (Chen et al., 1995).

1.2.2 Introduction of *Acanthopanax senticosus*

The reports about biological activities of Acanthopanax are versatile. It is regarded as an adaptogen which helps the body to cope with the effects caused by stress, anxiety, trauma and fatigue, and helps our body to maintain its best homeostatic state. It has been used to treat many kinds of stress-induced physiological changes as it was shown that Acanthopanax could modulate our body response to stress (Boon-Niermeijer et al., 2000), for instance it demonstrated protective effect of gastric ulcer on cold water-stressed mice (Fujikawa et al., 1996). This effect may be related to the ability of Acanthopanax to regulate the level of biogenic monoamines

including noradrenaline and dopamine in the brain (Fujikawa et al., 2002). Moreover, there are investigations showing that Acanthopanax could be used potentially in treating Diabetes Mellitus (DM), due to its antioxidant and anti-glycation activities (Lee et al., 2004a;Xi et al., 2008) or improving insulin resistance (Liu et al., 2005). The anti-hyperglycemic effect of Acanthopanax also attributed to the potential use in DM-related area (Park et al., 2006). Acanthopanax extract also exhibited anti-fatigue effect (Deyama et al., 2001), such as prolonging the time to exhaustion in chronic stressed mice (Nishibe et al., 1990) or increasing the exhaustion time of treadmill running and reduced the exercise-induced 5-hydroxytryptamine (serotonin) synthesis and tryptophan hydroxylase expression in the dorsal raphe (Rhim et al., 2007). Other activities such as neuroprotective effects (Fujikawa et al., 2005b;Fujikawa et al., 2005a;Jang et al., 2003;Bu et al., 2005) and effect on myocardial ischemia-reperfusion (Sui et al., 1994) were also noted.

Acanthopanax also possesses regulatory effect on the immune system. There are some reports about its anti-inflammatory activities, such as inhibiting nitric oxide synthase activity in RAW 264.7 macrophages (Lin et al., 2008b) and reducing the NO production in murine macrophages (Lin et al., 2007). The administration of Acanthopanax extract could also reduce the LPS-induced production of PGE_2, TNF-α and lower the COX-2 enzyme expression (Jung et al., 2003). Superoxide anion

production and hydrogen peroxide production from mouse peritoneal macrophages were suppressed by Acanthopanax extract treatment (Lin et al., 2008a) and the activities of antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase were also elevated after oral intake of Acanthopanax extract (Lee et al., 2004b). It was also reported that the polysaccharides from Acanthopanax could stimulate the proliferation and differentiation of B cells and the cytokines release from macrophages and it was proposed that the Acanthopanax polysaccharide may act through binding to the toll-like receptors on B cells and macrophages (Han et al., 2003).

There are some reports about the anti-tumor activities of Acanthopanax but the mechanisms of action described vary from one to another. A study in Japan investigated the effect of hot water extract of stem bark of Acanthopanax against stomach cancer. Their results showed that the Acanthopanax extract could inhibit the growth of KATO III cell line and induce apoptosis of it. The study also mentioned that one of its components, sesamin was responsible for the apoptosis inducing activity (Hibasami et al., 2000a). On the other hand, the anti-tumor effect of Acanthopanax was studied more extensively in mainland China. The polysaccharides from Acanthopanax were shown to be able to inhibit the growth of leukemia cell line K562 and murine sarcoma cells S180 in *in vitro* environment (Tong et al., 1994). It was

reported that Acanthopanax water extract was effective in inhibiting the growth of leukemia cells *in vitro*. Gel filtration column chromatography was applied to isolate the active constituents from the water extract. It was found that the anti-proliferative activity was associated with a protein component F2 and its size was about 64 kDa which demonstrated anti-proliferative effect on leukemia cell lines such as Raji and HL-60. After oral administration of F2 for ten days, the tumor size in tumor-bearing mice was significantly reduced compared to control group and the survival period of the tumor bearing mice was also prolonged after oral intake of F2 (Shan et al., 2004). Another *in vivo* study revealed that daily peritoneal injection of Acanthopanax extract could inhibit the growth lung cancer cells Lewis. Co-treatment of cyclophosphamide with Acanthopanax injection also demonstrated stronger anti-proliferative effects towards Lewis cells growth when compared with single cyclophosphamide treatment (Zhang et al., 2004). These studies showed that Acanthopanax extract was not only effective in inhibiting *in vitro* cancer cell growth, the tumor development in animal models was also repressed by Acanthopanax extracts.

In addition, there are also reports about the anti-metastatic activity of Acanthopanax extract. Intra venous injection of Acanthopanax water extract GF100 for 2 days before tumor cells inoculation was shown to be effective in lowering lung metastasis of colon tumor cell line colon 26-M3.1. Cytokine secretions such as IL-1β,

IL-12, TNF-α and IFN-γ were elevated and both macrophages and natural killer cells were activated upon the GF100 injection. The anti-metastatic activity could be abolished by removing NK cells in the *in vivo* model suggested that this anti-metastatic activity may be contributed by the modulating the immunity of the mice (Yoon et al., 2004).

A clinical study involving 73 lung cancer patients was carried out in China. The results suggested that daily Acanthopanax extract injection could raise the level of, IgA, IgG, IgM and NK cell activities while decreasing the TNF-α and TNF-β level after the treatment and the levels of IgA, IgG, IgM, NK cell activities, TNF-α and TNF-β in Acanthopanax treated patients were restored to levels closer to that of non-cancerous subjects (Huang et al., 2005). This study hinted the possibility that Acanthopanax may be useful as anti-cancer drug in clinical setting too.

1.2.3 Introduction of *Grifola frondosa*

Researches about Maitake mainly focus on the effect of its polysaccharide extract. Intraperitoneal injection of Maitake polysaccharide extract demonstrated inhibitory effect on the growth of sarcoma cells S180 in tumors and syngeneic systems like Meth A fibrosarcoma and MM46 carcinoma cells (Suzuki et al., 1984).

A (1→3)-branched (1→6)-β-glucan fraction termed as D-fraction isolated from the fruiting body of Grifola could act as biological response modifier and activated

the immunity of our body. D-fraction could be prepared from Maitake fruiting body by boiling with water followed by ethanol precipitation and DEAE ion-exchange chromatography. Immune cells such as macrophages, helper and cytotoxic T cells would be activated by the treatment with D-fraction. Oral administration of D-fraction could reduce tumor size in mice without causing any severe adverse effect (Hishida et al., 1988). It was shown that the co-treatment of D-fraction with Mitomycin-C (MMC) in tumor-bearing mice not only could reduce the immunosuppression caused by MMC, the anti-tumor efficacy was also higher than that of MMC treatment alone. The treatment of D-fraction together with MMC elicited a T-helper-1 dominant response by inducing the IL-12 release by macrophages which would be suppressed by MMC treatment alone (Kodama et al., 2005b). Another *in vivo* study revealed that i.p. injection of D-fraction in MM-46 tumor bearing mice could inhibit the growth of tumor. The intracellular TNF-α expression of splenic NK cells as well as the amount of TNF-α and IFN-γ released was elevated. IL-12, which plays a crucial role in stimulating NK cell activity, was prompted to release from both RAW 264.7 cells and peritoneal macrophages obtained from normal mice after the treatment with D-fraction. It was proposed that the tumor suppressing effect of D-fraction was contributed by the activation of NK cells (Kodama et al., 2002). The NK cell cytotoxicity towards YAC-1 cells and expression of CD223 on the surface of NK cells

was enhanced after D-fraction treatment. The detected IL-12 level in serum was elevated together with increased expression of IL-12 betaI receptor on NK cells surface. It is suggested that the activation of macrophages and increased level of IL-12 were associated with the NK cells stimulatory effect of D-fraction (Kodama et al., 2005a). It was shown in another study that D-fraction activated the T cells, especially CD4+ helper T cells rather than B cells in the lymph nodes of tumor-bearing mice. Release of Th-1 cytokines such as IL-12 , IL-18 and IFN-γ in spleen and lymph node were stimulated after D-fraction treatment, while Th-2 cytokine production such as IL-1β and IL-4 were inhibited. This indicated that the anti-tumor activities of D-fraction may be resulted from the establishment of Th-1 dominance immune response, which activates the cell-mediated immunity and cytotoxic T cells against the tumor cells (Inoue et al., 2002).

Despite the fact that most of the researches concluded the activation of immunity as the cause of anti-tumor effect of Maitake, other reports revealed that direct cytotoxicity may also take part in the anti-tumor effect of Maitake in some cases. For example, a novel polysaccharide-peptide GFPS1b isolated from Maitake was reported to induce apoptosis in a gastric carcinoma cell line SGC-7901. Many hallmarks of apoptosis such as phosphatidylserine externalization, DNA fragmentation, nuclear condensation, activation of caspase-3 activity could be observed after the treatment

with the polysaccharide-peptide. Bcl-2 expression was suppressed while the Bax pro-apoptotic protein expression was elevated in a dose-dependent manner after the treatment. It is believed that GFPS1b could elicit apoptotic response in the gastric carcinoma cells (Cui et al., 2007). Ethyl-acetate extract from the supernatant of the cultured broth from submerged Grifola culture exhibited cytotoxic activities towards hepatoma cells including Hep G2 and Hep 3B and cervical cancer HeLa cells. Further fractionation and purification indicated that o-Orsellinaldehyde as the active component. DNA laddering was observed and obvious sub-G1 peak was formed in cell cycle analysis assay suggested that the tumoricidal effect of o-Orsellinaldehyde could be mediated through the induction of apoptosis (Lin and Liu, 2006). However, these reports only comprise the minority when compared with the vast number of investigations about the immune mediated anti-tumor effect.

In addition, it was reported that the MD-fraction of Maitake exhibited anti-metastatic activity in an experimental murine model of lung metastasis. The NK cells activity was potentiated and IL-12 production from antigen presenting cells (APC) were also enhanced at the same time. B16/BL6 cell adhesion to LPS-activated murine lung vascular endothelial cells was inhibited by MD-Fraction. Therefore, it was proposed that MD-fraction would inhibit metastasis by the enhancement of NK cells and APC activity and suppressing the ICAM-1 so that the adhesion of tumor

cells to the vascular endothelial cells was hindered (Masuda et al., 2008).

1.3. Apoptosis and Cancer

Apoptosis or Programmed Cell Death is a way in which a cell destroys itself in a controlled process by eliminating vital biomolecules while maintaining cellular integrity. Apoptosis actually is a normal phenomenon in our body, which has its own physiological role. For example, it is an essential process in the development of multicellular organism, such as eliminating the tail of tadpole and removing the interdigital web of primate embryo (Meier et al., 2000). Apoptosis is also associated with the termination of unwanted cells in the immune system (Krammer, 2000). In addition, apoptosis acts as a natural ending of terminal differentiation process of continuously renewable cells in the epithelial surfaces and in the hematopoietic system (Chaturvedi et al., 1999). Other than a physiological process, apoptosis also acts as a self-destruction pathway for cells with severe and irreversible damage to its genome. This enables the conservation of the genome and prevents the accumulation of damaged genetic material which could be dangerous to the life of the organism (Miele, 2003).

Apoptosis has drawn the attention of scientists in cancer research recently. Cancers are disease of un-regulated clonal expansion of cells which results in the invasion of normal tissue finally. The force behind cancer development is the

mutations in genes that govern and control the proliferation of cells. The mutations in such genes empower the cells to have some propagation advantages over the others, and as a result, the outgrowth of cells with such advantages will be fostered. Therefore, the genesis and progress of cancer is actually evolutionary in nature and is the result of natural selection of cells with such propagative advantages over the others (Evan and Vousden, 2001). However, it seems that our body is aware of the danger caused by unlimited clonal expansion of cells and has a defense system against such unlimited proliferation. The proliferation of cells is guarded and restrained by the action of apoptosis. And most of the cancer has been associated with errors in the apoptotic machinery which allows the propagation of cells with altered genetic material despite of the genomic instability, such as the overexpression of anti-apoptotic Bcl-2 family member (Schmitt and Lowe, 2001). The proliferation of transformed cells leads to the accumulation of mutations which results in the development of cancer eventually.

In fact, the majority of non-surgical cancer treatment nowadays targeted at inducing a residual apoptotic response in the cancer cells. Radiotherapy would damage the DNA content of the cancerous tissues and lead to apoptosis. Chemotherapy aims at destroying or interfering the DNA or removing critical survival signals. Anti-angiogenic approach inhibits the blood vessels proliferation near tumor

and limits the blood supply hence the nutrients to the cancer cells and trigger apoptosis (Miele, 2003). A number of anti-neoplastic agents induce the synthesis of Fas ligand, such as doxorubicin, methotrexate, cisplatin, and bleomycin by activating the NF-κB pathway (Muller et al., 1997). Treatment of solid tumor with cytotoxic drugs such as cytarabine, 5-fluorouracil (5FU), fludarabine, cyclophosphamide, etoposide, and camptothecin also characterized with apoptotic features (Mesner, Jr. et al., 1997). Some common anti-cancer drugs that are used in chemotherapy clinically, like Paclitaxel or Docetaxel and Vincristine, would also induce apoptosis of the cancer cells (Kaufmann and Earnshaw, 2000).

In general, the cells in apoptosis could be characterized by a number of morphological features, for instance, cell shrinkage, membrane blebbing, chromatin condensation, formation of apoptotic bodies etc. These morphological changes represent a later stage of apoptosis which are the results of a series of biochemical events in the apoptotic cells. In other words, a number of distinctive events could also be observed at the molecular level (Saraste and Pulkki, 2000). Each normal cell has a set of genes, and therefore proteins for regulating the proliferation or committing to the cell suicide process. These proteins form the apoptotic machinery altogether, taking care of the self-destruction process step by step. The components of the apoptotic network are genetically encoded and considered to be usually in place in a

nucleated cell ready to be activated by a death inducing stimulus (Weil et al., 1996;Ishizaki et al., 1995). Basically, apoptosis could be sub-divided into two pathways: intrinsic and extrinsic. Although these two pathways are activated in different ways, they unite at certain point and lead to the same result: the death of the cell.

1.3.1 Caspases

Caspases are a group of enzymes which play a vital role in apoptosis. They participate in the signal transduction process as well as the execution of the apoptotic pathway. The word 'caspase' is derived from cysteine dependent aspartate protease. A highly conserved peptide sequence QACRG could be found in the active site of the caspases and the cysteine residue of the active site plays an important role in contributing to the proteolytic activities of caspases. Caspases are synthesized as inactive zymogens, which are called procaspases, with a very low basal level activity. Under certain appropriate condition, these procaspases could be self activated and then activate the procaspases downstream. Prodomain could be found in the N-terminal of these procaspases, followed by a large and small subunit (Earnshaw et al., 1999). The prodomain itself may serve a function as well, especially for recruitment purpose, such as the Dead Receptor Domain (DED) of procaspase-8 (Yao et al., 2007) or the Caspase Recruitment Domain (CARD) in the case of procaspase-9

(Bratton et al., 2001) . The prodomain is usually removed during the activation process and forms an active caspase which is a heterodimer consists of two large and two small subunits. Caspases could be further sub-categorized into effectors (caspase-3, caspase-6 and caspase-7) which are responsible for the massive proteolytic events or initiators (caspase-8, caspase-10, caspase-9 and caspase-2) which are responsible for signal transduction (Perl et al., 2005). The initiators are capable of autocatalytic activation while the executioners needed to be activated by the initiators and the interactions between those play an important role in the caspase-dependent apoptotic pathway (Gewies, 2003).

1.3.2 Intrinsic apoptotic pathway

Mitochondria play a central role in the intrinsic pathway in coordinating and propagating the various death signals arose within the cells. As response to different kinds of cellular stresses such as radiation, hypoxia, deprivation of nutrients, the transcription and expression of a number of apoptotic regulatory signals is attenuated, such as the anti-apoptotic proteins Bcl-2 and Bcl-X_L and pro-apoptotic protein Bax and Bak in the Bcl-2 family (Newmeyer and Ferguson-Miller, 2003). The pro-apoptotic proteins are involved in facilitating the opening of the voltage dependent ion channel in the outer membrane of mitochondria which leads to the depolarization of mitochondrial membrane (Tsujimoto and Shimizu, 2000). A sudden

increase in permeability to solute and the influx of water to mitochondria results in the rupture of outer mitochondrial membrane and leads to the release of cytochrome c, which is originally bound to the outer leaflet of inner mitochondrial membrane (Bernardi et al., 1999;Loeffler and Kroemer, 2000). Once released into the cytosol, cytochrome c would associate with Apaf-1 (Zou et al., 1997) to form a heptameric wheel-like structure known as apoptosome which would recruit procaspase-9 through its CARD domain and results in auto-activation of caspase-9 (Li et al., 1997;Acehan et al., 2002). As a result of caspase-9 activation, the caspase-3 would be activated and apoptosis would be executed. In addition to cytochrome c, other death signals would also be released as a result of mitochondrial permeability transition, including apoptosis inducing factor (AIF), endonuclease G (endoG) and Smac/DIABLO. AIF was associated with caspase-independent programmed cell death (Daugas et al., 2000). EndoG has been reported to induce CAD-independent internucleosomal DNA fragmentation (Schafer et al., 2004). Smac/DIABLO would bind to X-chromosome linked inhibitor of apoptosis (XIAP) and release caspases-3, -9 and -7 from its inhibition (Verhagen et al., 2000;Ekert et al., 2001).

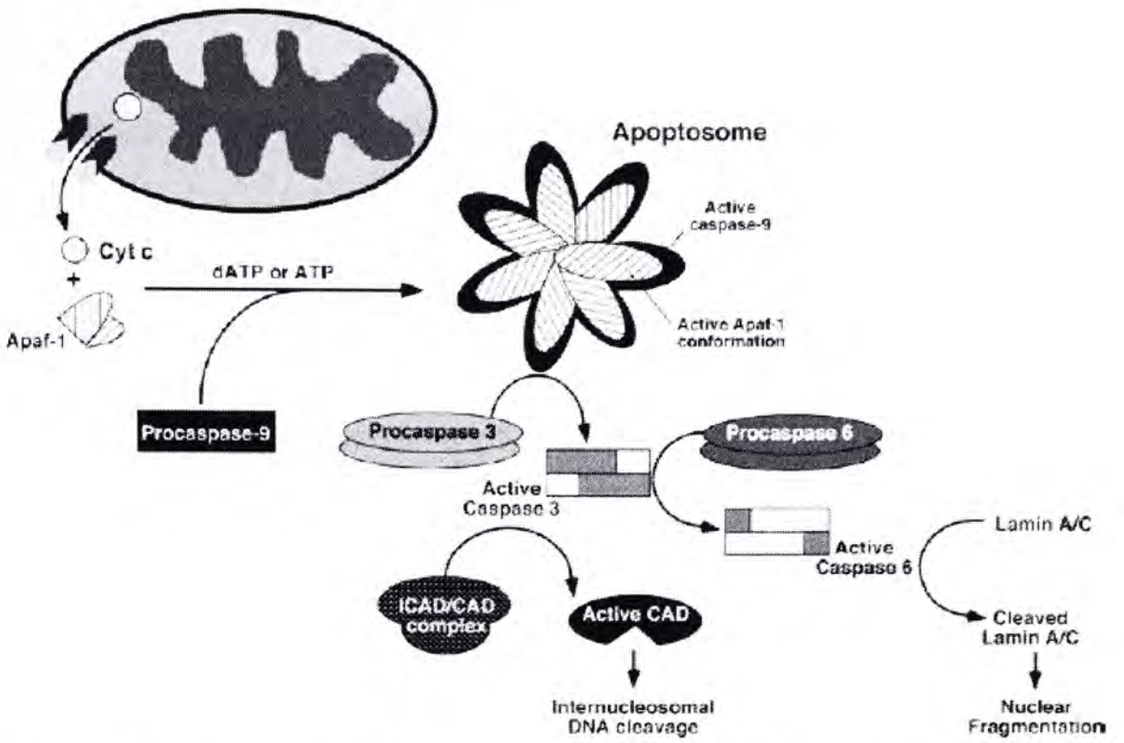


Fig.1.1 Schematic representation of intrinsic pathway of apoptosis. (adapted from Kaufmann, (2007))

1.3.3 Extrinsic apoptotic pathway

The extrinsic apoptotic pathway is initiated by the death receptors which are cell surface receptors and is activated upon the binding with specific extracellular death inducing signals like Tumor Necrosis Factor (TNF), Fas ligand and TNFR apoptosis-inducing ligand (TRAIL). The death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95 and the TRAIL receptor DR-4 and DR-5 (Ashkenazi, 2002). The cysteine rich extracellular domain of TNFR enables them to recognize and to bind with their specific ligands (Naismith and Sprang, 1998). Once Fas or TRAIL receptor is bound to its ligand, conformational change and oligomerization of death receptors take place and then

Fas-associating protein with death domain (FADD) in the cytoplasm will be recruited to the death domain (DD) of the cytoplasmic region of the death receptor and form a complex known as death inducing signaling complex (DISC). The DED domain in the adaptor FADD in turns recruits the DED-domain containing procaspase-8 through DED-DED interaction and sequesters procaspase-8 to the DISC. The localization of several procaspase-8 results in the autocatalytic activation and the formation of active caspase-8 (Budihardjo et al., 1999). If sufficient amount DISC could be formed, the caspase-8 may directly activate downstream caspase cascade, and this is called the type-I cells (Scaffidi et al., 1998), which do not require the manipulation of mitochondria. In type-II cells, the activation of the death receptor activation is not strong enough, and only a small amount of DISC is formed. As a result, the capase-8 activation does not create a cascade signal that is strong enough for the initiation of apoptosis on its own. The apoptotic signal is therefore amplified with the help from the mitochondria. A Bcl-2 family member Bid links the caspase signal with the mitochondrial pathway together. The caspase-8 cleaves Bid to its truncated form tBid. tBid could translocate into the mitochondrial and interact with other pro-apoptotic proteins Bax and Bak and results in the release of cytochrome c, Smac/DIABLO to cytosol and activate the intrinsic apoptotic pathway(Luo et al., 1998;Holdenrieder and Stieber, 2004). On the other hand, TNFR once activated will bind TNF-receptor

associated death domain (TRADD) instead (Hsu et al., 1995). The DD in TRADD will recruit other adaptor proteins such as receptor-interacting protein (RIP) and TNFR-associated factor II (Hsu et al., 1996). This complex will then associate with FADD through DD-interaction and bind with procaspase-8. However, the activation of TNFR would also free the NF-κB from its inhibitor and allows its translocation into the nucleus and results in the transcription of a series of mostly anti-apoptotic genes, including FLICE inhibitory protein (FLIP) (Kucharczak et al., 2003). The activation of TNFR would lead to apoptosis only if the transcribed survival signals are overwhelmed by the apoptotic response. As a tightly regulated process, other factors could modulate the cellular response to the death signal. For example, the presence of DcR1 (decoy death receptor 1), DcR2, DcR3, which are not armed with functional dead domain, could compete for the death signals (Meng et al., 2000). They act as the ligand sink and prevent the activation of death receptors. Similar regulation could be seen in the procaspase-8 activation. c-FLIP is a homologue of procaspase-8 but without catalytic activity, the presence of c-FLIP interferes and limit the activation of procaspase-8 (Kataoka, 2005).

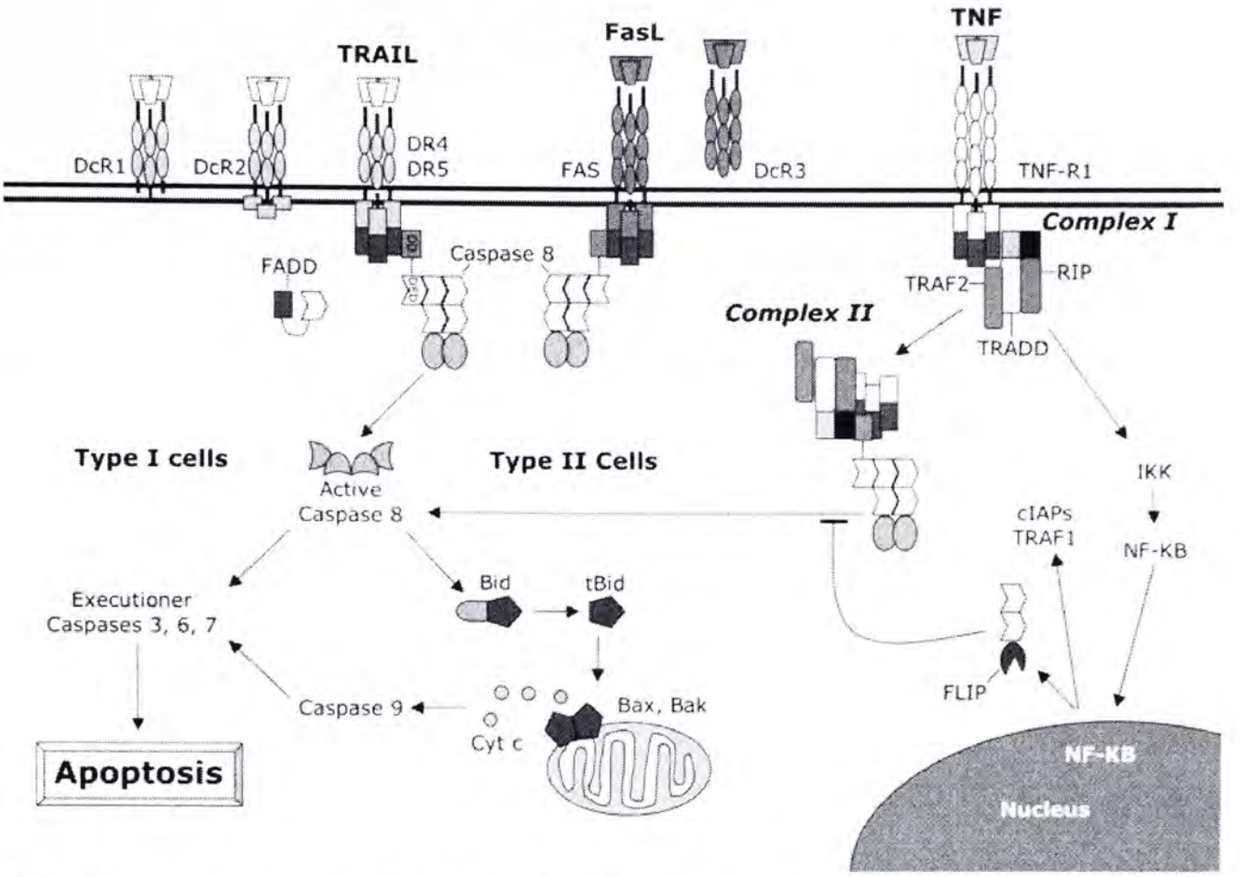


Fig. 1.2 Schematic representation of extrinsic pathway of apoptosis (Ricci and El Diery, (2007)

1.3.4 Execution of Apoptosis

Both intrinsic and extrinsic pathways lead to the activation of the effector caspases, and result in the observable changes in apoptotic cells. Activated caspase-3 cleaves the actin filament severing enzyme gelsolin and contributes to cytoplasmic blebbing (Kothakota et al., 1997). In addition, caspase-3, together with caspase-6, cleaves a series of intermediate filament proteins that are important in maintaining normal cell shape, including lamin A, lamin B, nuclear/mitotic apparatus protein NuMA and cytokeratin (Fischer et al., 2003;Ruchaud et al., 2002). Other than that, caspase-3 also targets on ICAD (inhibitor of caspase-activated deoxyribonuclease) therefore releases CAD which is responsible for digesting the DNA and brings about

the hallmark internucleosomal DNA fragmentation (Wyllie, 1980). The proteolytic activity of caspase also activates a number of kinases, including protein kinase Cδ (Emoto et al., 1995), which in turn activates a phospholipid scramblase catalyzing the transfer of phosphatidylserine (PS) from the inner to outer leaflet of plasma membrane (Frasch et al., 2000) and bring about another hallmark of apoptosis, PS externalization. PS externalization has its physiological role in our body, acting as the signal for macrophage phagocytosis for apoptotic cells (Fadok et al., 2000).

1.4 Cell cycle and cancer

Apoptosis ends the cells which undergo uncontrolled proliferation and expansion as a result of accumulated mutations in the genome. However, if apoptosis is the answer to mutation in every cell, then a large number of cells would have been killed and that would be massacre like. In fact, DNA in every cell is under constant attack and susceptible to damage. For instance, free oxygen radicals from respiration could lead to base damage and DNA breakage. External source like sunlight or ionizing radiation would also lead to DNA damage. To cope with such damaging stress, our body has a system of DNA process although it is not perfect. To allow time for DNA repair, cells respond to DNA damage by pausing the cell cycle progression during the cell cycle checkpoint. Cell cycle checkpoint is the signal transduction pathway that delays cell cycle progression or induces cell cycle arrest to ensure that the previous

process, such as DNA replication or mitosis is completed (Hartwell and Weinert, 1989). If the damage in DNA is too severe to be repaired, then apoptosis would take place to eliminate the cells with altered genetic information (Dupre and Gautier, 2007).

In every phase of cell cycle, there exists cell cycle checkpoints like G1/S checkpoint, intra-S-phase checkpoints or G2/M checkpoint. One of the common features of these checkpoints is that the transcription or the activity of cyclin dependent kinases (cdk) would be affected. Cdks are enzymes that play a crucial role in regulating cell cycle progression by forming complexes periodically with proteins known as cyclins. The periodic expression of cyclins defines the start and transition to succeeding cell cycle phases (Morgan, 1997). For instance, cdk 4 and cdk 6 together with their D-type cyclin partners are needed for progression through G1 while cdk 2 and cyclin E complex is required for G1 to S-phase transition. Progression through S phase needed the action of cdk 2 / cyclin A complex while cdk 1 and cyclin B is necessary for the occurrence of mitosis (Sherr, 1996).

Under the control of cell cycle checkpoints, cells respond frequently by arrest the cell cycle progression or die in response to DNA damage and the risk of cancer development will be lowered. However, mutations in the signal transduction pathway of cell cycle checkpoints would allow the survival or progression of cells with

genomic abnormalities and the likelihood of malignancies development will be much more enhanced (Kastan and Bartek, 2004). Therefore, if a compound could inhibit the activity of cdk or alter the expression of cyclins in tumor cells, the cell cycle may once again halted and stop the cancer development. Specific inhibitors of cdk, such as inhibitors of cdk 4 or cdk 6 could block the cells from progressing into S phase, as a result promoting cell cycle arrest or even lead to apoptosis in cancer cells (Weinberg, 1995).

1.5 Objective of this project:

The present study aims at investigating the anti-tumor effect of Acanthopanax on the human breast cancer cell lines MCF-7 and MDA-MB-231, and human hepatoma Hep G2 cells. The active fraction responsible for such activities will be studied and the mechanism behind the anti-tumor effect will also be addressed.

Chapter 2 Materials and Methods

2.1Materials

2.1.1. Culture medium

Fetal bovine serum (FBS) and Penicillin-Streptomycin (PS)

FBS and PS were purchased from GIBCO Invitrogen (Grand Island, NY, USA). The FBS was subjected to heat inactivation by incubating at 56 °C for 30 minutes before use. FBS and PS were stored at –20 °C.

RPMI 1640

RPMI (Rosewell Park Memorial Institute) 1640 powder containing phenol red, L-glutamine and 0.5 mM HEPES were purchased from GIBCO Invitrogen (Grand Island, NY, USA). 16.4 g of RPMI 1640 powder and 2 g of sodium bicarbonate were dissolved into 1 L of distilled water. The pH of the medium was adjusted to 7.2 with the addition of NaOH or HCl while stirring. Membrane filtration was performed with the use of 0.22 μm cellulose acetate membrane bottle-top filter and the filtered medium was stored at 4 °C.

DMEM

DMEM (Dulbecco's Modified Eagle's Medium) powder containing phenol red, D-glucose, L-glutamine and sodium pyruvate were purchased from GIBCO Invitrogen (Grand Island, NY, USA). 13.4 g of DMEM powder and 3.7 g of sodium

bicarbonate were dissolved in 1 ml of distilled water. The pH was adjusted to 7.2 with the addition of HCl or NaOH while stirring. Membrane filtration was performed with the use of 0.22 μm cellulose acetate membrane bottle-top filter and the filtered medium was stored at 4°C.

2.1.2 Buffers and reagents

MTT

MTT was purchased from Sigma (St. Louis, MO, USA). MTT solution was prepared by dissolving 50 mg MTT powder in 5 ml PBS.

XTT

XTT and PMS (Phenazine methosulfate) was purchased from Sigma (St. Louis, MO, USA). XTT solution was prepared by dissolving 15 mg of XTT powder into 50 ml of PBS. XTT solutions were kept in darkness in 4 °C while PMS was kept at darkness in -20 °C.

PBS

The composition of PBS (Phosphate-buffered Saline) was 2.7 mM KCl, 1.5 mM KH_2PO_4, 136 mM NaCl and 8 mM Na_2PO_4. The pH was adjusted to 7.4 and sterilized by autoclave and then stored at room temperature.

Flow cytometry

JC-1 and propidium-iodide were prepared by dissolving in DMSO and were stored at

-20 °C. These stock solutions are at least 1000 times more concentrated than the working concentration so that the influence of DMSO on the assay should be negligible.

Propidium-iodide (PI) was purchased from Sigma (St. Louis, MO, USA). PI powder was dissolved in distilled water at a concentration of 2 mg/ml for cell cycle analysis or at a concentration of 1 mg/ml for Annexin-V-PI labeling.

Annexin-V-FITC staining solution was purchased from BD Biosciences Pharmingen.

AV-PI staining buffer was prepared by 10 mM HEPES, 140 mM NaCl and 5 mM $CaCl_2$ and the pH was adjusted to 7.4.

DNA fragmentation

Reagents and chemicals being used in the DNA fragmentation experiment were listed in the Table 2.1 below. DNA 6 X loading dye and 100 base pair DNA marker was purchased from Fermentas (EU). Agarose was purchased from United States Biochemical Corporation (Cleveland, OH, USA).

Table 2.1 Reagents and buffers used in DNA fragmentation

Reagent / Buffer	Constituents and Concentration	Storage
DNA lysis buffer	50 mM Tris-HCl 20 mM EDTA 3 % (v/v) IGEPAL CA-630 ((Octylphenoxy)polyethoxyethanol) pH adjusted to 7.5	Room temperature
RNase A	10 mg/ml dissolved in dH2O	-20 °C
Proteinase K	20 mg/ml dissolved in dH2O	-20 °C

TE buffer	10 mM Tris-HCl 0.1 mM EDTA pH adjusted to 7.5	Room temperature
50X Tris-Acetate-EDTA (TAE) buffer, 1 L	2 M Tris base 57.1 ml Glacial Acid 0.5 M EDTA pH adjusted to 8.0	Room temperature

Western blotting

Reagents and buffers used in Western blotting experiment were listed in the Table 2.2 below. The antibodies against Bcl-2, procaspase-3 and PARP were purchased from Santa Cruz Biotechnology, Inc. Antibodies against procaspase-9 and caspase-9 were purchased from StressGene Biotechnology Corp. while the antibodies for cdk 2, Cyclin D_1 and β-actin were purchased from Cell Signaling Technology, Abcam plc and Sigma respectively.

Table 2.2 Reagents and buffers used in Western blotting

Reagent / Buffer	Constituents and Concentration	Storage
Total protein extraction lysis buffer	0.0625 M Tris-HCl 2 % (w/v) SDS 10 % Glycerol pH adjusted to 6.8	4 °C
4 X Separating gel buffer (500 ml)	90.8 g Tris base 2 g SDS pH adjusted to 8.8	4 °C
4 X Stacking gel buffer (500 ml)	30.3 g Tris base 2 g SDS pH adjusted to 6.8	4 °C
10 X SDS running buffer (1 L)	30.3 g Tris base 144 g Glycine 10 g SDS	Room temperature
10 X Tris-Buffered Saline	12.11 g Tris base	Room temperature

(TBS), (1 L)	87.66 g NaCl pH adjusted to 8.0	
TBS-T	1 X TBS 1 ml Tween-20	Room temperature
10 X Tris-glycine	30.3 g Tris base 144 g Glycine pH adjusted to 8.3	Room temperature
Electroblotting buffer (1L)	5.82 g of Tris base 2.93 g of glycine 1.875 ml of 20% SDS 200 ml of methanol pH adjusted to 9.2 - 9.4	Room temperature

Separating gel (12%) was freshly prepared right before electrophoresis. It was composed with 40 % acrylamide / bis-acrylamide (30 %) solution (BIO-RAD Laboratories), 25 % 4 X separating gel buffer, 0.05 % (w/v) ammonium persulphate (APS) (BIO-RAD Laboratories) and 0.12 % TEMED (BIO-RAD Laboratories).

Stacking gel (4.5%) was freshly prepared right before electrophoresis. It was composed of 15 % acrylamide / bis-acrylamide (30 %) solution, 25 % 4 X stacking gel buffer, 0.05 % (w/v) ammonium persulphate (APS) and 0.12 % TEMED.

2.1.3 Herbs

The dried root of *Astragalus membranaceus* (voucher no. 2005-2731b) was purchased from Inner Mongolia. Dried cortex of *Acanthopanax senticosus* (voucher no. 2005-2780) was purchased from Jilin. Dried fruiting body of *Grifola frondosa* was purchased from northern Guangdong (2005-2758).

2.2 Methods

2.2.1 Cell Cultures

2.2.1.1 Cell lines

Four cell lines, Hep G2, Hs68, MCF-7 and MDA-MB-231, were employed in this study. All of them were purchased from American Type Culture Collection (ATCC). Hep G2 is a human hepatocellular cell line (ATCC no. HB-8065) which does not contain a Hepatitis B virus genome. MCF-7 (ATCC no. HTB-22) is an estrogen-responsive breast cancer cell line, which simulates a relatively early stage of breast cancer. On the other hand, MDA-MB-231 (ATCC no. HTB-26) is a breast cancer cell line which does not express estrogen receptors, and represents a latter and more invasive stage of breast cancer. All of these three cell lines are adherent in nature, and were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin (complete medium) at 37°C and 5% CO_2 humidified atmosphere.

Hs68 (ATCC no. CRL-1635) is a normal foreskin fibroblast cell line, which is obtained from an apparently normal Caucasian newborn male. This cell line is also adherent in nature, and it was maintained in DMEM medium, supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin (complete medium) at 37°C and 5% CO_2 humidified atmosphere.

2.2.1.2 Cell culture techniques

The cells were cultivated in T_{75} culture flask with 10 ml complete medium. Subculture was performed when 70-80% confluence of T_{75} was reached. Hep G2, MCF-7, and MDA-MB-231 cells were subcultured every 3-4 days, while Hs68 cells were passed every 7-8 days. The used medium was discarded and the bottom of the flask was rinsed by PBS to remove any remaining medium. The cells were brought into suspension by the use of trypsin/EDTA solutions. Centrifugation was carried out at 375 xg for 3 minutes after the addition of complete medium. The cell pellets were then resuspended with complete medium and passed to a new culture flask.

2.2.1.3 Cryopreservation of cell lines

Frozen cell culture stock was kept in cell tank filled with liquid nitrogen for long term storage. Cells were brought into suspension by EDTA/trypsin, after washing with PBS. After centrifugation at 375 xg, the cell pellet was resuspended with 4 ml of fresh complete medium. The number of viable cells was determined by trypan blue exclusion test with a hemocytometer. 3 x 10^6 cells were resuspended in 1 ml of freezing medium consisted of 50% FBS (v/v), 40% complete medium and 10% DMSO (v/v). The cells were then transferred to a cryogenic vial (Nalgene, USA) and stored at -70 $^{\circ}$C overnight. The cells were then kept frozen in liquid nitrogen for future use.

Frozen cell lines could be resuscitated by thawing the cells in 37 °C water bath until a small amount of ice remained in the vial. Thawed cells were resuspended by 10 ml pre-warmed complete medium and were then incubated in a T_{75} flask in 37 °C with humidified atmosphere of 5% CO_2 for 24 hours. The culture medium was replaced with fresh complete medium on the next day.

2.2.2 Proliferation assay

2.2.2.1 MTT assay

MTT assay was used to determine the proliferation of cells treated with the herbal extracts. MTT [3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide, Sigma] would be reduced to purple formazan salt by the action of mitochondrial dehydrogenase found in living cells. The formation of the formazan salt could be monitored spectrophotometrically by measuring the absorbance at 540 nm.

Cells were harvested from the culture flask as described previously. The amount of viable cells was determined by the trypan blue exclusion test with the use of hemocytometer. For Hep G2, MCF-7 and MDA-MB-231, 5,000 cells were seeded in 100 μl of complete medium in each well of 96-well flat bottom culture plates (Iwaki, Japan), while 7000 Hs68 cells were seeded in each well of the 96-well flat bottom culture plates. The cells were incubated in 37 °C humidified atmosphere of 5 % CO_2 for 24 hours to allow time for the adherence of cell to the bottom of the well.

Complete medium containing various concentrations of herbal extracts were added to the well accordingly and the cells were further incubated in 37 °C humidified atmosphere of 5 % CO_2 for 48 hours.

After 48 hours of incubation, the culture medium was replaced by fresh complete medium and then 30 μl of 5 mg/ml MTT solution were added to each well. After 2 hour 37 °C incubation, the culture medium was again aspirated and the formazan product was dissolved by the addition of 100 μl DMSO. Absorbance at 540 nm was then measured by BMG FLUOstarOptima microplate reader (BMG LABTECH GmbH, Offenburg, Germany). The results were expressed as the percentage of absorbance at 540 nm of the vehicle control.

2.2.2.2 Isolation of PBMC

Buffy coat provided by the Hong Kong Red Cross was diluted with PBS in a ratio of 1 : 1. 20 ml diluted buffy coat was carefully pipetted onto an equal volume of Ficoll-Paque ™ Plus solution (GE healthcare, UK) in a 50 ml centrifuge tube so that two layers were formed. Centrifugation was carried out at 800 xg for 20 minutes at 18 °C. Three layers could be found after the centrifugation, plasma formed the top layer, while PBMC were located in the middle layer and the bottom layer was Ficoll-Paque ™ Plus solution and red blood cells. PBMC were then aspirated out with care and washed with PBS twice followed by centrifugation at 100 xg for 10 minutes at 18 °C.

The cell pellet was resuspended in 4 ml of complete RPMI medium and trypan blue exclusion test was performed to check the number of viable cells. The PBMC was then seeded into 96-well flat bottom culture plates (Iwaki, Japan) in a density of 4 x 10^6 per well. Since PBMC are cells in suspension, the proliferation of PBMC is monitored by the use of XTT instead of MTT.

2.2.2.3 XTT

After 48 hours of treatment with herbal extracts, the proliferation of PBMC was determined with the use of XTT assay. Active XTT reagent (sodium 3′-[1-(phenylaminocarbonyl)- 3,4- tetrazolium]-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate) was prepared by mixing 300 μg/ml XTT in PBS with PMS in a ratio of 400 : 1. 50 ml of active XTT reagent was added to each well. After 4 hours of incubation at 37 °C, proliferation of PBMC could be verified by measuring absorbance at 450 nm with a microplate-reader.

2.2.3 DNA fragmentation

As one of the hallmarks of apoptosis, DNA fragmentation assay was carried out to confirm the apoptotic effect of Acanthopanax extract on breast cancer cells. MCF-7 and MDA-MB-231 cells were seeded on a 100 mm^2 culture dish (Iwaki, Japan) at a density of 1 x 10^5 cells/ml. 24 hour incubation at 37 °C with humidified atmosphere of 5 % CO_2 was allowed for the adherece of the cells to the bottom of the dish and the

culture media was then replaced by complete RPMI medium containing various concentrations of Acanthopanax extract. After 48 hours of treatment, the culture medium together with the floating cells were collected and adherent cells were harvested with EDTA/trypsin treatment. The collected cells were then centrifuged at 800 xg for 3 minutes and the cell pellet was washed twice with PBS followed by centrifugation at 800 xg for 3 minutes. The cell pellet was then subject to 200 μl of lysis buffer and incubated at 37 °C for 15 minutes. The lysed cells were then centrifuged at 6000 xg for 5 minutes and the DNA-containing supernatant was transferred to a microcentrifuge tube containing 50 μl of 5% SDS solution and stored at 4 °C until use.

10 μl of 10 mg/ml RNase A solution was added to each microcentrifuge tube and incubated at 56 °C for 90 minutes in order to digest the cellular RNA. Then, 20 μl of 10 mg/ml Proteinase K solution was added and incubated for another 90 minutes to remove the protein debris in the samples. DNA in the samples was precipitated by the addition of 30 μl of 3 M sodium acetate solution and 750 ml of absolute ethanol, followed by centrifugation at 20,000 xg for 30 minutes. The supernatant was discarded and the DNA precipitate was washed with 1 ml of 75 % ethanol and absolute ethanol, followed by centrifugation at 20,000 xg for 5 minutes. Supernatant was again discarded and the DNA pellet was air-dried. 30 ml of TE buffer was then

added to each DNA sample and incubated at 37 °C for 30 minutes. DNA extracted was then added to a 1.5 % (w/v) agarose gel with 0.5 mg/ml Ethidium Bromide incorporated. Electrophoresis was then carried out at a constant voltage of 100 V for about 45 minutes and the DNA bands were visualized under ultraviolet illumination.

2.2.4 Flow cytometry

Cells with appropriate treatment were subjected to the analysis of flow cytometer system FACS CANTO II (Becton Dickinson). For every set of experiment, forward scatter (FSC) and side scatter (SSC) were used to identify and gate the target cell population by their size and granularity. 10,000 events of desired cell population were collected for each sample. WinMDI version 2.9 was employed to analyze the data collected from flow cytometry.

2.2.4.1 Detection of mitochondrial membrane depolarization by the use of JC-1

MCF-7 and MDA-MB-231 cells were seeded in a 6-well culture plate (Iwaki, Japan) in a density of 2×10^5 per well and incubated for 24 hours for the adherence of the cells. The culture media were then replaced by RPMI complete medium with various concentrations of Acanthopanax extracts. After 48 hours of treatment, both floating and attached cells were collected and were centrifuged at 800 xg for 3 minutes. The cell pellets were resuspended and washed for two times with PBS, followed by centrifugation at 800 xg for 3 minutes. The cells were then resuspended

with JC-1 solution, containing 1 µl of JC-1 and 500 µl of PBS. After incubation at 37 ^{o}C for 15 minutes, the cells were subjected to flow cytometric analysis with an excitation at 488 nm. Fluorescence signal FITC and PE channels were collected for the detection of mitochondrial membrane depolarization.

2.2.4.2 Annexin-V-FITC PI labeling of apoptotic cells

One of the earliest features of apoptosis is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the cell membrane. And the externalization of PS molecules could be characterized with double staining with Annexin-V-FITC and PI.

MCF-7 and MDA-MB-231 cells at a density of 2 x 10^5 per well were seeded in a 6-well culture plate (Iwaki, Japan) and incubated at 37 ^{o}C humidified atmosphere of 5 % CO_2 for 24 hours for the adherence of cells. The culture media were then replaced by complete RPMI medium containing various concentrations of Acanthopanax extracts. After 48 hours of treatment, both floating and attached cells were collected and centrifuged at 800 xg for 3 minutes. Cell pellets were resuspended and washed with PBS for two times, followed by centrifugation at 800 xg for 3 minutes. The cell pellets were resuspended with the AV-PI staining solution and incubated at room temperature and in darkness for 15 minutes. The cells were then subjected to flow cytometric analysis with an excitation at 488 nm. Fluorescence signals in FITC and

Per-CP Cys-5 channels were gathered for characterizing apoptotic cell population.

2.2.4.3 Cell cycle analysis

MCF-7 and MDA-MB-231 cells were seeded in 6-well culture plates (Iwaki, Japan) at a density of 2 x 10^5 per well. After incubating at 37 °C with humidified atmosphere of 5 % CO_2 for 24 hours, the culture media were replaced by RPMI complete medium containing various concentrations of Acanthopanax extracts. After 48 hours of treatment, both the floating and attached cells were collected and centrifuged at 800 xg for 3 minutes. The cells were then washed twice with PBS, followed by centrifugation at 800 xg for 3 minutes. The cell pellets were then fixed with 1 ml of 70 % ethanol at 4 °C overnight. Propidium-iodide staining solutions containing 43 μg/ml propidium-iodide dye and 1 mg/ml RNase A in PBS were freshly prepared before the assay. Fixed cells were centrifuged at 800 xg for 3 minutes and the cell pellets were resuspended in 460 μl of propidium-iodide staining solution and incubated at 37 °C in darkness for 30 minutes. The cells were then subjected to flow cytometric analysis with an excitation at 488 nm. Fluorescence signals in the PI channel were collected.

2.2.5 Western blotting

2 x 10^6 MCF-7 and MDA-MB-231 cells were seeded in 100 mm^2 culture dish (Iwaki, Japan) and incubated for 24 hours for cell adherence. Culture media were then

replaced by complete RPMI medium containing various concentrations of Acanthopanax extracts, and incubated for 48 hours.

2.2.5.1 Total protein extraction

After 48 hours of treatment, medium together with floating cells were aspirated and attached cells were harvested by adding 1 ml of EDTA/trypsin solution. Floating and attached cells were then pooled and centrifuged at 800 xg for 3 minutes. The cell pellets were then washed with PBS for two times, followed by centrifugation at 800 xg for 3 minutes. The cell number was then determined by the use of hemocytometer. For every one-million of cells, 100 ml of lysis buffer were added and incubated at room temperature for 30 minutes. The lysed cells were then boiled at 100°C for 10 minutes, followed by centrifugation at 20,812 xg for 7 minutes. Protein containing supernatants were then collected and stored at -20 °C until use.

2.2.5.2 Determining protein concentration

The protein concentrations in the cell lysates were determined colorimetrically by Bicinchoninic acid (BCA) assay. Protein samples were ten-fold diluted by mixing 1 ml of protein samples with 9 ml PBS in a 96-well culture plate. 200 ml of BCA reaction mixture, which consisted of a mixture of BCA solution and $CuSO_4 \cdot 5H_2O$ in a ratio of 50 : 1, were added to each protein sample. Triplicates were performed for each protein sample to ensure an accurate result. After incubating at 37 °C for 30

minutes, absorbance at 540 nm was measured with a microplate reader. Bovine serum albumin (BSA) was employed as a protein standard to construct a standard curve with protein concentration ranged from 0 – 10 mg/ml. Protein concentrations in the cell lysates were calculated by referring to the standard curve.

2.2.5.3 SDS-PAGE

Mini-Protean 3 cell (BIO-RAD Laboratories) was employed in the experiments. SDS-PAGE was set up according to protocol stated in the user manual, with 4.5 % stacking gel and 12.5% separating gel used. 25 μg of protein was mixed with an equal volume of 2X loading gel containing SDS and β-mercaptoethanol and the protein samples were denatured by 3-minute boiling. Protein samples and low range protein marker were then loaded into the wells. Electrophoresis was performed at a constant voltage of 120 V for about 90 minutes. The stacking gel was then cut out and soaked in the transfer buffer for equilibration.

2.2.5.4 Electroblotting

Trans-Blot SD® Electrophoretic Transfer Cell (BIO-RAD Laboratories) was used in electroblotting. A 0.22 μm PVDF (polyvinylidene fluoride) membrane was soaked in the transfer buffer for 15 minutes to ensure complete wetting before electroblotting. Two extra-thick filter papers were also saturated with transfer buffer by soaking for 15 minutes. The transfer system is set up by aligning materials onto the

platinum anode as the following sequence: cathode, pre-soaked extra thick filter paper, PVDF membrane, polyacrylamide gel, pre-soaked extra-thick filter paper, anode. Air bubbles in-between the various layers were carefully excluded by slowly rolling a test tube over the transfer sandwich. Electroblotting was then performed at a voltage of 15 V for about 30 minutes.

2.2.5.5 Probing

After electroblotting, the PVDF membrane was soaked in 10 ml of 5% non-fat milk (TBS-T) for 2 hours at room temperature for non-specific blocking. The membrane was then probed with primary antibody at specific dilutions with non-fat milk at 4 ^{o}C overnight. After washing with TBS-T for three times for 15 minutes, secondary antibody was added and incubated for an hour at room temperature. The membrane was again washed with TBS-T twice for 15 minutes to remove the unbound antibodies and the membrane was ready for enhanced chemiluminescence assay.

2.2.5.6 ECL

The PVDF membrane was treated with 1 ml of ECL reagent (Amersham Pharmarcia Biotech) prepared by mixing ECL reagent 1 and ECL reagent 2 at a ratio of 1 : 1. After 3 minutes of reaction, the fluorescent signal could be detected by exposing the membrane to a Fuji Medical x-ray film (super Rx, Fuji) for various time

intervals. The x-ray film was then developed by a film processor (M35 X-MOAT, Kodak).

2.2.6 Preparation of herbal extracts

2.2.6.1 Preparation of extract of *Astragalus membranaceus* and *Grifola frondosa*

About 100 g of herbs were extracted by boiling at 100 °C under reflux with 1 L of different solvents (distilled water, 50 % and 95 % ethanol) for an hour twice by an electric heat mantle. The extracts were then centrifuged at 1000 xg for 10 minutes and the supernatants were frozen at -70 °C overnight. The frozen extracts were treated with liquid nitrogen before subjecting to lyophilization and then were stored in a dessicator.

2.2.6.2 Preparation of *Acanthopanax senticosus* aqueous extract

About 100g of Acanthopanax was extracted by boiling at 100 °C under reflux with distilled water for an hour twice by an electric heat mantle. The extract was then centrifuged at 1000 xg for 10 minutes to precipitate the non-soluble materials. The supernatant was then frozen at -70 °C overnight. The frozen extract was treated with liquid nitrogen before subjecting to lyophilization and then was stored in a dessicator.

2.2.6.3 Partition of Acanthopanax

Acanthopanax was first grated by razor blade and then was soaked in methanol for six days. The methanol extract was subjected to rotary evaporator and the residue

was re-dissolved in distilled water. The water fraction was then partitioned with n-hexane in a separating funnel. The two solvents were mixed mildly to prevent emulsification. The partition system was left undisturbed until the formation of two distinct layers again. The hexane layer was then collected and replaced by fresh hexane. This process is repeated until the hexane layer remains colorless at the end of partition.

The water layer was then subsequently partitioned with three other organic solvents in the order of chloroform, ethyl-acetate and water-saturated n-butanol as described previously. The organic solvent extracts were treated with sodium carbonate and then filtered to remove the water contaminants. Filtered extracts then were subjected to rotary evaporator. And the residues were kept in the vacuum chamber for 48 hours to ensure the removal of all of the solvents. The dry mass of the partition extracts were then weighed.

2.2.6.4 Column purification of ethyl-acetate fraction

About 4 g of ethyl-acetate fraction was applied onto 400 g of silica gel column chromatography (size~ 4.8 cm x 4.8 cm x 50 cm). A discontinuous gradient elution at a flow rate of 6 ml/min was performed with a mobile phase of hexane and ethyl-acetate. 1.5 L of 5 : 3 of hexane and ethyl-acetate, then 1 L of 10 : 9 hexane and ethyl-acetate, followed by 2 L of 2 : 5 hexane and ethyl-acetate. Then the column was

eluted with 1 L of ethyl-acetate. Finally, 3 L methanol was applied to the column. The eluates were collected by a fraction collector and elutes with similar chemical profile were pooled together to generate a sub-fraction.

2.2.6.5 Analytical thin layer chromatography

Chemical constituents in various extracts from Acanthopanax were revealed by performing thin layer chromatography (TLC). Silica gel 60 F254 on aluminum sheet (20 x 10 cm, MERCK, Darmstadt, Germany) was employed as the stationary phase in TLC study. A solvent system consisted of hexane, ethyl-acetate and formic acid at a ratio of 5 : 3 : 0.05 was employed as the mobile phase. Isofraxidin was used as the reference compound, as suggested in the Pharmacopoeia of the People's Republic of China (2005). Glass chamber was pre-equilibrated with the mobile phase for 15 minutes in order to create a closed environment saturated with the mobile phase. The TLC plate was then placed into the chamber. The TLC plate was then allowed to dry out in a well ventilated area after development. The bands were observed at three different conditions, UV longwave (365 nm), UV shortwave (254 nm) and spraying with 10 % (v/v) sulphuric acid in 95 % ethanol and heated to 110 ^{o}C for 2 minutes and viewed under visible light. All the solvents used including *n*-hexane, chloroform, ethyl-acetate, *n*-butanol, formic acid and methanol were analytical reagents and were purchased from LAB-SCAN (Thailand).

2.2.7 Statistical Analysis

Results of the assays were expressed as mean ± S.D. Statistical significance was determined by Student's unpaired t-test. Difference with a p value less than 0.05 would be considered as statistical significant.

Chapter 3 Results

3.1 Extractions

Components of Astragalus and Maitake were extracted with distilled water, 50 % ethanol or 95 % ethanol while compounds of Acanthopanax were extracted by reflux with distilled water for 2 hours only. The yield in terms of weight for the three herbs after extraction was listed in Table 3.1.

Table 3.1 Extraction yield of Astragalus, Maitake and Acanthopanax

Solvent	Astragalus	Maitake	Acanthopanax
	Percentage Yield		
Water	36.67%	36.8%	10.9%
50 % EtOH	22.8%	9.45%	/
95 % EtOH	16.6%	8.7%	/

The herbs were refluxed with the solvents for 2 hours. The percentage yield was calculated by dividing the dry mass of the extract by the mass of raw herb used (100g)

3.2 Anti-proliferative effect of herbal extracts on cancer cell lines

The effects of the Astraglus extracts on the proliferation of various cancer cell lines were studied with MTT assay (Fig. 3.1). The aqueous, 50 % ethanol and 95 % ethanol extracts did not show any statistically significant inhibition on the growth of the three tested cell lines at a concentration up to 800 μg/ml (Fig. 3.1).

The results of MTT test of Maitake extracts were shown in Fig. 3.2. Similarly, the

aqueous, 50 % ethanol and 95 % ethanol extracts did not show any statistically significant inhibition on the growth of the three tested cancer cell lines at a concentration up to 800 μg/ml.

The results of of MTT assay with the Acanthopanax water extract were shown in Fig. 3.3. The aqueous extract exhibited suppressive effect on the growth of MCF-7, MDA-MD-231 and Hep G2 cells in a dose-dependent manner. Statistically significant inhibitory effect could be observed from a concentration of 300 to 800 μg/ml. As can be seen, MCF-7 and MDA-MB-231 were the most susceptible cell lines. On the other hand, the Acanthopanax water extract also demonstrated a dose-dependent inhibitory effect on the growth of Hs68 human normal skin fibroblast cell line. Yet, the inhibitory effect was the least in the normal cell line Hs68.

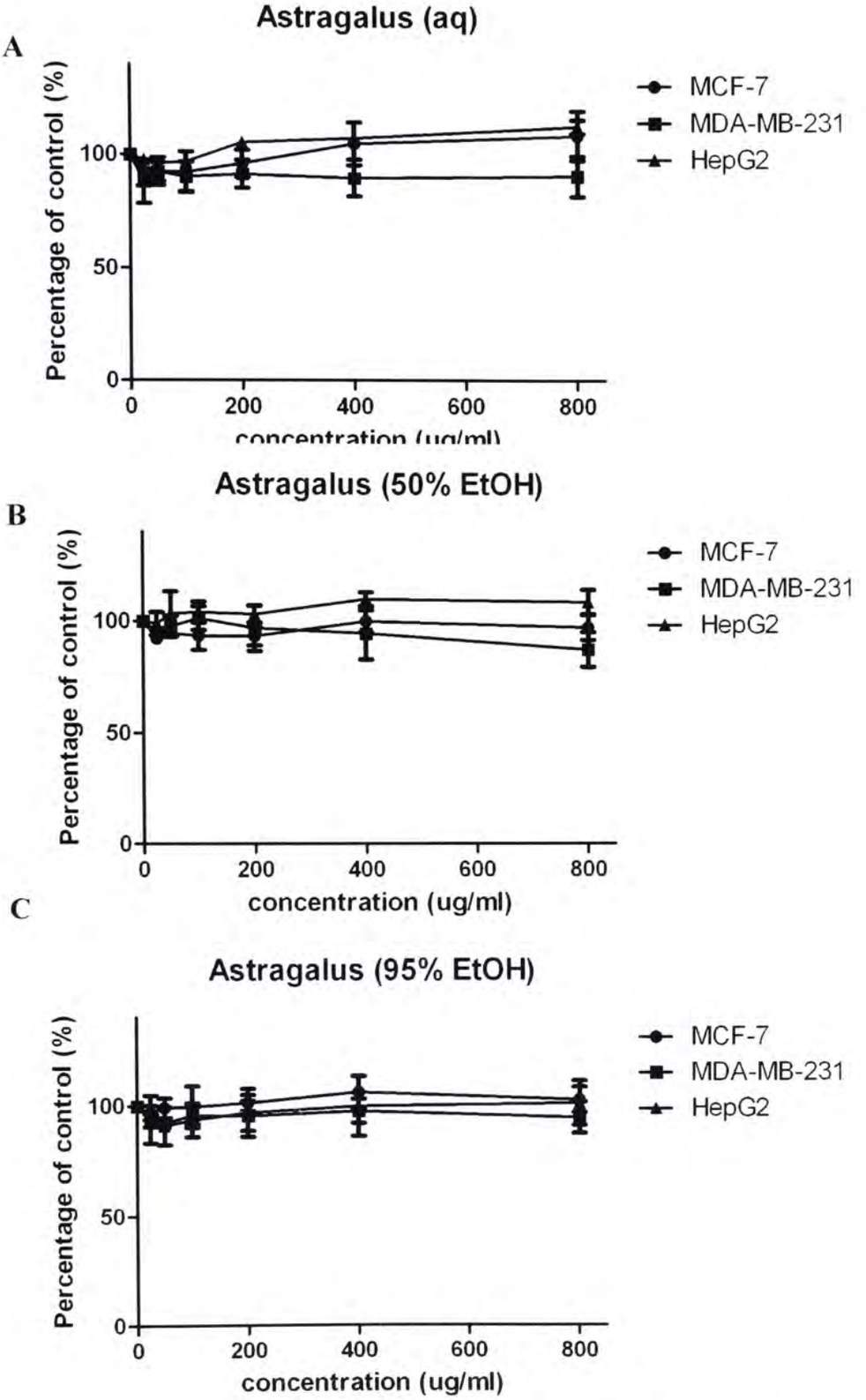


Fig. 3.1 Effect of Astragalus aqueous (A), 50 % EtOH (B), 95 % EtOH (C) extracts on the proliferation of MCF-7, MDA-MB-231 and Hep G2 cells. Cells in a density of 5 x 10^5 / ml were treated with various concentrations of Astragalus extracts for 48 hours at 37 °C, 5 % CO_2. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations.

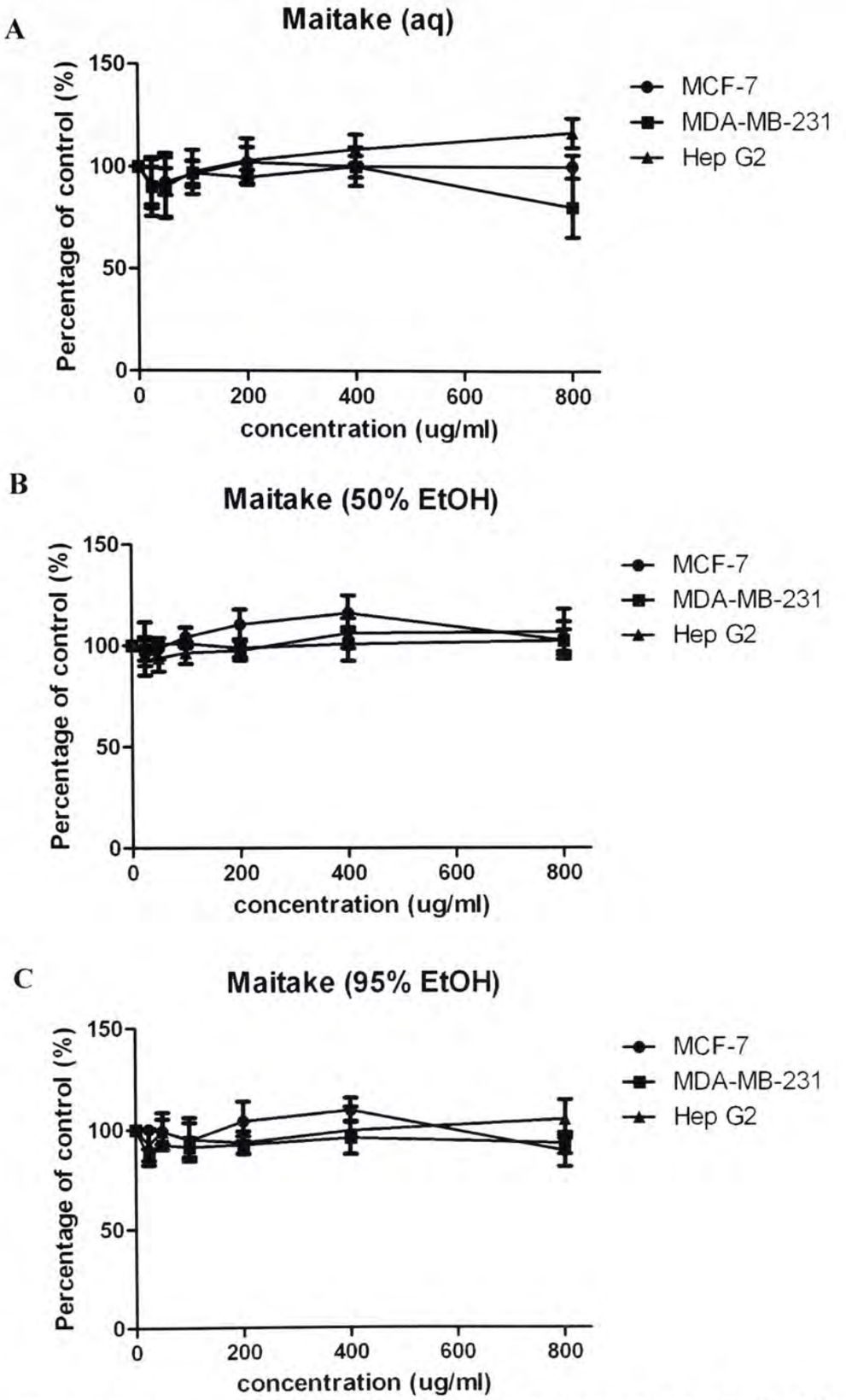


Fig. 3.2 Effect of Maitake aqueous (A), 50 % EtOH (B), 95 % EtOH (C) extracts on the proliferation of MCF-7, MDA-MB-231 and Hep G2 cells. Cells in a density of 5 x 10^5 / ml were treated with various concentrations of Maitake extracts for 48 hours at 37 °C, 5 % CO_2. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations.

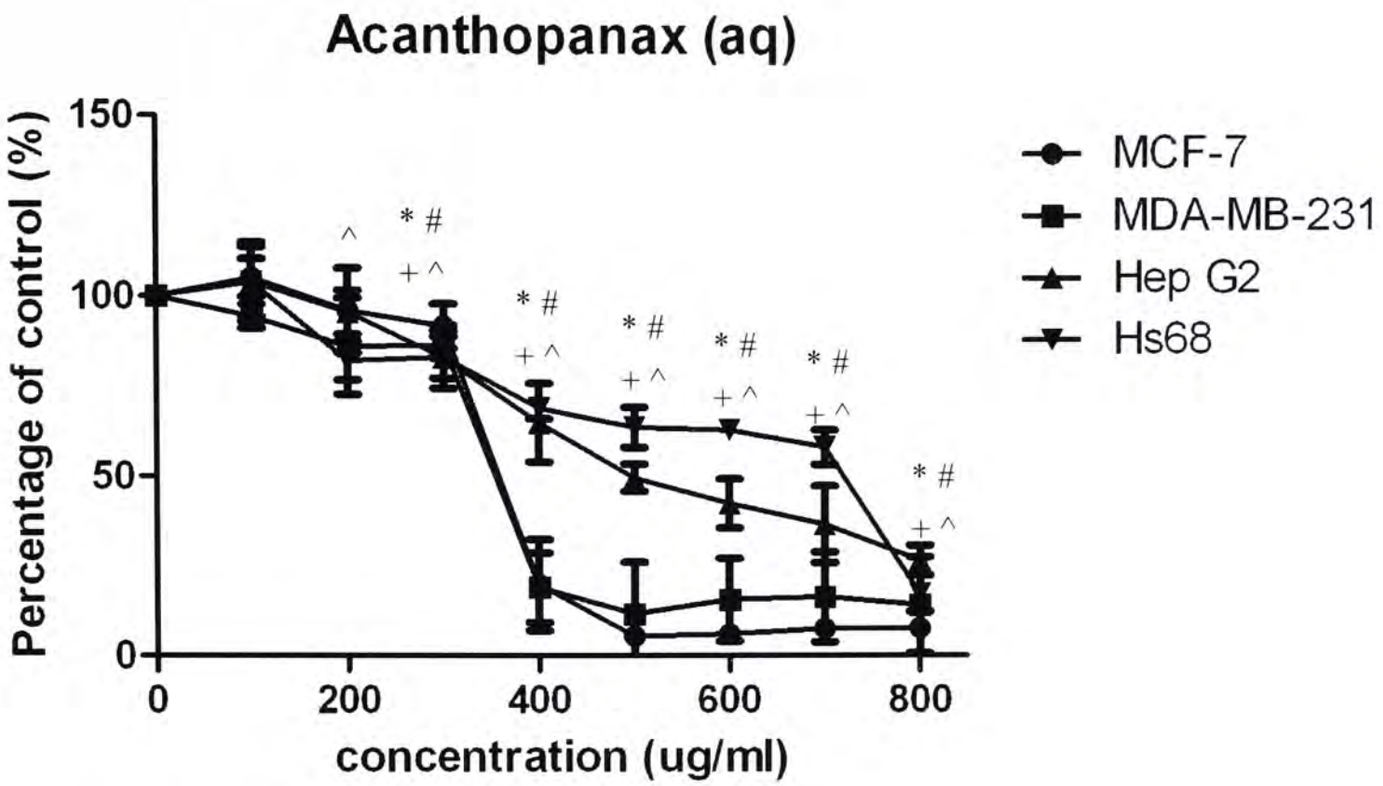


Fig. 3.3 Effect of Acanthopanax aqueous extract on the proliferation of MCF-7, MDA-MB-231, Hep G2 and Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7, MDA-MB-231 and Hep G2) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 ^{o}C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Acanthopanax aqueous extract for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Difference between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7, #$p < 0.05$ for MDA-MD-231, +$p < 0.05$ for Hep G2 cells and ^$p < 0.05$ for Hs68 cells.

3.3 Partition of Acanthopanax methanol extract

The Acanthopanax was treated with methanol for six days at room temperature and the methanol extract was further partitioned with four organic solvents in a sequence of *n*-hexane, chloroform, ethyl-acetate and *n*-butanol. The yield of the partition was shown in Table 3.2 and the chemical profiles of the partition fractions were studied by TLC. Three conditions were used to reveal the bands on the TLC plate. The TLC plates were viewed under UV at 365 nm to elicit the natural fluorescence of compounds. Besides, since the silica gel plate that we used would emit fluorescence under illumination of UV at 254 nm, compounds that absorb UV at 254 nm would therefore appear as dark spots on a bright green background. This method is known as fluorescence quenching. In addition, sulfuric acid was sprayed over the TLC plate and used as a universal reagent to help visualizing compounds including glycosides, flavonoids, steroids, triterpenes etc. The chromatographs were shown in Fig. 3.4. It could be observed in the chromatographs that the bands are localized on the upper part of the plate in less polar fraction like hexane fraction while bands could only be found in the lower part of the plate in the butanol fraction. However, overlapping of the bands could be seen among the four fractions.

Table 3.2 Partition yield of Acanthopanax

Solvent	Weight (g)	Percentage yield (w/w)
Methanol	85.78	9.03 %
Hexane	11.69	13.63 %
Chloroform	2.25	2.62 %
Ethyl-acetate	8.39	9.78 %
n-butanol	14.21	16.57 %

The methanol extract of Acanthopanax was partitioned with hexane, chloroform, ethyl-acetate and butanol. The yield of methanol extract was calculated by dividing the dry mass of the fraction by the mass of the raw herb used in partition (950g) while the partition yield was calculated by dividing the dry mass of the fraction by the mass of methanol extract (85.78g).

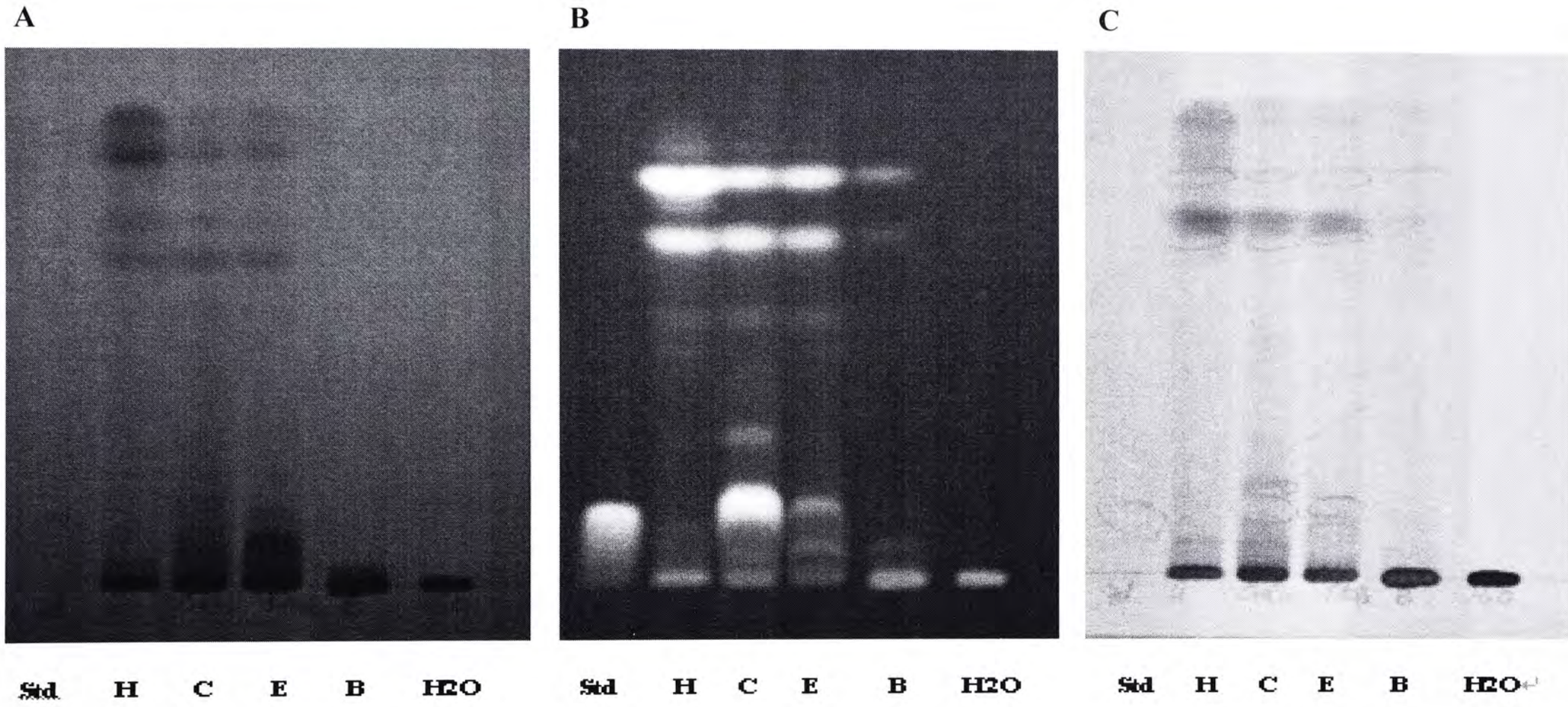


Fig. 3.4 The chromatograms of various partition extracts of Acanthopanax. The plate was observed under (A) UV 254 nm, (B) UV 365 nm and (C) Sprayed with H_2SO_4 in 10% EtOH. Isofraxidin was used as the chemical standard. The symbols are: Std: standard; H: n-Hexane; C: Chloroform; E: Ethyl-acetate; B: n-Butanol; H_2O: Water

Stationary phase: Silica gel 60 F_{254}. Mobile phase: Hexane-Ethyl-acetate-Formic acid (5:3:0.05)

3.4 Anti-proliferative effect of Acanthopanax partition fractions on breast cancer cells

The effects of the partition fractions on the growth of breast cancer cell lines MCF-7 and MDA-MB-231 cells were investigated with MTT assay. The results were shown in Fig. 3.5 and Fig. 3.6. All four fractions showed anti-proliferative effects on both breast cancer cell lines though with different potency. Both chloroform and ethyl-acetate fractions exhibited statistically significant anti-proliferative effect on MCF-7 and MDA-MB-231 cells, starting from a concentration of 50 μg/ml to 200 μg/ml. For hexane fraction, statistically significant inhibitory effect on MCF-7 cells growth was observed at a concentration start from 50 μg/ml onwards while such significant inhibitory effect on MDA-MB-231 cells growth could only be seen at a concentration higher than 100 μg/ml. For butanol fraction, statistically significant anti-proliferative effect on MCF-7 and MDA-MB-231 cells was not noted until the concentration reached 150 μg/ml.

3.5 Column chromatography of ethyl-acetate fraction

The ethyl-acetate fraction was further purified by employing column chromatography of silica gel. Discontinuous gradient elution was performed. Analytical TLC was employed for investigating the chemical profiles of the eluents. Eluents of similar profiles were pooled together as a sub-fraction. The TLC chromatographs of the sub-fractions were shown in Fig. 3.7. It can be observed that compounds of different

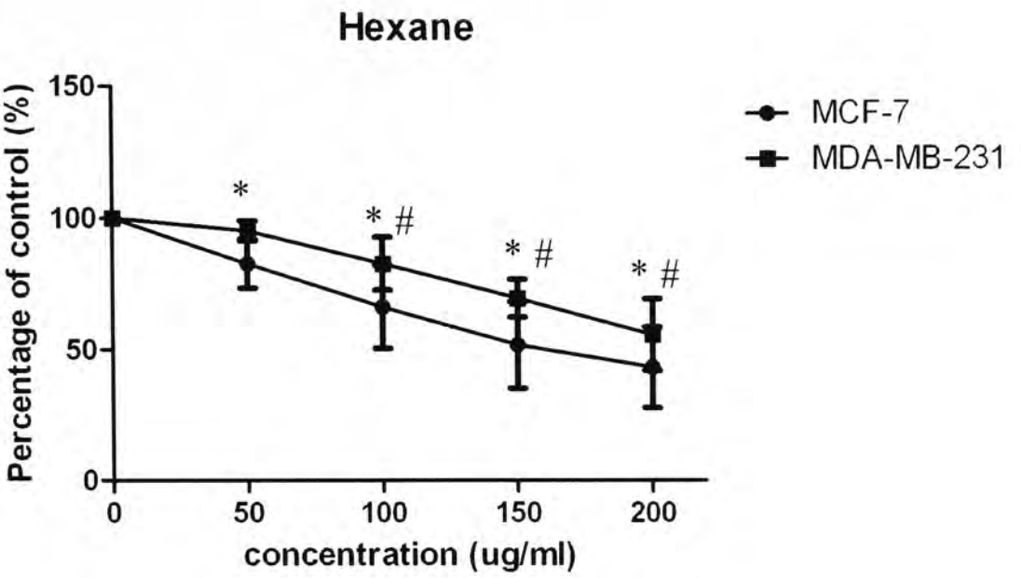
polarities were separated and localized in different sub-fractions and the overlapping of the bands was less extensive when compared with that in partition. The yields of the sub-fractions were listed in Table 3.3.

Table 3.3 Yield of sub-fractions of column chromatography

Fractions	Yield (mg)	Percentage Yield (%)
Fa	182	4.55
Fb	183	4.575
Fc	262	6.55
Fd	93	2.325
Fe	183	4.575
Ff	124	3.1
Fg	314	7.85
Fh	103	2.575
Fi	2255	56.375

Ethyl-acetate fraction of Acanthopanax was further purified by silica gel column chromatography. Elutes with similar chemical profiles were pooled together as a sub-fraction. The percentage yield of various sub-fractions was calculated by dividing the dry mass of the sub-fractions by the mass of ethyl-acetate extract used (4g).

A



B

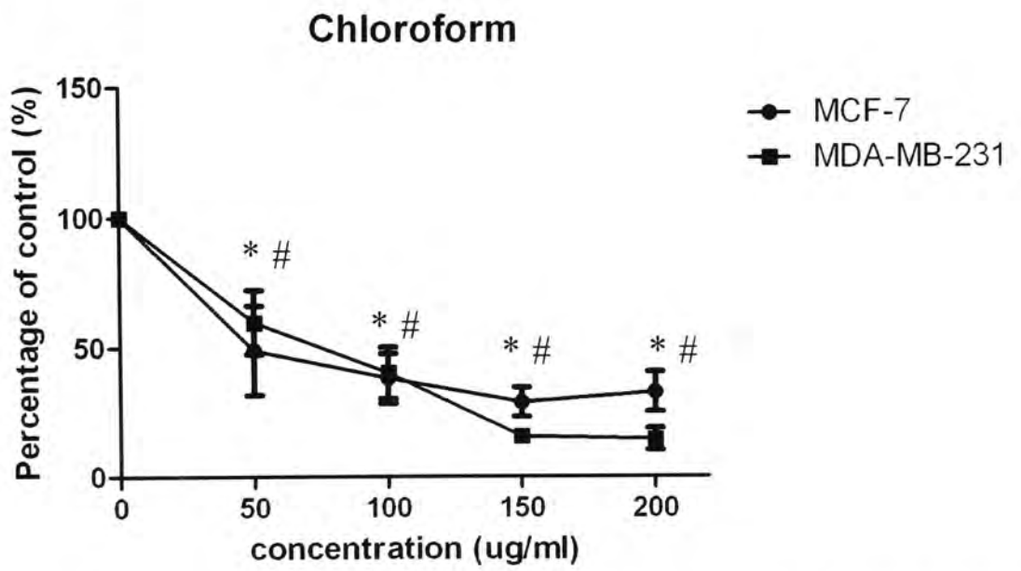
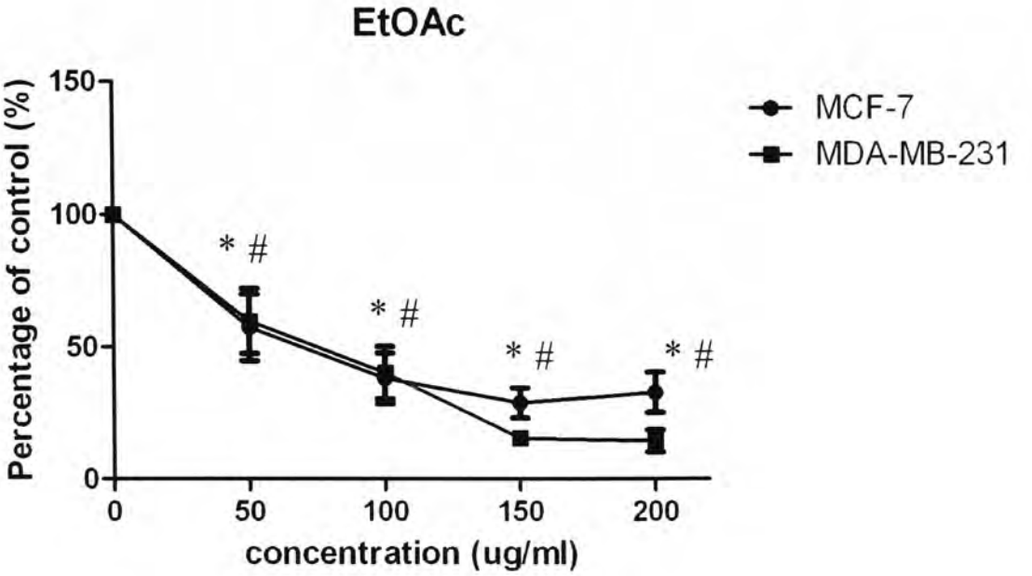


Fig. 3.5 Effect of partition extract (A) Hexane; (B) Chloroform on the proliferation of MCF-7 and MDA-MB-231 cells. Cells in a density of 5 x 10^5 / ml were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of partition fractions for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Difference between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



B

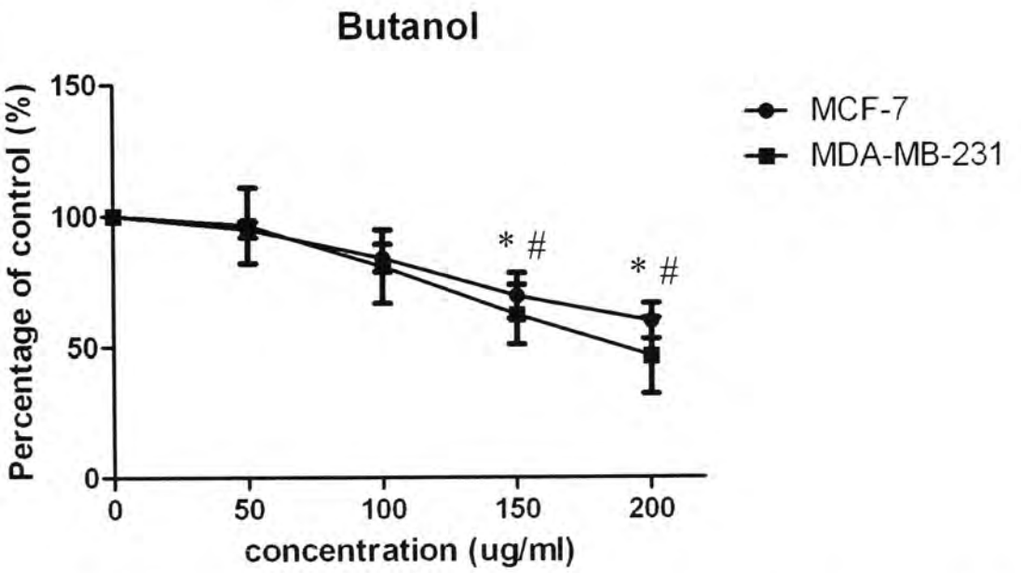


Fig. 3.6 Effect of partition extract (A) Ethyl-acetate; (B) Butanol on the proliferation of MCF-7 and MDA-MB-231 cells. Cells in a density of 5 x 10^5 / ml were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of partition fractions for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Difference between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.

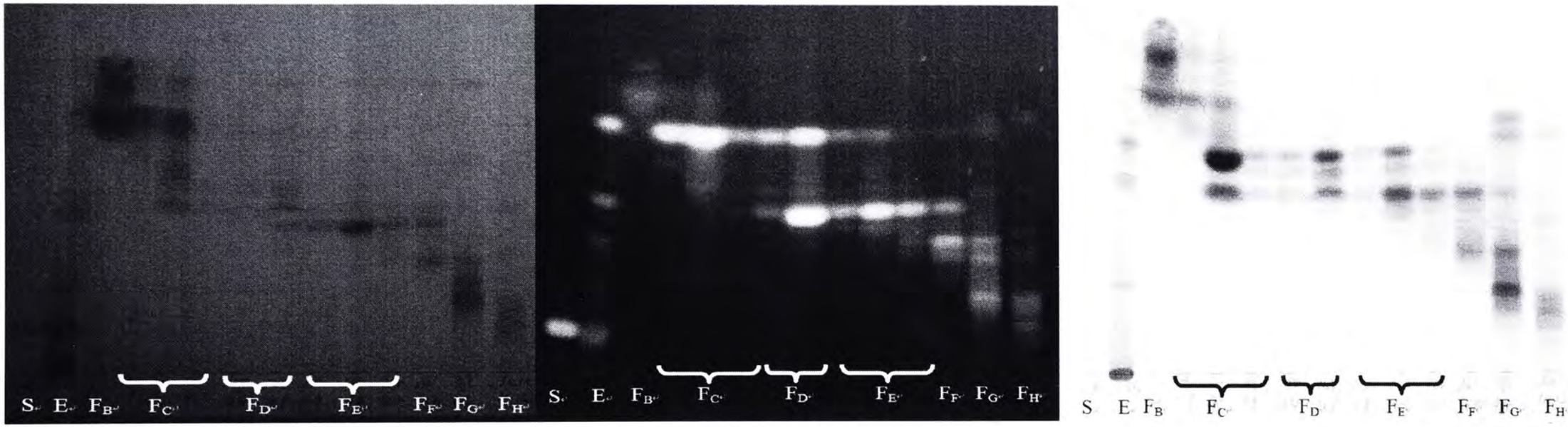


Fig. 3.7 The chromatograms of sub-fractions Fb - Fh of Acanthopanax. The plate was observed under (A) UV 254 nm, (B) UV 365 nm and (C) Sprayed with H_2SO_4 in 10% EtOH. Isofraxidin was used as the chemical standard. The symbols are: S: standard; E: Ethyl-acetate.

Stationary phase: Silica gel 60 F_{254}. Mobile phase: Hexane-Ethyl-acetate-Formic acid (5:3:0.05)

3.6 Anti-proliferative effect of various sub-fractions on breast cancer cells

The effect of the sub-fractions generated from column chromatography towards the growth of the breast cancer cell lines were tested with MTT assay. The anti-proliferative effect of various fractions ranged from 25 μg/ml to 150 μg/ml was tested. Hs68 normal human cell line was used as a reference to study the effect of the sub-fractions on normal human cells.

MTT results for fraction Fa were shown in Fig. 3.8. Up till a concentration of 150 μg/ml, no significant inhibition on the MCF-7 or MDA-MB-231 cells could be observed.

The results of MTT assay for fraction Fb were shown in Fig. 3.9. A concentration dependent anti-proliferative effect could be observed. Fb inhibited the growth of MCF-7 cells significantly starting from a concentration of 50 μg/ml while it only significantly reduced the growth of MDA-MB-231 cells above 100 μg/ml.

The results of MTT assay of fraction Fc were shown in Fig. 3.10. Fc exhibited a dose-dependent anti-proliferative effect on both MCF-7 and MDA-MB-231 cells. Statistically significant inhibitory effect on the growth of MCF-7 cells was demonstrated at a concentration as low as 25 μg/ml while it only exhibited significant suppressive effect on MDA-MB-231 cells starting from a concentration of 50 μg/ml. Fc also inhibited the growth of Hs68 cells, but significant inhibitory effect was only observed at 150 μg/ml which was the highest concentration tested.

Results of MTT assay of fraction Fd were shown in Fig. 3.11. Dose-dependent inhibitory effect on the growth of both MCF-7 and MDA-MB-231 cells could be seen. For MCF-7 cells, this suppressive effect was statistically significant starting from 50 μg/ml while a significant inhibition could already be seen at 25 μg/ml for MDA-MB-231 cells. However, Fd also suppressed the growth of Hs68 cells and the inhibition was considered as significant at 100 μg/ml.

Results of MTT test of fraction Fe were shown in Fig. 3.12. Significant growth suppressive effect on both breast cancer cell lines was observed at concentrations above 25 μg/ml. On the other hand, Fe also exhibited significant inhibitory effect on the proliferation of Hs68 cells at concentrations above 50 μg/ml.

Results of MTT test of fraction Ff and Fg were shown in Fig. 3.13. and Fig. 3.14 respectively. Both of them elicited significant inhibitory effect on the growth of MCF-7 and MDA-MB-231 cells starting from a concentration of 25 μg/ml. On the other hand, statistically significant suppressive effect on the proliferation of Hs68 cells could only be seen at concentration of 150 μg/ml which was the highest concentration tested.

MTT results of Fh and Fi were shown in Fig. 3.15 and Fig. 3.16 respectively. Fh exhibited dose-dependent inhibitory effect on both breast cancer cell lines with statistical significance starting from 25 μg/ml. For Fi, significant suppressive effect on

the growth of both breast cancer cell lines could be seen at concentrations above 50 μg/ml.

IC_{50} of the various sub-fractions were summarized in Table 3.4.

Table 3.4 Summary of IC_{50} of various sub-fractions (include SD)

Fraction	Human breast cancer cell line			
	MCF-7		MDA-MB-231	
	IC_{50} (μg/ml)	Cell viability (%) at IC_{50}	IC_{50} (μg/ml)	Cell viability (%) at IC_{50}
Fb	54 ± 6.5	50	84 ± 7.6	50
Fc	42 ± 7.0	50	65 ± 1.0	50
Fd	67 ± 8.5	50	33 ± 1.3	50
Fe	27 ± 9.1	50	48 ± 1.5	50
Ff	36 ± 6.1	60	70 ± 9.0	55
Fg	28 ± 8.7	50	45 ± 8.8	50
Fh	45 ± 7.0	60	60 ± 8.1	65

The human breast cancer cells were treated with different sub-fractions of Acanthopanax for 48 hours at 37 °C, 5 % CO_2. The IC_{50} was then determined by MTT assays by 3 independent experiments. (Mean ± S.D., n = 3)

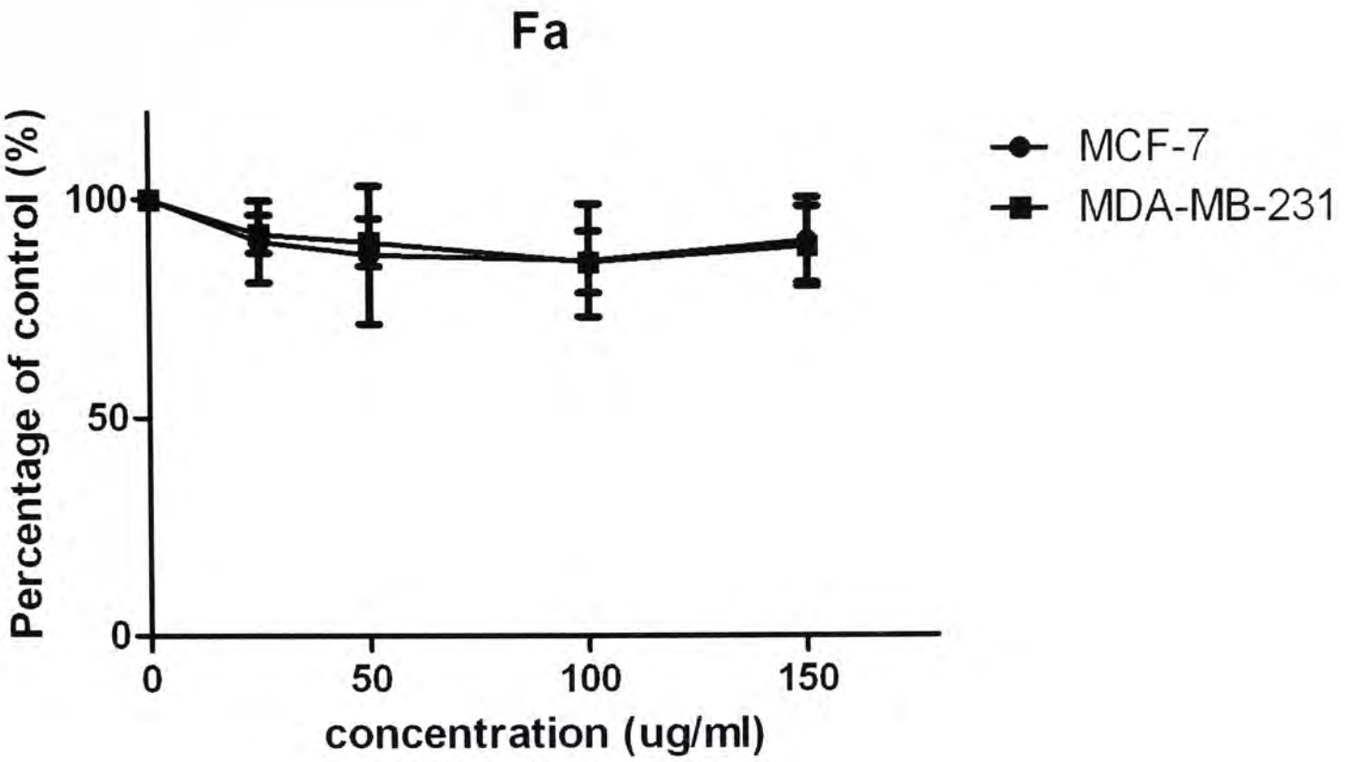


Fig. 3.8. Effect of sub-fraction Fa of Acanthopanax on the proliferation of MCF-7 and MDA-MB-231 cells. Cells in a density of 5 x 10^5 / ml were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fa for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (mean ± S.D. of 3 independent experiments)

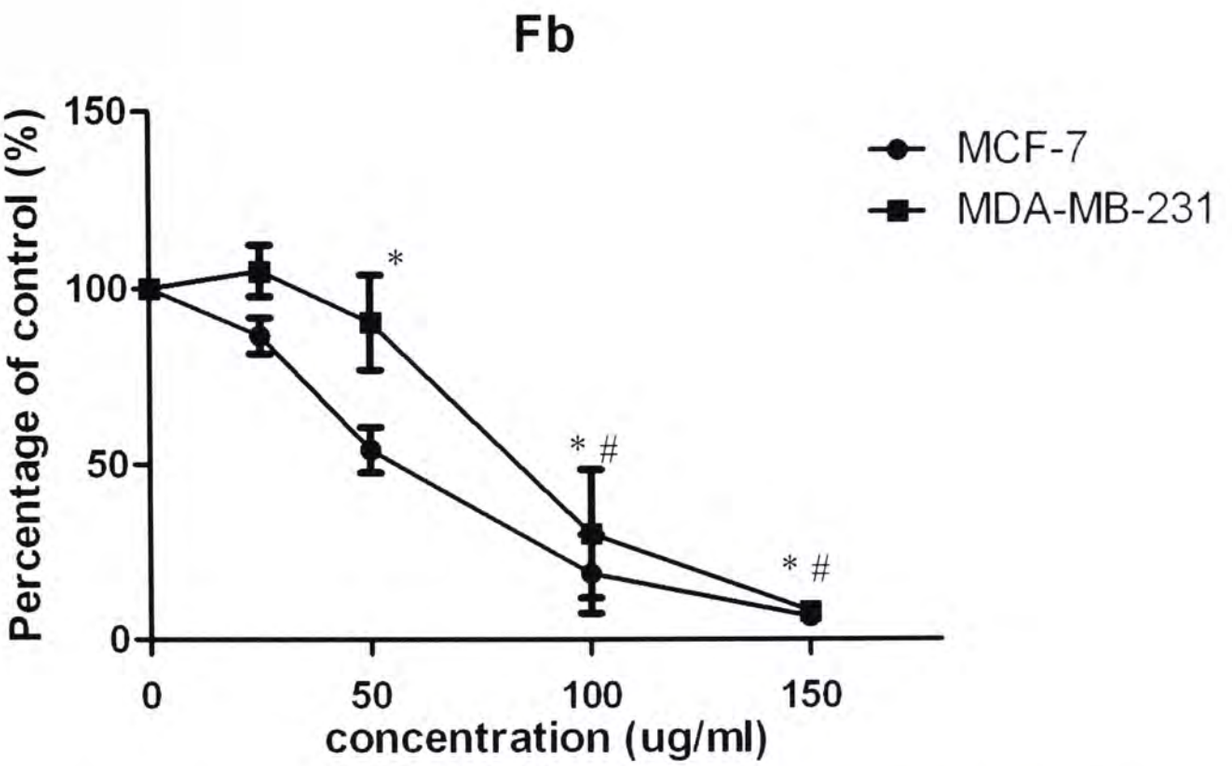
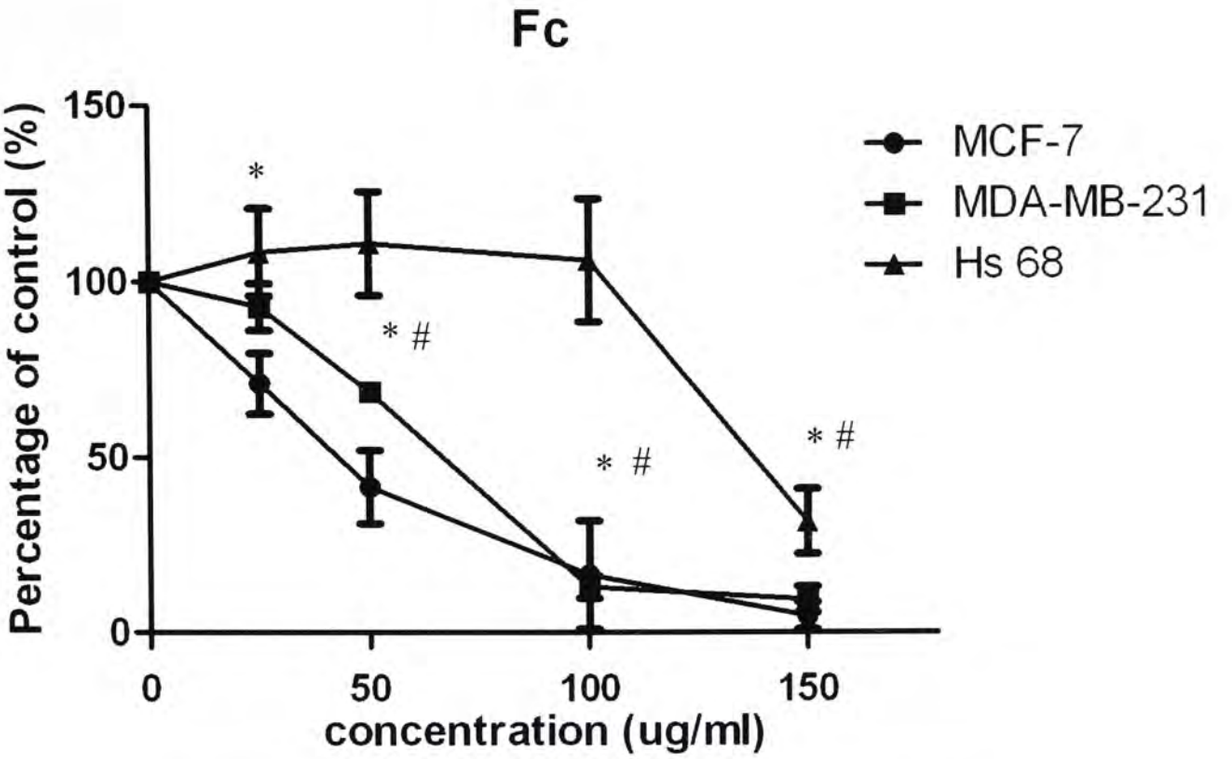
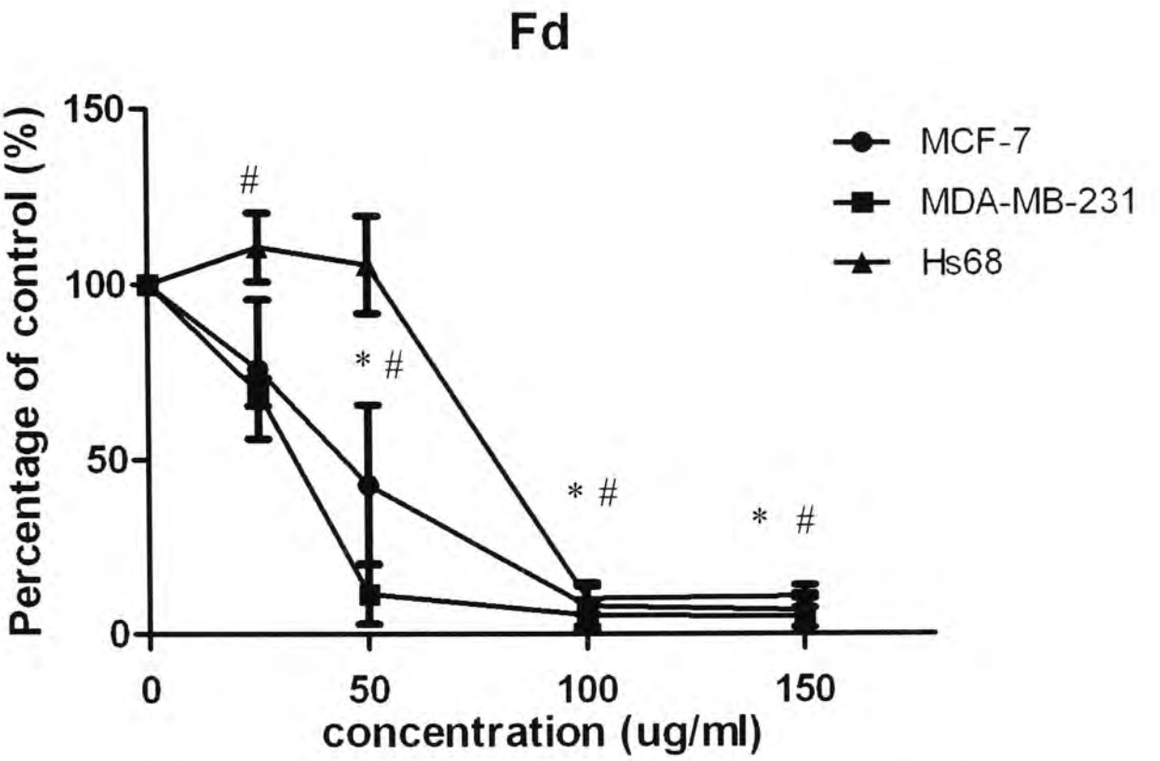


Fig. 3.9. Effect of sub-fraction Fb of Acanthopanax on the proliferation of MCF-7 and MDA-MB-231 cells. Cells in a density of 5 x 10^5 / ml were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fb for 48 hours. Cell viability was then determined by MTT. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



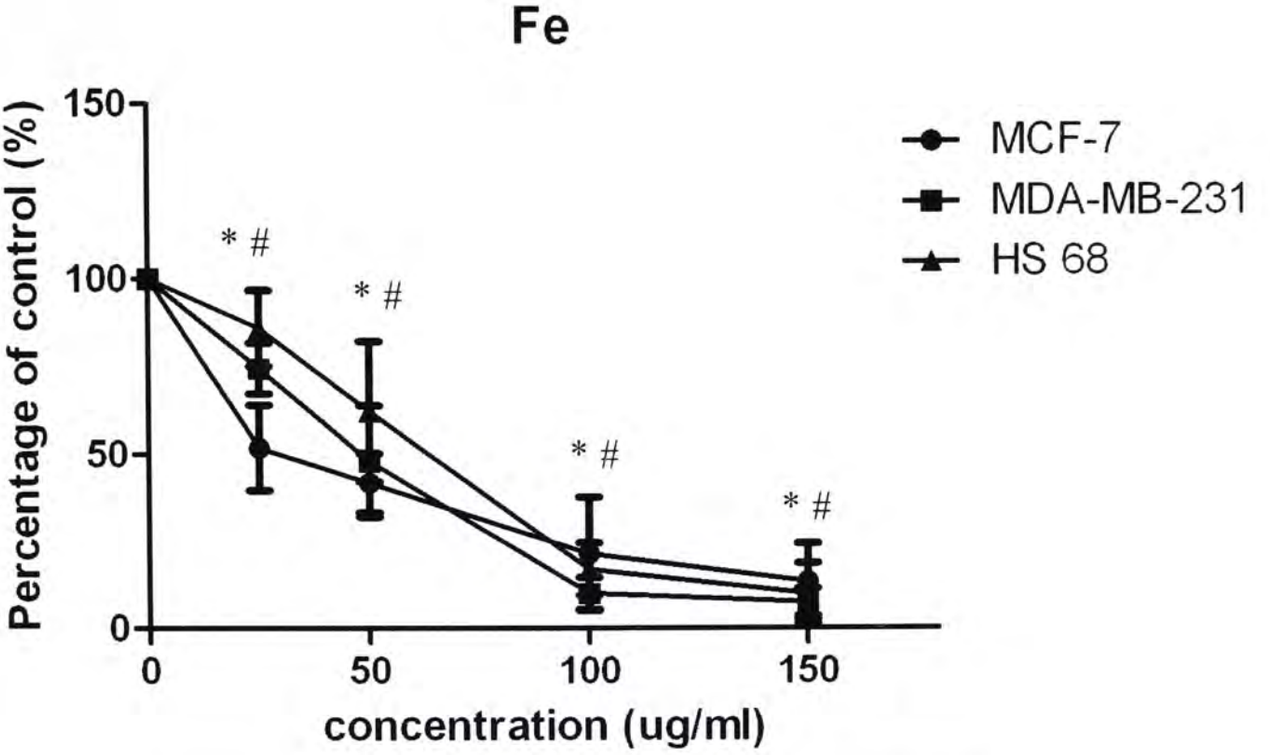
	Concentration (μg/ml)	MCF 7 vs HS 68	MDA-MB-231 vs Hs68
P value	25	>0.05	>0.05
	50	<0.01	<0.01
	100	<0.01	<0.01
	150	>0.05	>0.05

Fig. 3.10 Effect of sub-fraction Fc of Acanthopanax on the proliferation of MCF-7, MDA-MB-231 and Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7 and MDA-MB-231) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fc for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3-5 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



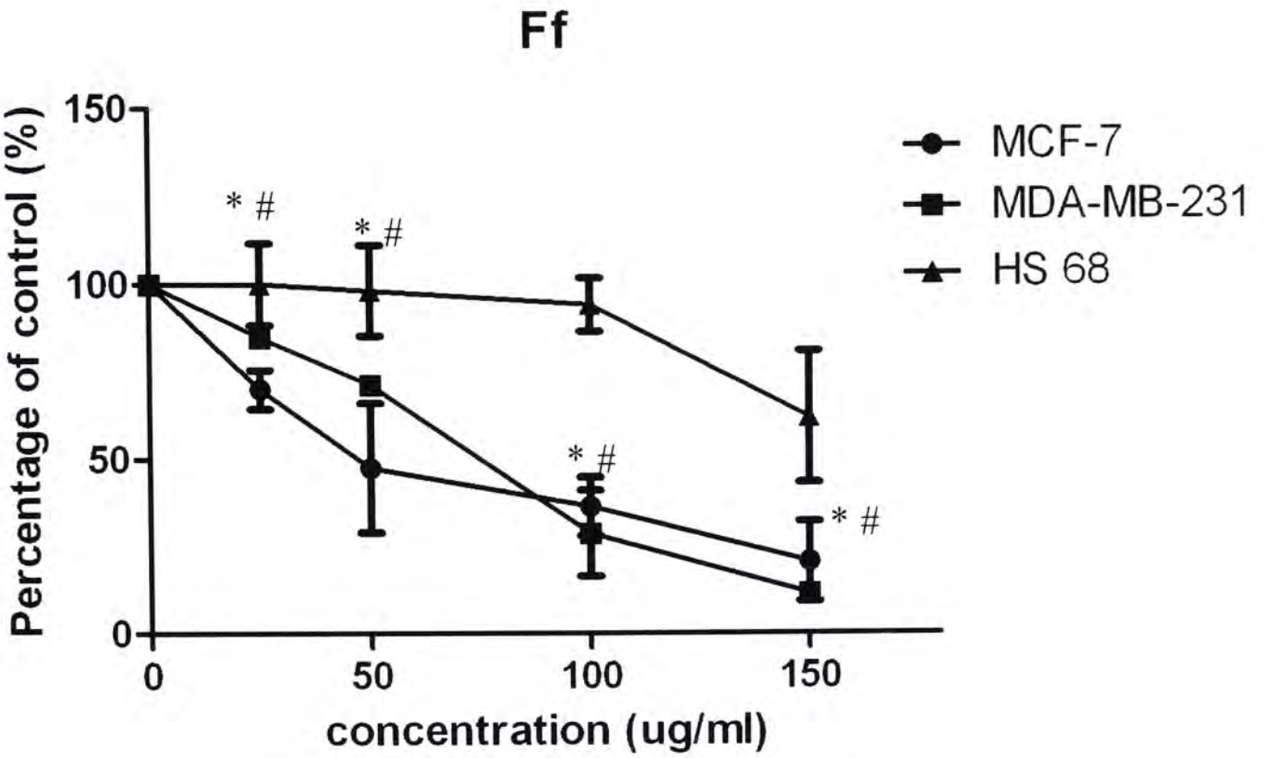
	Concentration (μg/ml)	MCF 7 vs HS 68	MDA-MB-231 vs Hs68
P value	25	<0.01	<0.01
	50	<0.01	<0.01
	100	>0.05	>0.05
	150	>0.05	>0.05

Fig. 3.11 Effect of sub-fraction Fd of Acanthopanax on the proliferation of MCF-7, MDA-MB-231 and Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7 and MDA-MB-231) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fd for 48 hours. Cell viability was then determined by MTT assy. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3-5 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



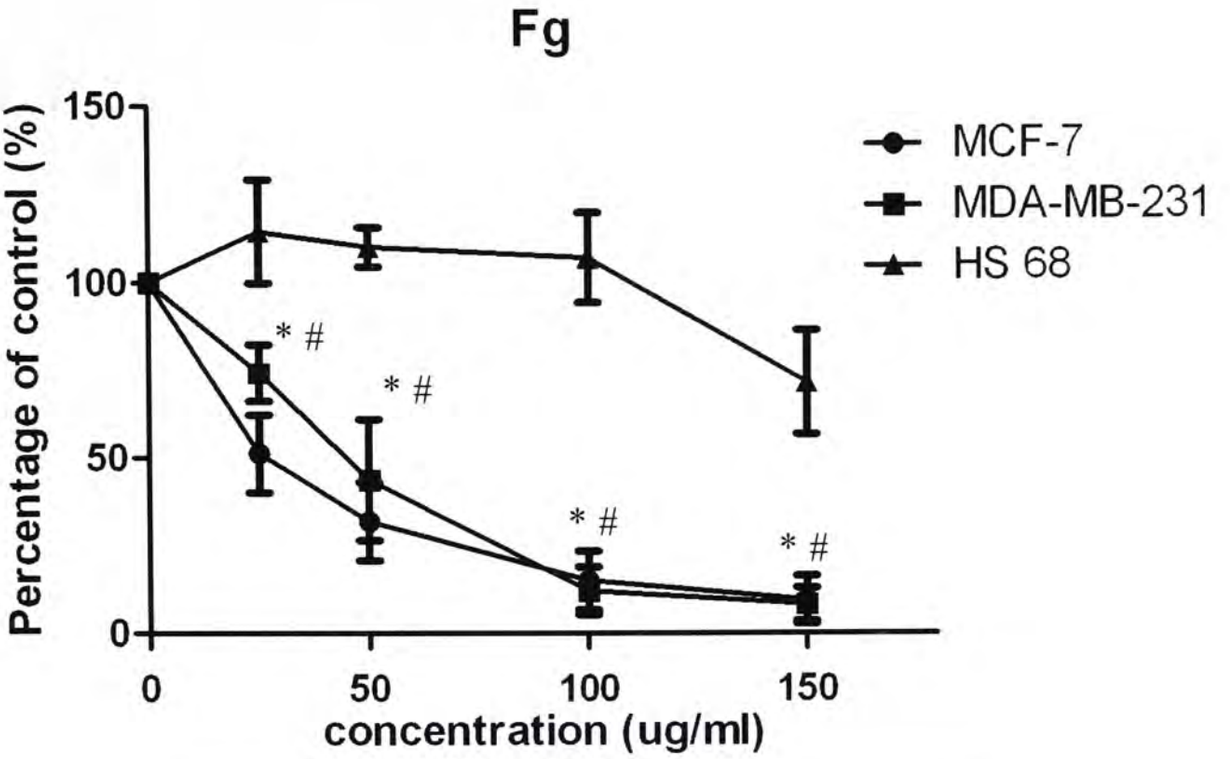
	Concentration (μg/ml)	MCF 7 vs HS 68	MDA-MB-231 vs Hs68
P value	25	<0.05	>0.05
	50	>0.05	>0.05
	100	>0.05	>0.05
	150	>0.05	>0.05

Fig. 3.12 Effect of sub-fraction Fe of Acanthopanax on the proliferation of MCF-7, MDA-MB-231 and Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7 and MDA-MB-231) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fe for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3-5 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



	Concentration (μg/ml)	MCF 7 vs Hs68	MDA-MB-231 vs Hs68
P value	25	<0.01	<0.01
	50	<0.01	<0.01
	100	<0.01	<0.01
	150	<0.01	>0.05

Fig. 3.13 Effect of sub-fraction Ff of Acanthopanax on the proliferation of MCF-7, MDA-MB-231, Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7 and MDA-MB-231) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Ff for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3-5 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.



	Concentration (μg/ml)	MCF 7 vs Hs68	MDA-MB-231 vs Hs68
P value	25	<0.01	<0.01
	50	<0.01	<0.01
	100	<0.01	<0.01
	150	<0.01	<0.01

Fig. 3.14 Effect of sub-fraction Fg of Acanthopanax on the proliferation of MCF-7, MDA-MB-231 and Hs68 cells. Cells in a density of 5 x 10^5 / ml (MCF-7 and MDA-MB-231) or 7 x 10^5 / ml (Hs68) were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fg for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3-5 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.

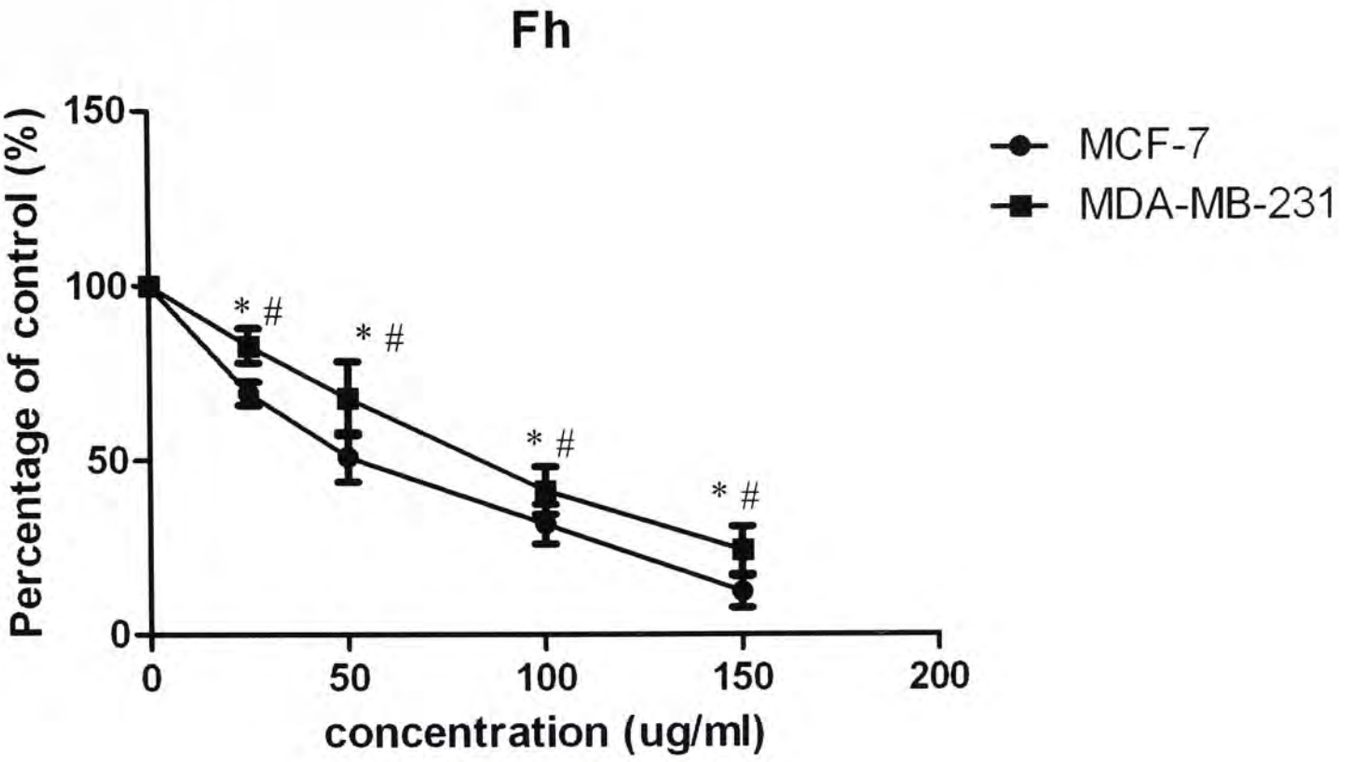


Fig .3.15 Effect of sub-fraction Fh of Acanthopanax on the proliferation of MCF-7 and MDA-MB-231 cells. Cells were seeded in 96-well plate and incubated at 37 ^{o}C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fh for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.

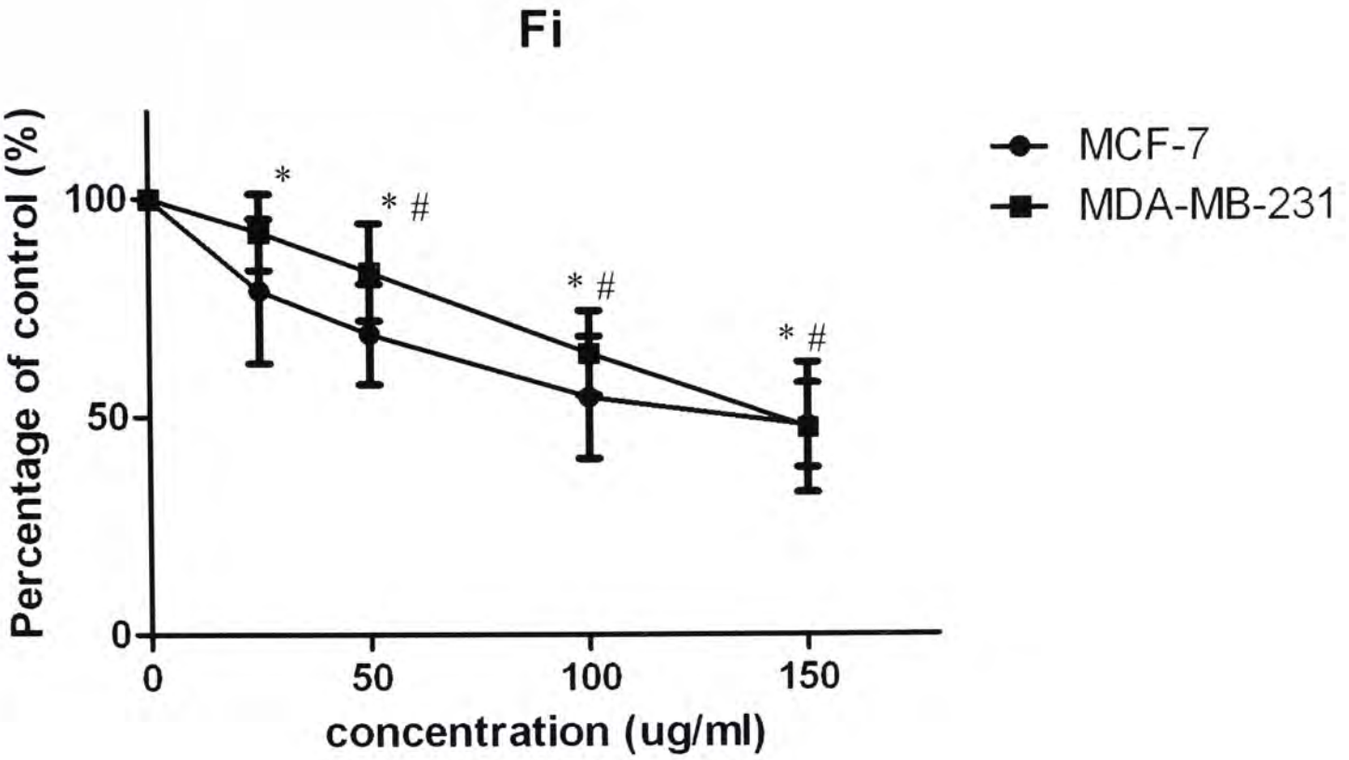


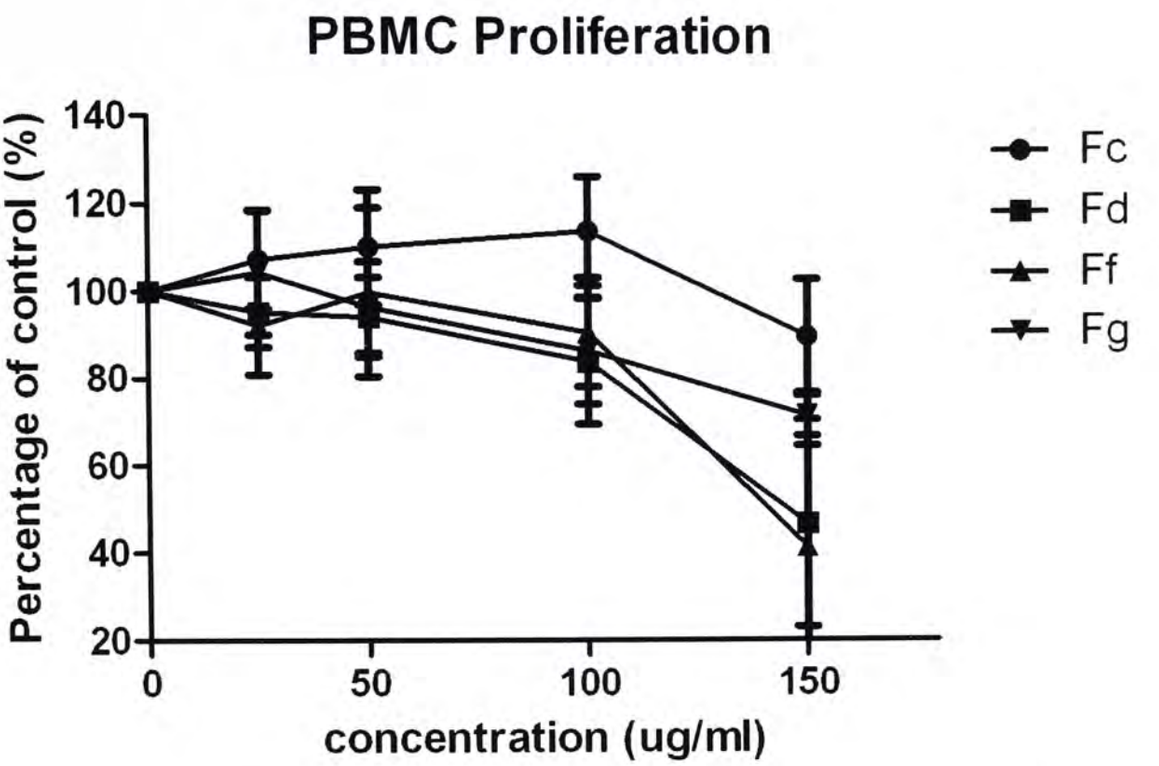
Fig. 3.16. Effect of sub-fraction Fi of Acanthopanax on the proliferation of MCF-7 and MDA-MB-231 cells. Cells were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fi for 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$ for MCF-7 cells, #$p < 0.05$ for MDA-MD-231 cells.

3.7 Effect of sub-fractions on PBMC proliferation

PBMC isolated from buffy coat were treated with fractions Fc, Fd, Ff and Fg for 48 hours and results of XTT assay were shown in Fig. 3.17. Among the tested sub-fractions, Fc exhibited the least effect on the growth of PBMC.

3.8 Kinetic study of anti-proliferative effect of Fc

MCF-7 and MDA-MB-231 cells were treated with Fc for 24 and 48 hours to study the effect of Fc on the proliferation of breast cancer cells at different time points. The MTT results were shown in Fig. 3.18. Fc demonstrated time-dependent effect on MCF-7 and MDA-MB-231 cells. Statistical significance could be found at 50 μg/ml and 100 μg/ml.



	Concentration (μg/ml)	Fc	Fd	Ff	Fg
P value	25	> 0.05	> 0.05	> 0.05	> 0.05
	50	> 0.05	> 0.05	> 0.05	> 0.05
	100	> 0.05	> 0.05	> 0.05	> 0.05
	150	> 0.05	<0.01	<0.01	<0.01

Fig. 3.17 Effect of sub-fractions on the proliferation of PBMC. Cells were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of sub-fractions for 48 hours. Cell viability was then determined by XTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Statistical significance between extract treated and control was determined by Student's unpaired t-test and the results were listed on the table above.

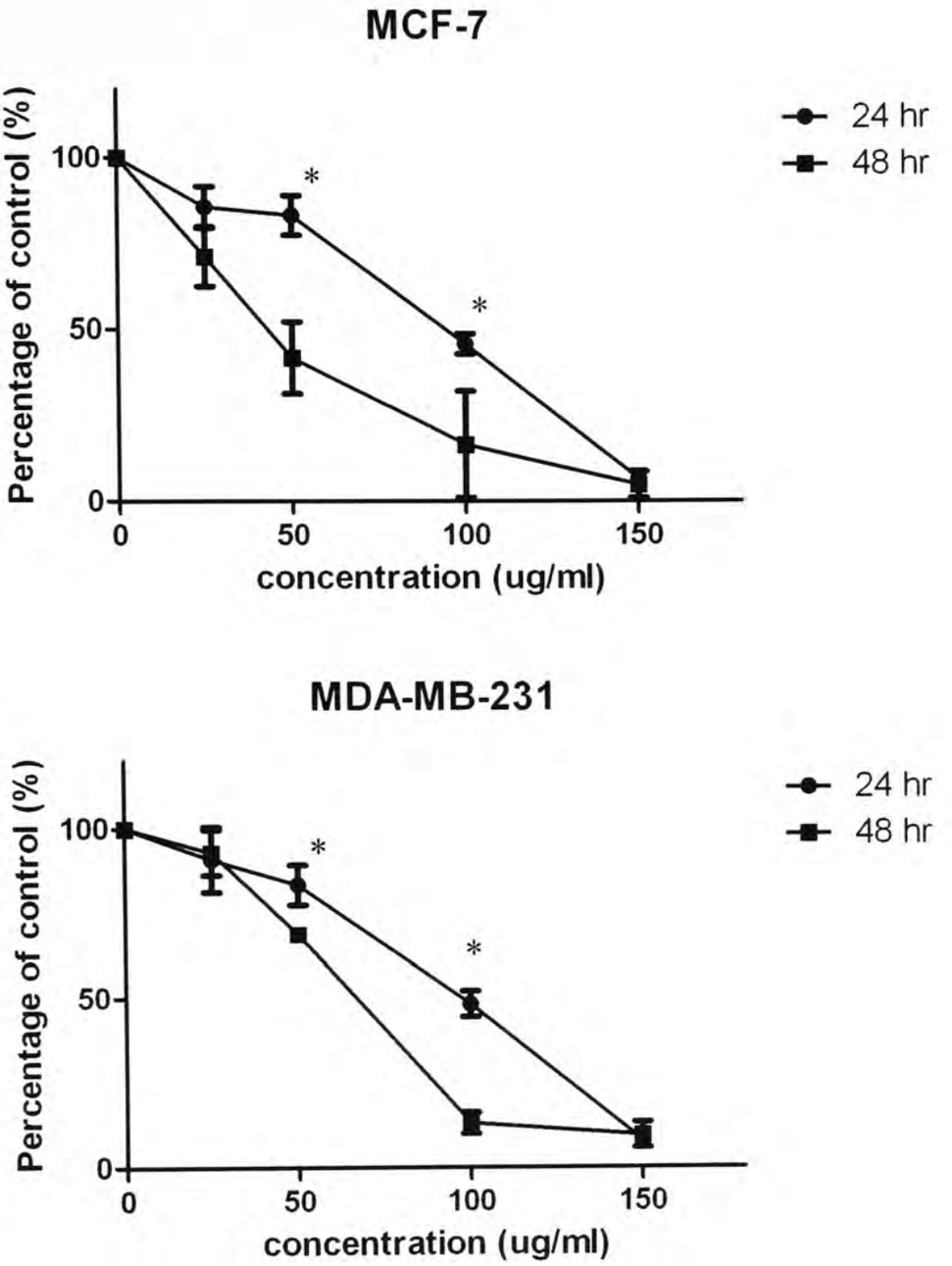


Fig. 3.18. Effect of sub-fraction Fc of Acanthopanax on the proliferation of MCF-7 and MDA-MB-231 cells after 24 and 48 hours of treatment. Cells were seeded in 96-well plate and incubated at 37 °C, 5 % CO_2 overnight. The cells were then treated with various concentrations of Fc for 24 or 48 hours. Cell viability was then determined by MTT assay. Results were expressed as percentage of absorbance with respect to control (100%). Results are mean ± S.D. of 3 determinations. Statistical significance between 24 and 48 hour treatment was determined by Student's unpaired t-test. *$p < 0.05$

3.9. Flow cytometric analysis

The underlying mechanisms of the anti-proliferative effect of sub-fraction Fc towards the breast cancer cell lines were further investigated by using of flow cytometry. Aspects including mitochondrial membrane potential change, cell cycle change and externalization of PS molecules were studied.

3.9.1 JC-1 staining

Various concentrations of Fc were used to treat MCF-7 and MDA-MB-231 cells for 48 hours and the changes in mitochondrial membrane potential were studied by staining the cells with JC-1 and analyzed with flow cytometry. The data were then interpreted with software WinMDI 2.9. Results of JC-1 staining towards MCF-7 and MDA-MB-231 cells were shown in Fig. 3.19 and Fig. 3.20 respectively. The results revealed that 48-hour treatment of Fc would induce mitochondrial membrane depolarization in both MCF-7 and MDA-MB-231 cells when compared with the control. The increase in percentage of cells with depolarized mitochondrial membrane was found to be dose-dependent.

3.9.2 Annexin-PI labeling

MCF-7 and MDA-MB-231 cells were treated with Fc for 48 hours and then labeled with FITC-conjugated Annexin V and PI. Results were presented as bivariant dot plot in Fig. 3.21 and Fig. 3.22 for MCF-7 and MDA-MB-231 cells respectively. Treatment of Fc could induce a shift in cell population from AV, PI double negative area to AV

positive, PI negative and AV, PI double positive area sequentially in MDA-MB-231 cells. Although Fc could also create numerical increase in apoptotic region in MCF-7 cells, the pattern is somehow different from that observed in MDA-MB-231 cells. No discrete population could be seen in AV positive and PI negative area and there seems no track of movement from AV positive and PI negative area to AV, PI double positive area.

3.9.3 Cell cycle analysis

DNA was extracted from breast cancer cells treated with Fc for 48 hours and then subjected to cell cycle analysis. Data were presented as histograms in Fig. 3.23 and Fig. 3.24 for MCF-7 and MDA-MB-231 cells respectively. For MCF-7 cells, no obvious sub-G1 peak could be observed, even if 80 μg/ml of Fc were added. In contrast, a dose-dependent increase in percentage of cells under G1 phase could be observed upon the treatment of Fc. At the same time, percentage of cells in S or G2/M phase decreased accordingly after the treatment with Fc. On the other hand, treatment of MDA-MB-231 cells with Fc resulted in an increase in sub-G1 peak and a dose-dependent effect could be seen. Numerical data were shown in Table 3.5.

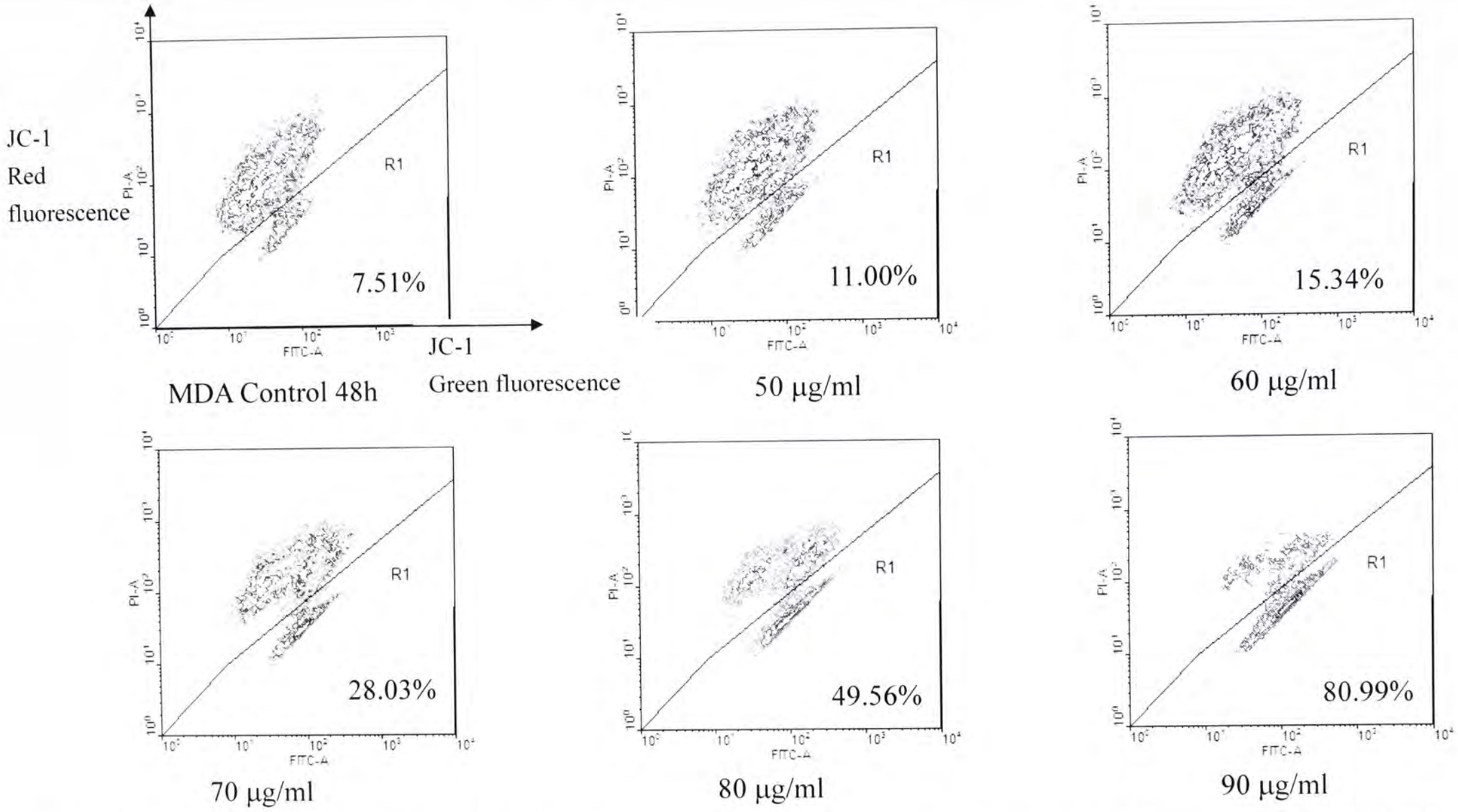


Fig. 3.19 Effect of sub-fraction Fc on MDA-MB-231 cells mitochondrial membrane potential. MDA cells (2×10^5) were seeded into 6-well plate and incubated overnight at 37°C, 5 % CO_2. Sub-fraction Fc of concentrations from 50 μg/ml to 90 μg/ml were added to the wells and incubate for another 48 hours at 37°C, 5 % CO_2. Cells were then collected and stained with JC-1 and then subjected to flow cytometry. Emission from FITC and PI channels were collected. Cells in region R1 were considered to have depolarized mitochondrial membrane.

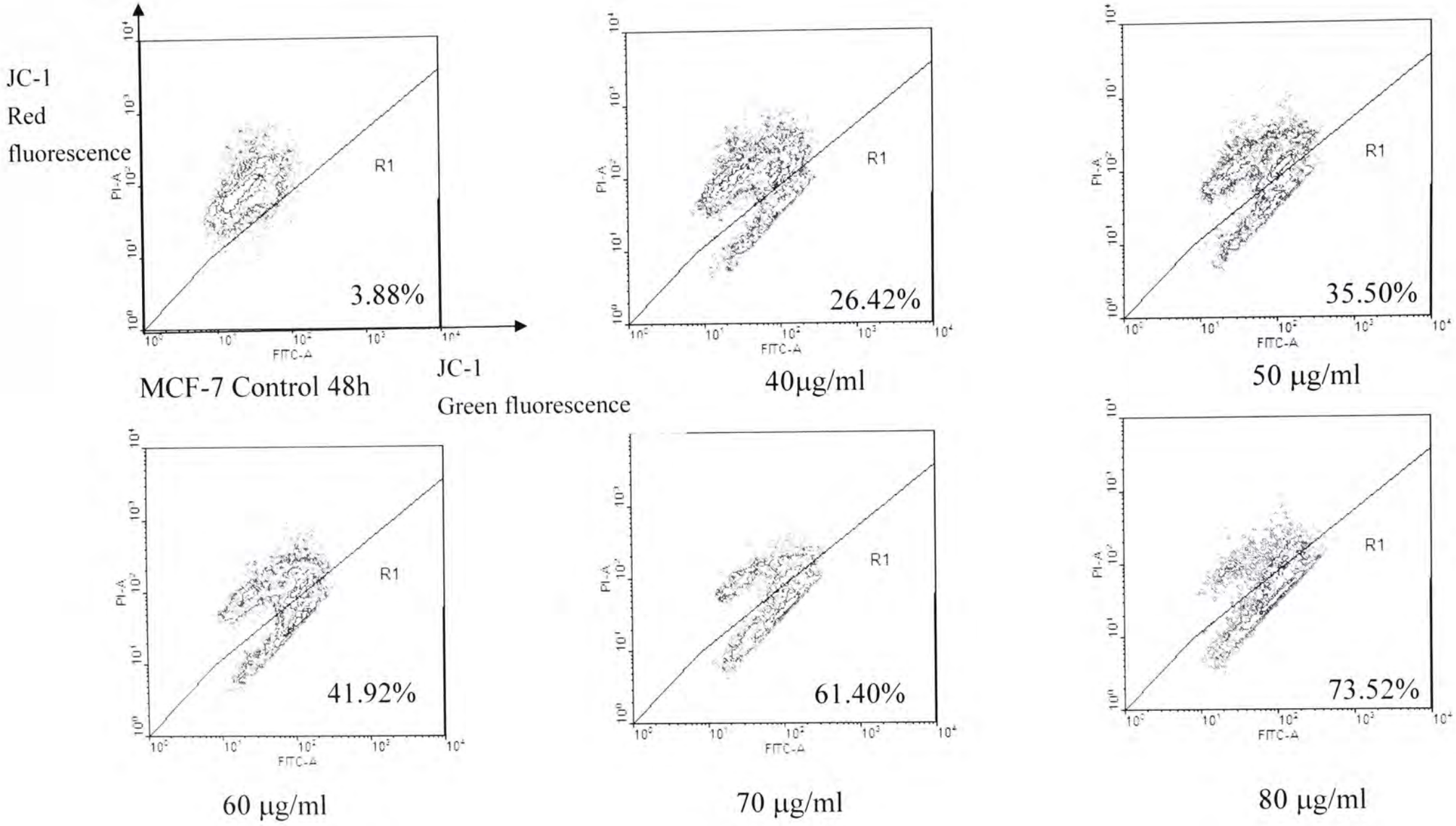


Fig. 3.20 Effect of sub-fraction Fc on MCF-7 cells mitochondrial membrane potential. MCF-7 cells (2 x 10^5) were seeded into 6-well plate and incubated overnight at 37°C, 5 % CO_2. Sub-fraction Fc of concentrations from 50 μg/ml to 90 μg/ml were added to the wells and incubate for another 48 hours at 37°C, 5 % CO_2. Cells were then collected and stained with JC-1 and then subjected to flow cytometry. Emission from FITC and PI channels were collected. Cells in region R1 were considered to have depolarized mitochondrial membrane.

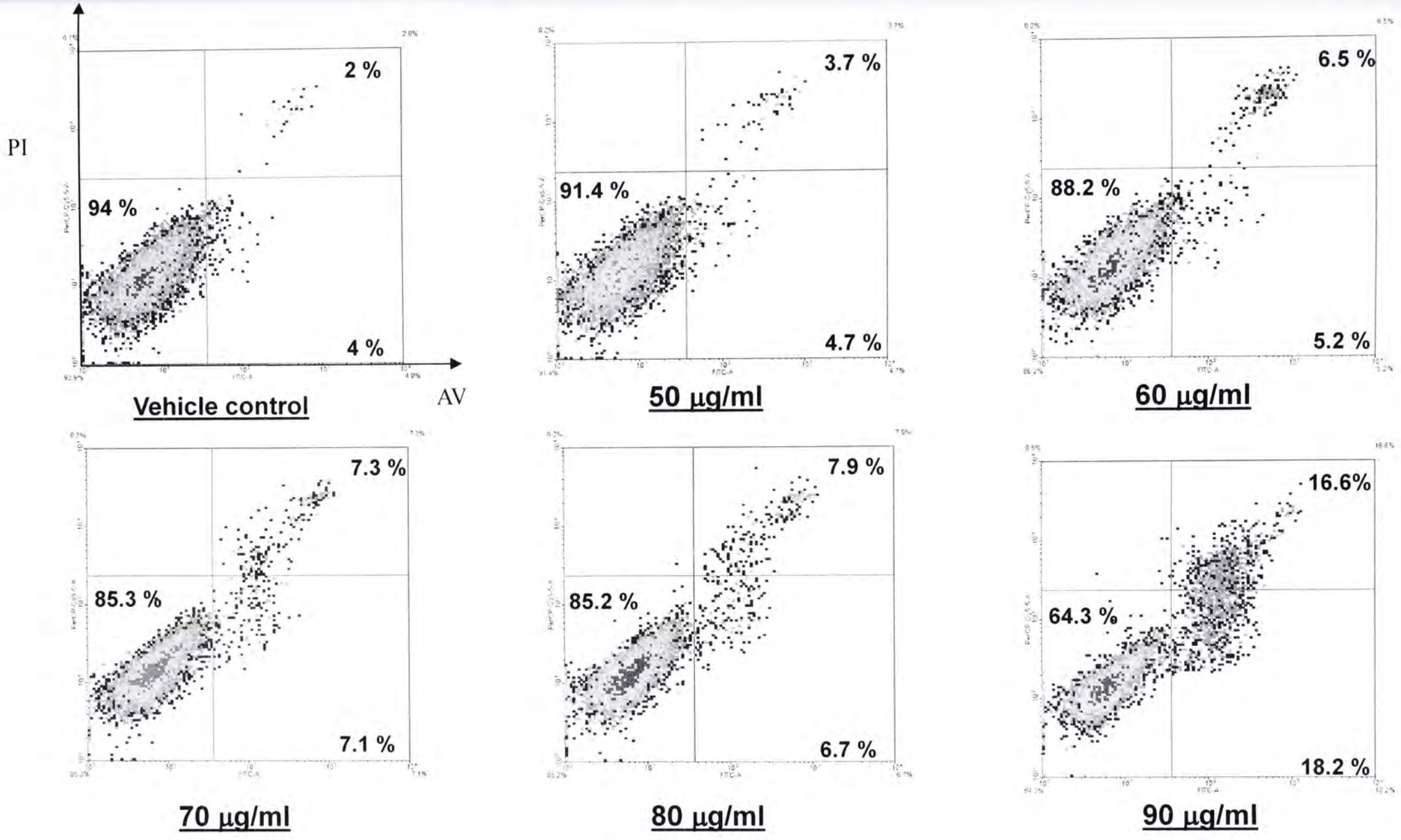


Fig. 3.21 Effect of sub-fraction Fc on the induction of apoptosis in MDA-MB-231 cells. MDA-MB-231 cells (2 x 10^5) were seeded into 6-well plate and incubated overnight at 37°C, 5 % CO_2. Fc of concentrations from 50 µg/ml to 90 µg/ml were added to the wells and incubate for another 48 hours. Cells were then collected and stained with Annexin-V (5 µl/test) and PI (5 µg/ml) and then subjected to flow cytometry. Emission from FITC and PerCP-Cy5.5 channels were collected

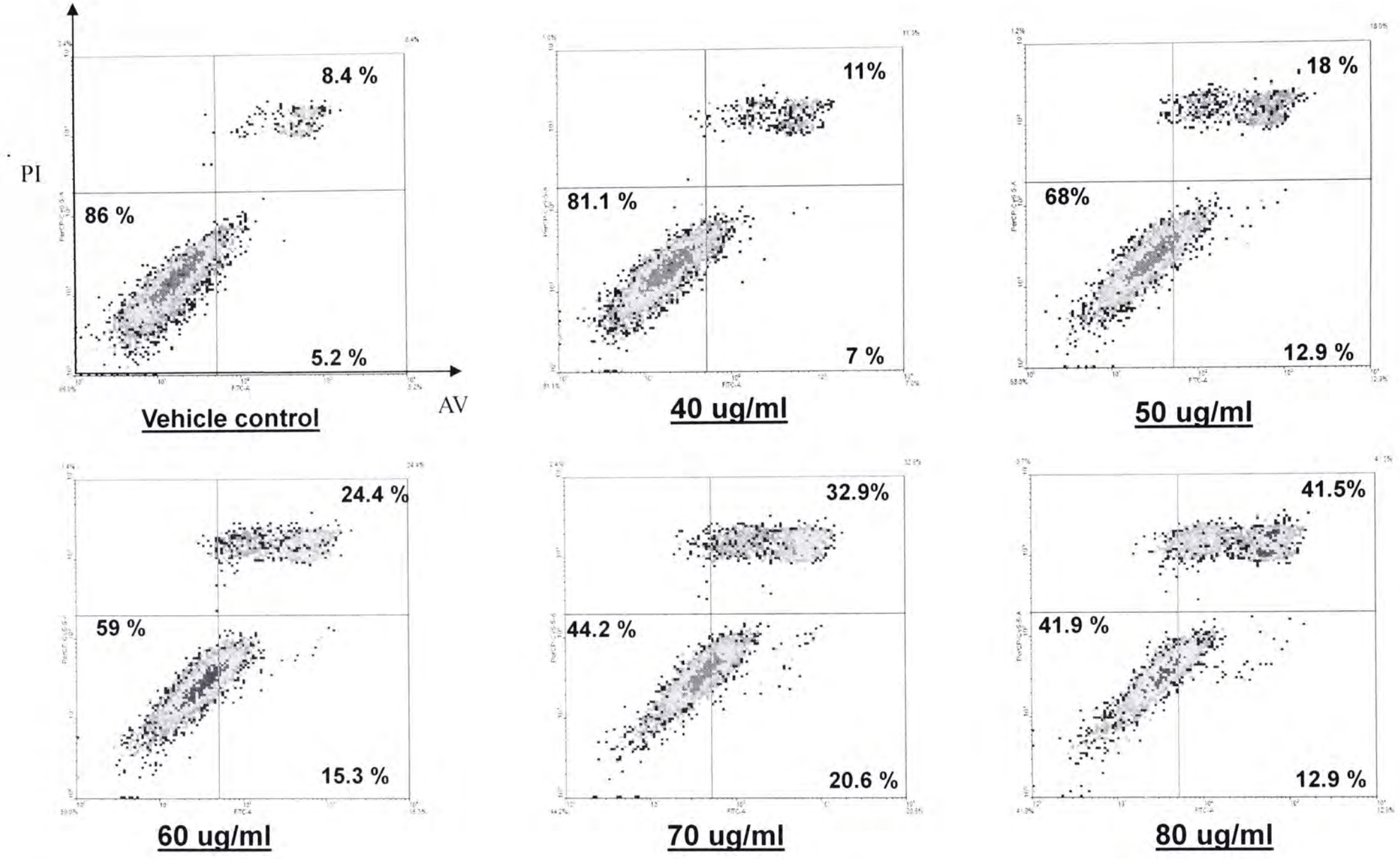


Fig. 3.22 Effect of sub-fraction Fc on the induction of apoptosis in MCF-7 cells. MCF-7 cells (2 x 10^5) were seeded into 6-well plate and incubated overnight at 37°C, 5 % CO_2. Fc of concentrations from 40 μg/ml to 80 μg/ml were added to the wells and incubate for another 48 hours. Cells were then collected and stained with Annexin-V (5 μl/test) and PI (5 μg/ml) and then subjected to flow cytometry. Emission from FITC and PerCP-Cy5.5 channels were collected.

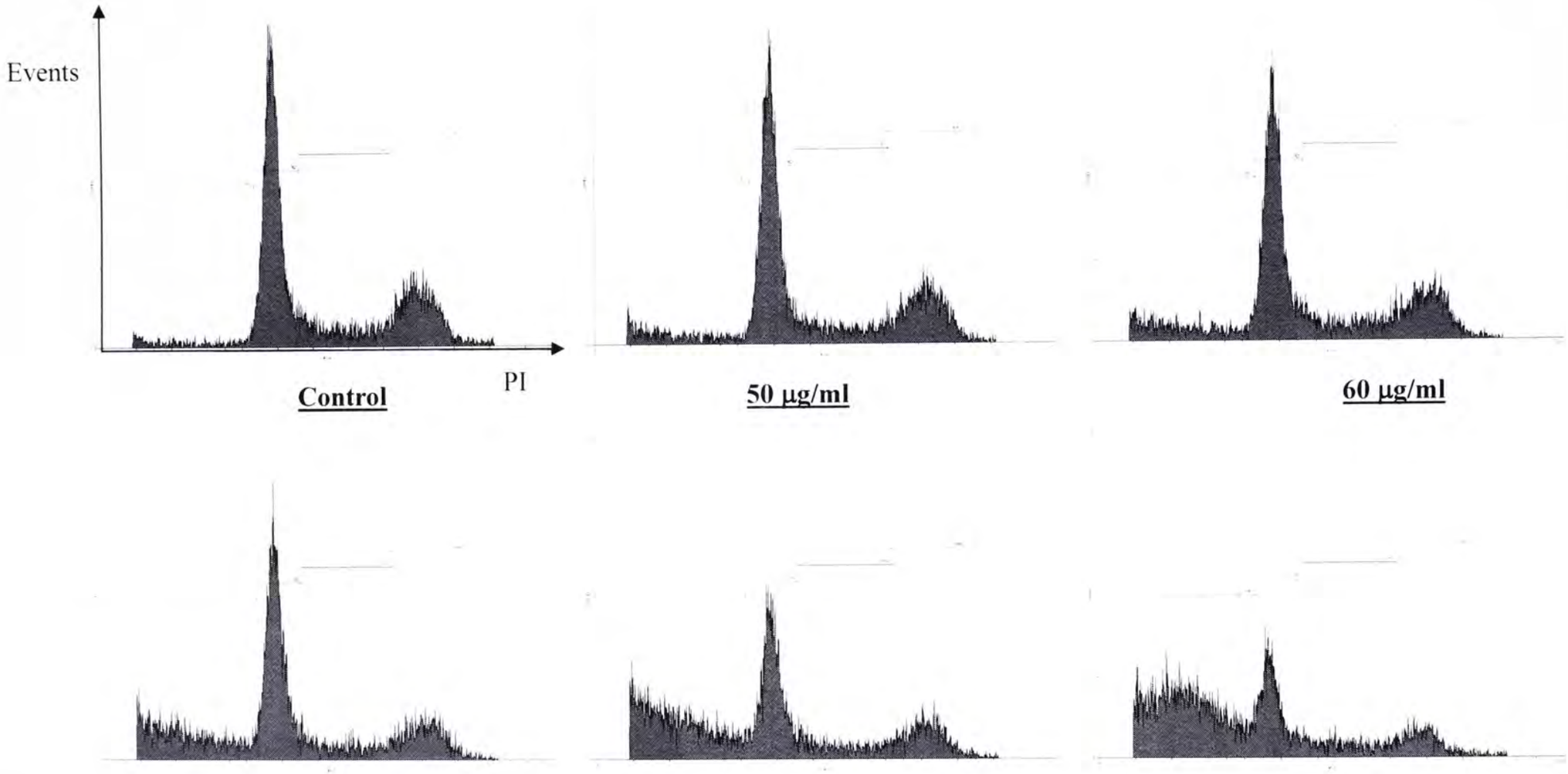


Fig. 3.23 Effect of sub-fraction Fc on MDA-MB-231 cell cycle progression. Cells (2 x 10^5) seeded in 6-well plate were treated with various concentrations of Fc for 48 hours. Cells harvested were fixed with ethanol and stained with PI (43 µg/ml). Cell cycle distributions were determined by flow cytometric analysis. Histograms presented are representative of three individual experiments.

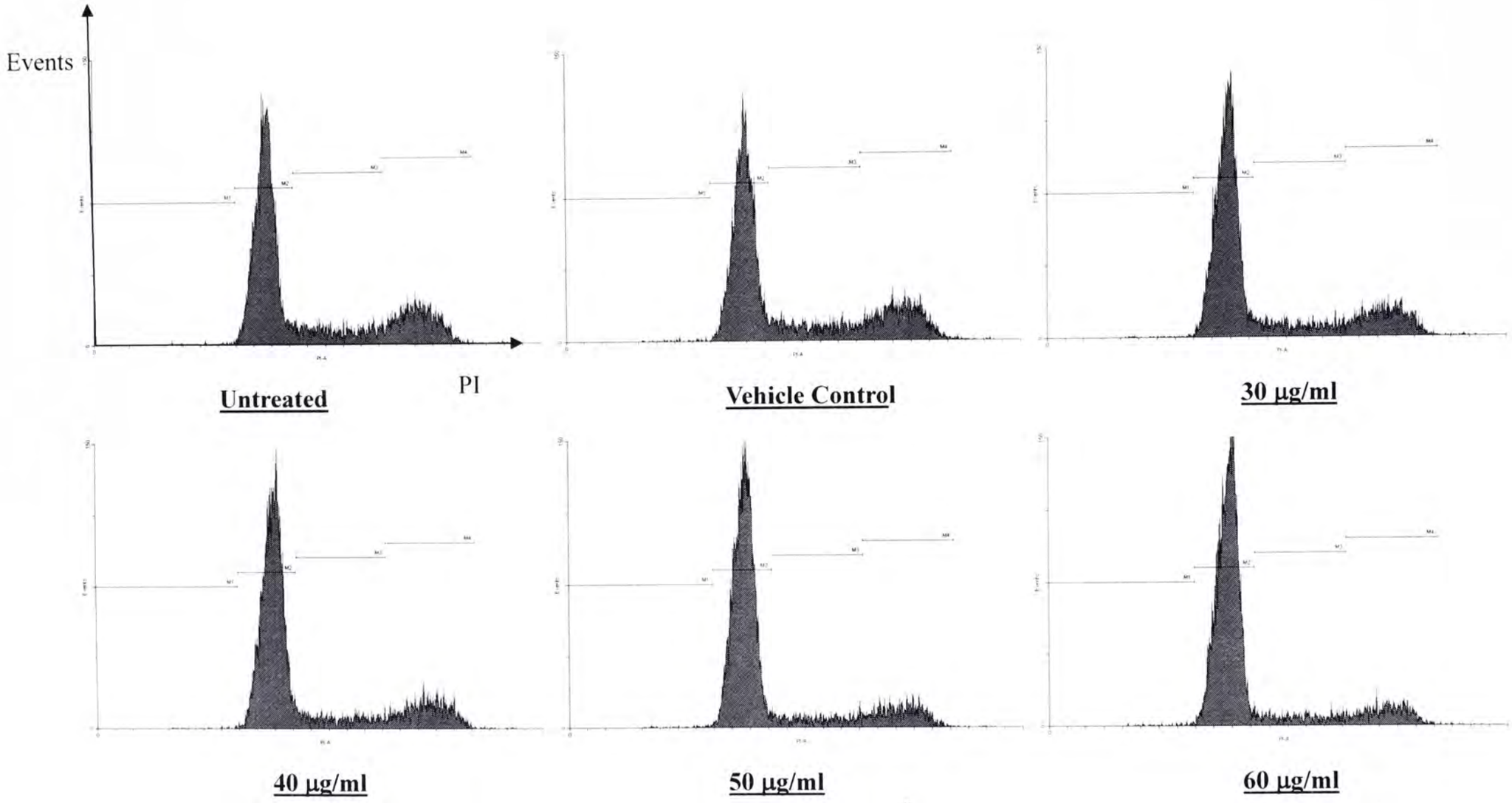


Fig. 3.24 Effect of sub-fraction Fc on MCF-7 cell cycle progression. Cells (2 x 10^5) seeded in 6-well plate were treated with various concentrations of Fc for 48 hours. Cells harvested were fixed with ethanol and stained with PI (43 µg/ml). Cell cycle distributions were determined by flow cytometric analysis. Histograms presented are representative of three individual experiments.

Table 3.5 Summary of cell cycle analysis

	Sub-G1	G_0/G1	S	G2/M
Control	4.84 ± 1.31	54.24 ± 2.34	16.78 ± 1.92	23.98 ± 1.29
50 μg/ml	7.01 ± 3.68	54.05 ± 4.16	15.03 ± 1.71	23.84 ± 0.41
60 μg/ml	9.68 ± 4.77	52.05 ± 4.94	14.06 ± 1.01	23.79 ± 2.28
70 μg/ml	24.27 ± 5.82*	42.57 ± 8.46*	10.45 ± 1.2*	22.38 ± 5.08
80 μg/ml	34.75 ± 5.05*	34.20 ± 3.09*	20.78 ± 4.45	15.65 ± 4.44
90 μg/ml	54.60 ± 4.42*	21.91 ± 3.10*	10.53 ± 1.60*	12.62 ± 2.59*

Cell cycle distribution of MDA-MD-231 cells treated with sub-fraction Fc were determined by PI staining and flow cytometric analysis. Results presented are representative of 3 individual experiments (Mean ± S.D., n = 3). Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$

	Sub-G1	G_0/G1	S	G2/M
Control	0.57 ± 0.16	62.18 ± 0.73	15.33 ± 1.01	21.93 ± 0.19
Vehicle Control	0.56 ± 0.03	62.08 ± 2.03	15.32 ± 2.13	22.01 ± 1.76
30 μg/ml	0.43 ± 0.14	69.43 ± 1.11*	12.01 ± 1.48	18.04 ± 2.57
40 μg/ml	0.48 ± 0.03	71.21 ± 1.84*	10.69 ± 1.58*	17.52 ± 1.39*
50 μg/ml	0.61 ± 0.16	76.72 ± 3.45*	9.3 ± 2.17*	13.35 ± 1.52*
60 μg/ml	0.62 ± 0.12	79.16 ± 3.96*	8.12 ± 2.79*	12.06 ± 1.36*

Cell cycle distribution of MCF-7 cells treated with sub-fraction Fc were determined by PI staining and flow cytometric analysis. Results presented are representative of 3 individual experiments (Mean ± S.D., n = 3). Statistical significance between extract treated and control was determined by Student's unpaired t-test. *$p < 0.05$

3.10 DNA fragmentation assay

After 48 hours of treatment with Fc, genomic DNA was extracted from MCF-7 and MDA-MB-231 cells and analyzed with gel electrophoresis. Results of DNA fragmentation assay of MCF-7 and MDA-MB-231 cells were shown in Fig. 3.25 and Fig. 3.26 respectively. No DNA ladder could be observed in Fc treated MCF-7 cells although the highest concentration added was as high as 80 μg/ml. In contrast, internucleosomal DNA fragments could be seen in Fc treated MDA-MB-231 cells. And a dose-dependent effect was obvious at high concentrations (70 and 80 μg/ml).

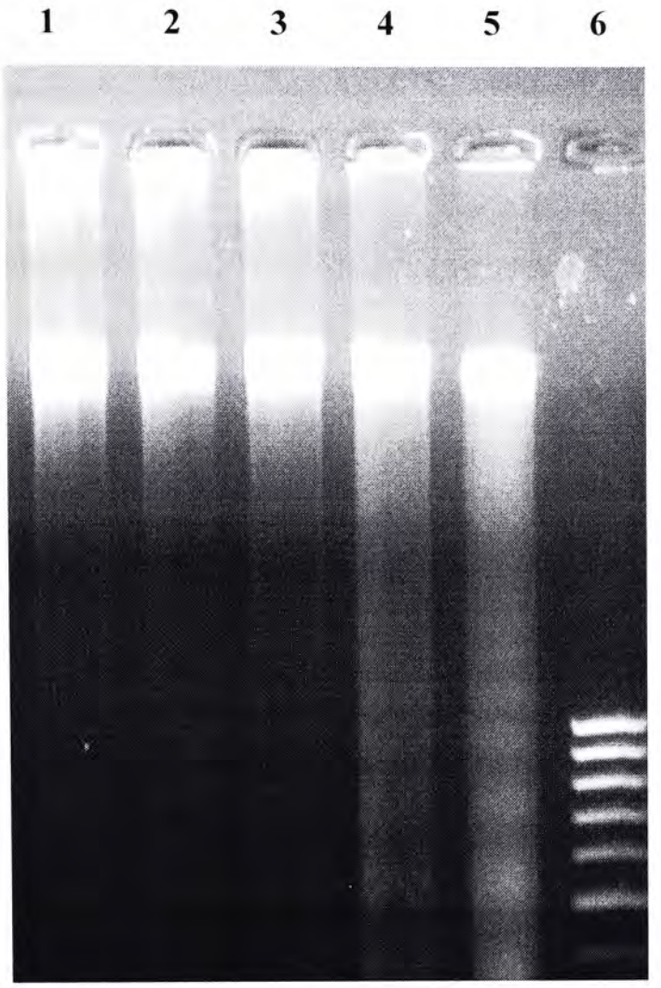


Fig. 3.25 DNA fragmentation analysis of MDA-MB-231 cells after 48 hours of treatment with various concentrations of sub-fraction Fc of Acanthopanax. Genomic DNA was prepared as described in Materials and Methods and analyzed with 1.5 % agarose gel electrophoresis. The figure presented is representative of the results of three individual experiments.

Lane 1 – vehicle control; Lane 2 – 5: Fc of concentrations 50, 60, 70 and 80 μg/ml respectively; Lane 6 – Marker

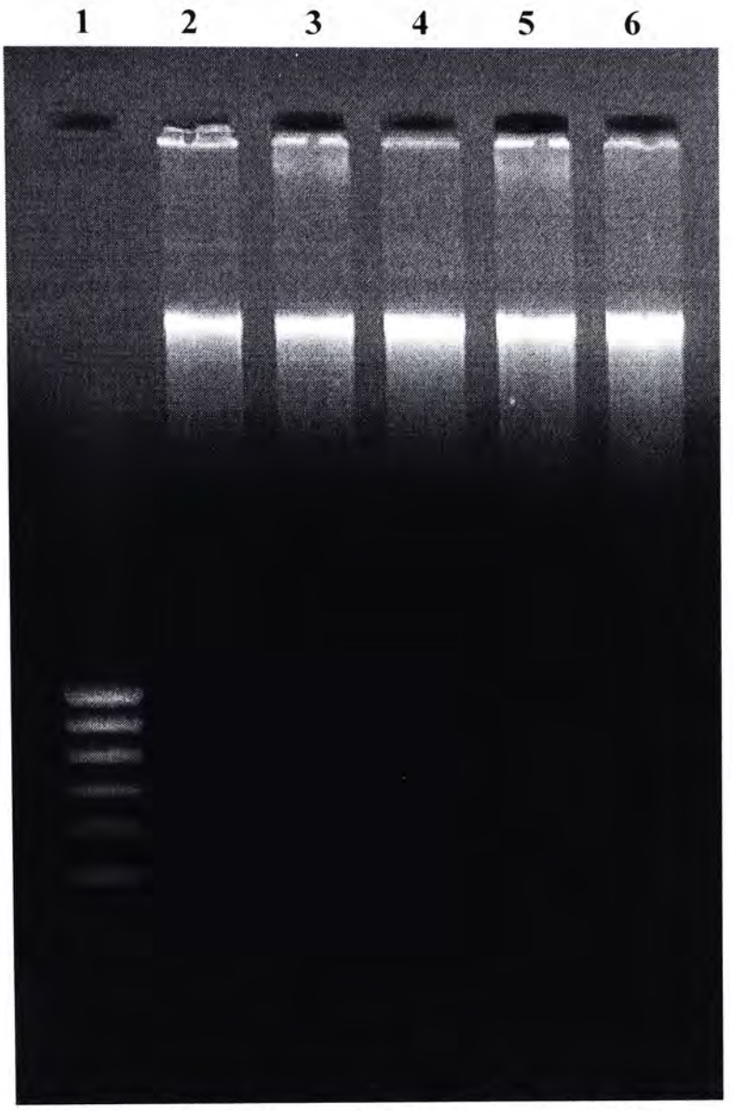


Fig. 3.26 DNA fragmentation analysis of MCF-7 cells after 48 hours of treatment with various concentrations of sub-fraction Fc of Acanthopanax. Genomic DNA was prepared as described in Materials and Methods and analyzed with 1.5 % agarose gel electrophoresis. The figure presented is representative of the results of three individual experiments.

Lane 1 – Marker ; Lane 2 – Vehicle control; Lane 3 – : Fc of concentrations 50, 60, 70 and 80 μg/ml respectively.

3.11 Western blotting

According to the results of flow cytometry analysis and DNA fragmentation assay, the amounts of various proteins in MCF-7 and MDA-MB-231 cells were studied with the help of Western blotting.

Since the results mentioned above suggested that Fc may induce apoptosis in MDA-MB-231 cells, some apoptotic regulators were probed with specific antibodies and the results were shown in Fig. 3.27 to 3.30. The expression of anti-apoptotic protein Bcl-2 was decreased upon the treatment with Fc for 48 hours. As shown in Fig. 3.27, the amount of Bcl-2 decreased as the concentration of Fc added was increased. Besides, the treatment of MDA-MB-231 cells with Fc could induce the cleavage of procaspase-9 into caspase-9 since the amount of procapase-9 decreased upon Fc treatment while the amount of caspase-9 increased accordingly (Fig. 3.28). And a dose-dependent manner could be observed, the higher the concentration used, the more the cleavage of procaspase-9. Accompanying this was the decrease in procaspase-3 in Fc treated MDA-MB-231 cells. A concentration dependent effect could be observed in the decrement of procaspase-3 (Fig. 3.29). In addition, the amount of PARP found was also lowered after the exposure to Fc. The band of PARP detected was reduced in a dose-dependent manner.

On the other hand, previous results suggested the possibility of G0/G1 cell cycle

arrest maybe induced by Fc. As a result, proteins which play important roles in regulating cell cycle were probed in MCF-7 cells and the results were shown in Fig. 3.31 and Fig. 3.32. Both cyclin D_1 and cdk 2 were required for the progression from phase G1 phase to S phase. Upon the addition of Fc, the amounts of both cyclin D_1 and cdk 2 were found to be decreased in a dose-dependent manner. This suggested that Fc could induce G0/G1 cell cycle arrest in MCF-7 cells via modulation of cyclin D_1 and cdk 2 protein expression.

Bcl-2

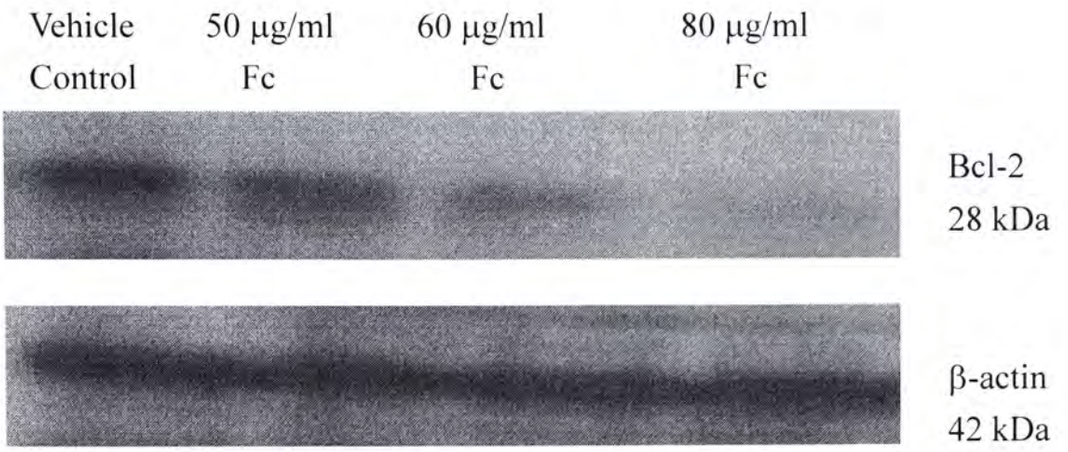


Fig. 3.27 Effect of sub-fraction Fc of Acanthopanax on the expression of Bcl-2 protein in MDA-MB-231 cells. $2x10^6$ MDA-MB-231 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-Bcl-2 antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

Procaspase-9 and caspase-9

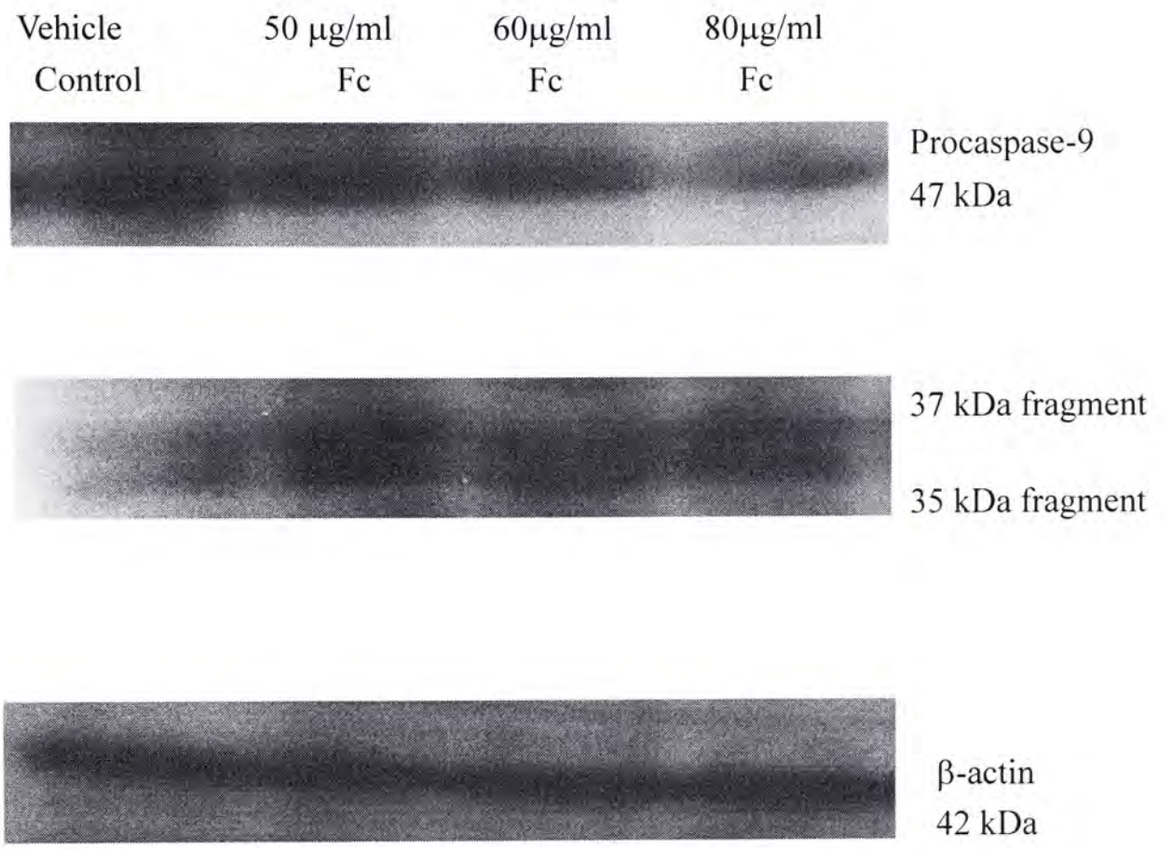


Fig. 3.28 Effect of sub-fraction Fc of Acanthopanax on the expression of procaspase-9 and caspase-9 in MDA-MB-231 cells. $2x10^6$ MDA-MB-231 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-procaspase-9 and anti-caspase-9 antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

Procaspase-3

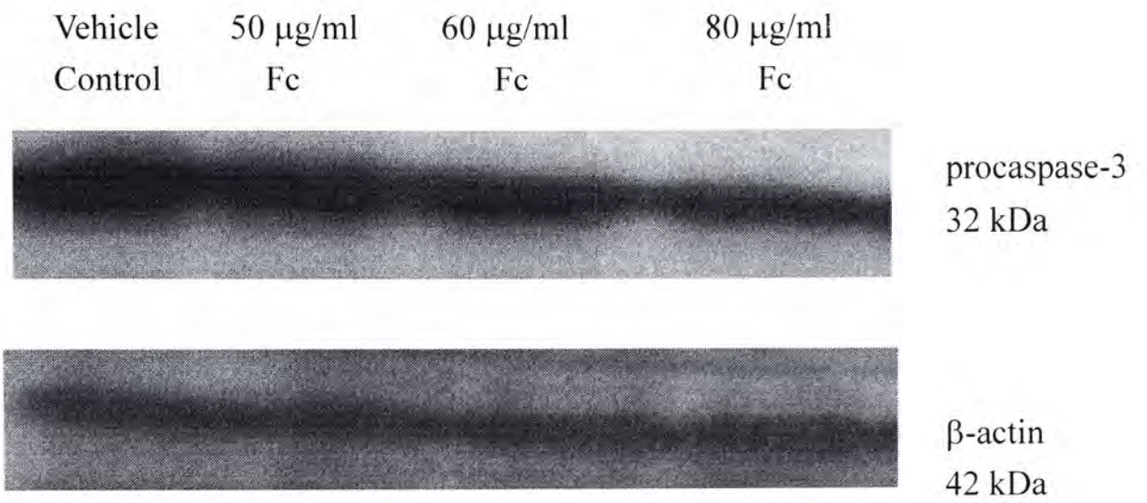


Fig. 3.29 Effect of sub-fraction Fc of Acanthopanax on the expression of procaspase-3 in MDA-MB-231 cells. $2x10^6$ MDA-MB-231 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-procaspase-3 antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

PARP

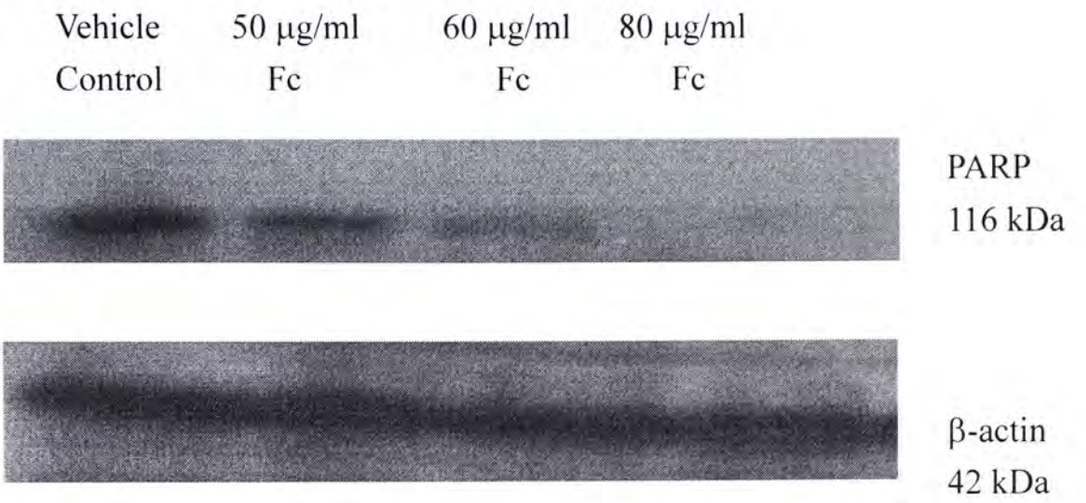


Fig. 3.30 Effect of sub-fraction Fc of Acanthopanax on the expression of PARP in MDA-MB-231 cells. $2x10^6$ MDA-MB-231 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-PARP antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

Cyclin D_1

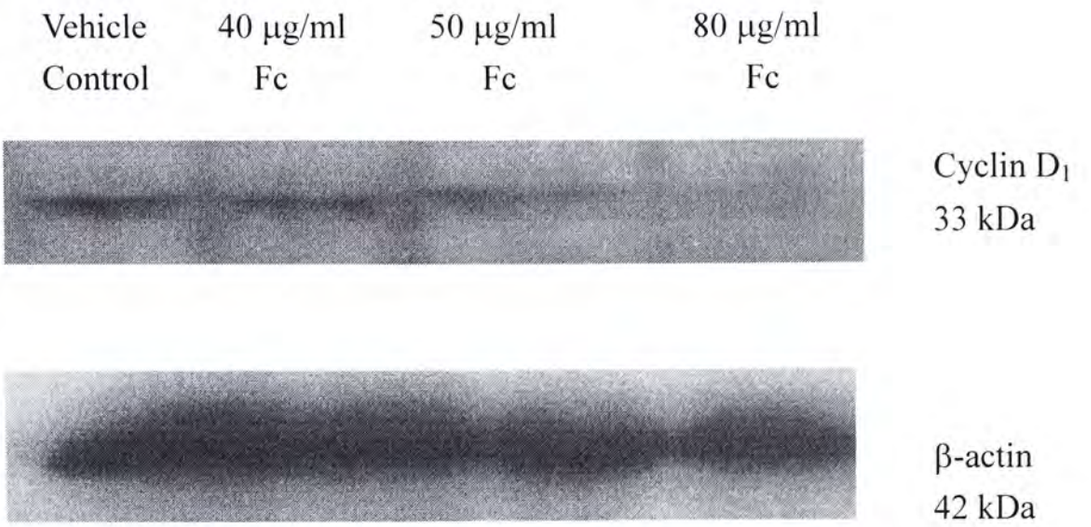


Fig. 3.31 Effect of sub-fraction Fc of Acanthopanax on the expression of cyclin D_1 in MCF-7 cells. $2x10^6$ MCF-7 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-cyclin D_1 antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

Cdk 2

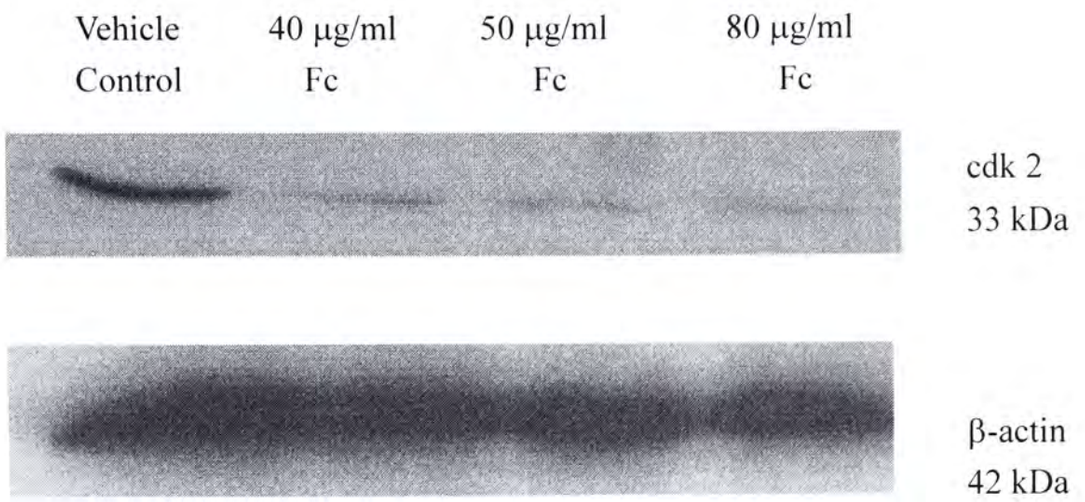


Fig. 3.32 Effect of sub-fraction Fc of Acanthopanax on the expression of cdk 2 in MCF-7 cells. $2x10^6$ MCF-7 cells were seeded in 100 mm^2 dish overnight. The cells were then treated with various concentrations of Fc and incubated for another 48 hours. Both floating and adherent cells were collected for analysis. Cell lysates were prepared and normalized for total protein content. Normalized lysates were analyzed by SDS-PAGE and immunoblotting using anti-cdk 2 antibodies. Vehicle control was prepared by treating cells with 0.5% DMSO, which is equivalent to the DMSO content in Fc treatment groups. β-actin was used for internal normalization. Results presented are representative of 3 individual experiments.

Chapter 4 Discussion

Astragalus and Maitake have been long known to be effective in treating tumor. There are lots of studies about the effects of Astragalus and Maitake towards our immune system and how these effects could benefit our body defense against the invasion of cancer cells. It has been proposed that the anti-tumor effect of Astragalus and Maitake was mediated through boosting the host immunity. However, there are only limited reports about the cytotoxic action of Astragalus and Maitake towards cancer cells. Although there are investigations reporting that apoptosis induction may play a part in the anti-tumor effect of Astragalus or Maitake, the cytotoxic effect of Astragalus or Maitake has not been fully investigated, therefore this raised our interest about the study of the cytotoxic effect of Astragalus and Maitake.

On the other hand, the anti-tumor effect of Acanthopanax is less well-characterized. Previously, there are reports about the cytotoxic effect of Acanthopanax extracts towards cancer cells such as stomach cancer KATO III cells or other leukemia cell lines as described above. There were no reports about the anti-tumor activities of Acanthopanax towards breast cancer or hepatoma. Moreover, the detailed mechanism behind the anti-tumor activity of Acanthopanax was not clear at the moment. Therefore, we would like to study the direct cytotoxic effect of these three herbs towards cancer cells. One human hepatoma cell line, Hep G2 and two

human breast cancer cell lines, MCF-7 and MDA-MB-231 were employed in this investigation. The two breast cancer cell lines used represent different stages of breast cancer. Estrogen receptor positive MCF-7 cells are regarded as prototype of early stage of breast cancer which is hormone-sensitive while estrogen receptor negative MDA-MB-231 cells reflect a latter stage of breast cancer which is more invasive and not hormone-sensitive.

The results of MTT assay was employed as the screening test for checking the anti-proliferative effect of the extracts towards different cancer cell lines since it is well-established that MTT could correlate the number of living cells with the formazan salt formed (Alley et al., 1988;Mosmann, 1983). Therefore, it is a rapid, easy and valid assay in studying the anti-proliferative effect of various herbal extracts.

Previously, different classes of compounds, including polysaccharides, flavanoids, saponins, sterols, courmarins and amino acids, were isolated from Astragalus, Maitake and Acanthopanax (Zhang et al, 2002 ; Wang et al, 2007; Wang et al, 2002). These compunds vary in polarity due to their differences in chemical structure. Gernerally speaking, compounds with higher polarities, like polysaccharides, flavonids or amino acids, are more likely to be found in the aqueous or 50 % ethanolic extract. On the other hand, compounds like saponins, sterols or courmarins are less polar, and therefore maybe found mainly in 50 % or 95 %

ethanolic extracts.

The results of MTT assays of aqueous extracts of Astragalus and Maitake revealed that both extracts did not have much impact on the proliferation on the three tested cancer cell lines since none of the extracts exhibited significant effect on the growth of the cancer cells. Therefore, we tried to draw out the constituents with lower polarity by extracting the herbs with 50 % and 95 % ethanol. However, no significant inhibitory effect on cell viabilities in the tested cells was seen even if we changed the extraction solvent. Therefore, we concluded that aqueous, 50 % and 95 % ethanol extracts of Astragalus and Maitake did not possess cytotoxic effect towards the three tested cell lines, below concentration of 800 μg/ml.

In contrast, the aqueous extract of Acanthopanax exhibited suppressive effect on MCF-7, MDA-MB-231 and Hep G2 cells in a dose-dependent manner. The aqueous extact of Acanthopanax reduced the proliferation of MCF-7, MDA-MB-231 and Hep G2 cells to 50 % at concentrations about 355 μg/ml, 355 μg/ml and 400 μg/ml respectively. The anti-proliferative effect of Acanthopanax extract towards breast cancer cells was relatively stronger. In fact, statistically significant difference could be found between the anti-proliferative effect on breast cancer cells and Hep G2 cells, in concentrations from 300 μg/ml to 800 μg/ml. This suggested that breast cancer cells were more sensitive to the suppressive effect of Acanthopanax when compared with

Hep G2 cells. In regard of this, we have decided to concentrate our effort in studying the anti-proliferative effect of Acanthopanax extract on breast cancer cells. In addition, the anti-proliferative effect of Acanthopanax extract on Hs68 cell line which is an untransformed human fibroblast cell line was also tested and the 50 % inhibition of proliferation of Hs68 cells was seen at a concentration of 720 μg/ml, which was much higher than that on cancer cells. Acanthopanax aqueous extract suppressed proliferation of breast cancer cells at a higher extent when compared with normal cells. The differential effect of Acanthopanax on breast cancer cells and normal cells suggests that the anti-proliferative effect of Acanthopanax extract may possess selectivity to certain extent.

Acanthopanax was extracted and fractionated previously by other groups. Hot methanol extract of Acanthopanax was fractionated by partitioning in a sequence with ether, chloroform and then butan-1-ol (Deyama et al., 2001). Therefore, similar protocol was adopted except that we added another organic solvent ethyl-acetate to the fractionation protocol, just like many other herbs that were fractionated before (Chiu et al., 2006). The addition of ethyl-acetate into the fractionation protocol could further sub-localize the compounds and achieving a more detailed fractionation. The results in other investigations showed that sometimes the ethyl-acetate fraction exhibited promising anti-tumor activities, and the ethyl-acetate may play a vital role

in the whole fractionation protocol (Chiu et al, 2006). Another modification we made to the fractionation protocol is that we replaced the petroleum ether as suggested in other studies with hexane (Wu et al., 2008). The replacement is due to the fact that petroleum ether is a mixture of volatile hydrocarbon rather than a single compound which is mainly composed of alkanes such as pentane, hexane and heptane. Considering the fact that petroleum ether and hexane share similar polarity, replacing petroleum ether with hexane would make the fractionation protocol more reproducible.

Among the four partition fractions, chloroform and ethyl-acetate demonstrated the strongest anti-proliferative effect on the breast cancer cell lines. Chloroform fraction reduced the proliferation of MCF-7 and MDA-MB-231 cells to half at 50 μg/ml and 80 μg/ml respectively while ethyl-acetate fraction reduced the proliferation of both breast cancer cell lines to half at 70 μg/ml. Other fractions, such as hexane fraction containing less polar compounds and butanol fraction consisting of compounds with higher polarity, inhibited the proliferation of cancer cells less effectively. Therefore, we may say that compounds responsible for the anti-tumor activity of Acanthopanax should have a polarity similar to that of chloroform and ethyl-acetate. Considering the fact that the yield of ethyl-acetate fraction (9.78%) was much higher than that of chloroform fraction (2.62%), constituents in ethyl-acetate

fraction may play a more important role in the anti-tumor effect of Acanthopanax. Therefore, the ethyl-acetate fraction rather than chloroform fraction was chosen for further investigation.

Constituents in ethyl-acetate fraction were further isolated by employing silica gel as the stationary phase of column chromatography. Discontinuous gradient elution was performed. Mobile phase of hexane and ethyl-acetate in a ratio of 5 : 3 was used at the start and then replaced by a more polar solvent system consisting of 10 : 9 hexane and ethyl-acetate, and then eluted with pure ethyl-acetate and methanol. The yield of the various sub-fractions was listed in Table 3.4. Fi was the sub-fraction with the highest yield, which was more than 50 %, followed by Fg and Fc. MTT assay was used to investigate the anti-proliferative effect of the sub-fractions. And the results of Fi revealed that Fi did not have much effect on breast cancer cell proliferation with an IC_{50} of higher than 150 μg/ml. Since Fi is the last fraction eluted from the column, Fi contains compounds with relatively higher polarity. As indicated in the comparison of the anti-tumor activities of partition fractions, polar compounds in Acanthopanax extract exhibited weaker anti-tumor activity relative to their counterpart with lower polarity. Therefore, the weak anti-tumor activity of Fi found is actually consistent with the results of anti-proliferative effect of the partition fractions.

The anti-proliferative effect on MCF-7 and MDA-MB-231 cells were

investigated for every fraction. The MTT results of the sub-fractions were shown in Fig. 3.8- Fig. 3.16. One point worth noting is that the IC_{50} of the sub-fractions on MCF-7 cells are lower than their IC_{50} on MDA-MB-231 cells in most cases when we compared the anti-proliferative effect of the same fraction on the two breast cancer cell lines. It seems that MCF-7 cells are more susceptible to the growth-inhibitory effect of Acanthopanax extract.

All the sub-fractions exhibited anti-proliferative effect on the growth of breast cancer cells, although they varied in potency. In general, all the sub-fractions would inhibit the growth of both breast cancer cell lines significantly at a concentration of 50 μg/ml, except for Fa and Fb. Among the sub-fractions, Fg demonstrated the strongest anti-proliferative effect on MCF-7 and MDA-MB-231 cells. Surprisingly, when we compared the anti-proliferative effect of ethyl-acetate fraction with those of sub-fractions generated from column chromatography, the difference between them is not obvious. This may be explained by the fact that most of the sub-fractions possess certain degree of anti-tumor activities. This means that there are multiple compounds contributing to the overall anti-tumor activities of Acanthopanax rather than one single compound with outstanding anti-tumor activity. This accounts for the majority of anti-tumor effect of the herb. Although the largest sub-fraction Fi did not exhibit strong anti-tumor activities, the interactions of other sub-fractions could be

complicated and the possibility of synergistic effect with each other should not be excluded and thereby contributing to the comparatively strong anti-tumor effect of ethyl-acetate fraction.

Other than potency, the selectivity of the anti-tumor drug is also of a great concern. Obviously, it will have little practical use if a drug could not differentiate between normal cells and malignant cells. In order to see whether the extract exhibited differential effect on the growth of malignant cells and normal cells, the effects of the sub-fractions Fc, Fd, Fe, Fg and Ff on the proliferation of normal human fibroblast cells Hs68 were also investigated and the results were compared with the anti-proliferative effect on the two breast cancer cell lines to see if there is any statistical significance.

Fc reduced proliferation of Hs68 cells to 50 % at 138 μg/ml and statistical significance ($p < 0.01$) could be found between the effects of Fc on the two breast cancer cell lines and Hs68 cells from concentration of 50 μg/ml to 100 μg/ml. Although the proliferation of Hs68 cells was reduced to about 30 % of control, Fc could already suppress the growth of MCF-7 and MDA-MB-231 cells to 10 % of control at 100 μg/ml while the proliferation of Hs68 cells stayed about the same as control at such a concentration. Therefore, we can say that Fc exhibited selective anti-proliferative effect on breast cancer cells, at least at concentrations below 100

μg/ml. For Fd, statistically significant difference in anti-proliferative effect between breast cancer cells and Hs68 cells could only be seen at concentrations from 25 to 50 μg/ml. On the other hand, both Ff and Fg also demonstrated selective anti-proliferative effect on breast cancer cells. Ff showed significant difference in inhibiting MDA-MB-231 and Hs68 cells in concentrations from 25 μg/ml to 150 μg/ml while significant difference could be seen between MCF-7 and Hs68 cells at all tested concentrations. Similarly, significant difference between the suppressive effect of Fg on the growth of breast cancer cells and Hs68 cells could be found at all tested concentrations. In contrast, sub-fraction Fe did not exhibit selective anti-proliferative effect on breast cancer cells. There was no significant difference between the anti-proliferative actions on breast cancer cells and Hs68 cells at all tested concentrations, if we compare their MTT results. In short, sub-fractions Fc, Fd, Ff and Fg had shown selectivity in inhibiting human breast cancer cells growth over normal fibroblast cells Hs68 cells.

Yet, to further confirm that the sub-fractions possess selectivity in inhibiting human breast cancer cells growth over normal cells, we have used another type of normal human cells (PBMC). Previously, other groups have also employed PBMC in comparing the cytotoxicity of anti-tumor compounds on tumor cells and normal cells (Lopez-Gonzalez et al., 2004;Sun et al., 2005;Yan et al., 2008;Sreenivasan et al.,

2006b). PBMC from buffy coats of healthy donors could be isolated by differential centrifugation using Ficoll- Paque ^{TM} Plus solution and therefore PBMC are a rapid and convenient source of normal human cells. The major cell populations found in PBMC include B cells, T cells, natural killer cells and monocytes which are the immune cells circulating in our cardiovascular system. The proliferation of PBMC also reflects how our immunity responds to the drug treatment. Mitogenic effect on PBMC is considered as a sign of activation of immune cells while suppressive effect on PBMC proliferation is regarded as immunosuppression. Therefore, studying the effect of herbal extract on PBMC proliferation could also give us a hint and insight on how the extract affects the immune system. Taken the fact that immunosuppression is one of the common side effects of cancer chemotherapy, testing the effect of anti-tumor drug on immune cells is reasonable and vital.

We have investigated the proliferation of PBMC under the treatment of sub-fractions Fc, Fd, Ff and Fg, which have demonstrated to have specific anti-tumor effect when Hs68 cells were used as normal cell control. Since PBMC are suspension cells rather than adherent in nature, XTT assay was used to replace MTT assay for monitoring the proliferation of PBMC. XTT assay is similar to MTT assay in nature, both of them measure the colorimetric change accompanying the conversion of tetrazolium salt into fomazan salt by mitochondrial enzymes. Since the formazan salt

generated in XTT salt reduction is positively charged and therefore water-soluble, an aspiration step could be skipped for XTT assay. As PBMC are suspension in nature, it is possible that PBMC would be removed together with the medium during the aspiration step required in MTT assay. Therefore, replacement of MTT with XTT assay could reduce the experimental error due to cell removal and gave a more accurate result.

The results of XTT assay were summarized in Fig. 3.17. Fd, Ff and Fg exhibited suppressive effect on the proliferation of PBMC with statistical significance at a concentration of 150 μg/ml. Fd and Fg reduced the proliferation of PBMC to more than 50 % at concentrations below 150 μg/ml. Although Fg only reduced the PBMC proliferation for about 30 %, significant inhibitory effect could still be seen. In contrast, Fc did not have any statistically significant effect on the growth of PBMC at all concentrations tested. PBMC proliferation remained comparable to that of control at 150 μg/ml of Fc while more than 90 % inhibition was seen at such concentration on the growth of human breast cancer cells. This suggested that Fc demonstrated selective inhibitory effect on human breast cancer cells.

From the MTT and XTT results, we can see that the anti-tumor activities of the various sub-fractions differed from each other in terms of potency and selectivity. Sub-fractions like Fe possessed potent anti-proliferative effect on both breast cancer

cell lines but it did not demonstrate much selectivity when we compared its effect with Hs68 cells and PBMC. Taking the potency of anti-proliferative effect, selectivity and percentage yield into consideration, Fc could be one the sub-fractions that gave the most promising anti-tumor activity. Therefore, the mechanism underlying the anti-proliferative effect of Fc was further investigated. Nevertheless, the anti-tumor activity of other sub-fractions, such as Fg and Ff are still possible targets for future investigations.

The previous studies about the anti-tumor effect of Acanthopanax extract proposed that Acanthopanax extract was able to induce apoptosis in tumor cells, such as stomach cancer cell line KATO III (Hibasami et al., 2000b). With reference to this, we explored the possibility that Fc may also suppress the human breast cancer cells through activating the apoptotic pathway.

To investigate whether Fc could induce apoptosis in breast carcinoma, Fc treated breast cancer cells were characterized with some common features or hallmarks of apoptosis through flow cytometric analysis owing to the its high efficiency of studying biochemical changes in a large number of cells within a short period of time. In many other studies, apoptosis is accompanied with the depolarization of mitochondrial membrane (Lu et al., 2008;Yao et al., 2008;Baxa et al., 2005). JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a

membrane-permeable lipophilic fluorochrome that is commonly used in evaluating the mitochondrial membrane potential ($\Delta\psi_m$). JC-1 exists in two forms, dependent on its concentration. It would exist as monomer under low concentration and green fluorescence would be emitted. On the other hand, aggregate (J-aggregates) formation will be favored at a high concentration and the formation of aggregate is accompanied by a red-spectral shift which could be measured in the red channel of flow cytometer. On the other hand, the dye concentration found in cells is dependent on the uptake of the dye into the mitochondria. JC-1 penetrates membrane of the living cells as monomers, and the uptake of JC-1 into the mitochondria is driven by the $\Delta\psi_m$. Polarized mitochondrial membrane of living cells favors the entry of JC-1 and therefore a high JC-1 concentration is reached in mitochondria of living cells, results in a red-spectral shift due to the formation of the J-aggregates. On the contrary, dead cells or apoptotic cells (intrinsic pathway), would have a depolarized mitochondrial membrane, results in less rapid accumulation of JC-1 inside the mitochondria. Since most of the dye remains as monomers, apoptotic or dead cells exhibited a higher emission of green fluorescence.

From Fig. 3.19 and 3.20, the breast cancer cells population shifted to area with higher level of green fluorescence emission upon treatment with Fc, and a dose-dependent manner could be observed. This shows that Fc could induce

mitochondrial membrane depolarization in both MCF-7 and MDA-MB-231 cells. However, mitochondrial membrane depolarization is not a unique feature of apoptosis, similar event could also be seen in necrotic cells. Therefore, to confirm the activation of apoptotic pathway, phosphatidylserine externalization was studied.

Phosphatidylserine (PS) externalization is one of the earliest morphological features that can be observed in apoptotic cells. The externalization of PS, which normally confined to the inner leaflet of the cell membrane, serves to provide an 'eat me' signal to the scavengers like macrophages which rapidly recognize and engulf the apoptotic cells to prevent onset of inflammation process (Hengartner, 2001).

The exposure of PS to the extracellular environment could be labeled by a protein known as Annexin–V. It is a vascular anti-coagulant protein in humans with high affinity towards the PS molecules. The use of Annexin-V conjugated to FITC allows cytometric detection of apoptosis by the fluorescence emission.

Since necrotic cells would also expose PS due to the loss of membrane integrity, a co-staining with a vital dye propidium-iodide would be required for differentiating cells in early apoptosis from that in necrosis. Propidium-iodide is a DNA fluorescent intercalating agent which stains the DNA when the membrane integrity is lost. As cells in early apoptosis would express PS molecule to the extracellular environment meanwhile maintaining an intact cell membrane, they could be identified as cell

population of Annexin-V positive and PI negative and located in the lower right area.

As shown in Fig. 3.22, the treatment of MDA-MB-231 cells with Fc resulted in the movement of cells from the area of viable cells in the lower left corner, to the area of cells in early apoptosis and late apoptosis. As we increased the dose of Fc, the percentage of cells found in apoptotic area was also increased, indicating the concentration dependency. More importantly, the tracks of cell movement from the viable area to the apoptotic area could be seen clearly. This result provides strong evidence that Fc cause apoptosis in MDA-MB-231 cells.

On the other hand, though a dose-dependent increase in cells in the apoptotic area could also be seen in MCF-7 cells treated with Fc, the track of movement of cells was absent in the graph. It seems that the MCF-7 cells instantly transferred to the top right corner without by-passing the area considered as early apoptosis. In other words, the PS cannot not be detected when the cell integrity was still maintained. It is doubtful whether MCF-7 cells underwent apoptosis after the treatment of Fc. In contrast, the apoptotic effect of Fc on MDA-MB-231 cells is more promising comparatively.

Flow cytometric analysis on the DNA content of the malignant cells treated with Fc was also performed to see if Fc had any effect on the cell cycle regulations of the breast cancer cells. Propidium-iodide is a fluorochrome that would bind

stoichiometrically to DNA molecules, and as a result reflect the DNA content of the cells. In this study, PI and flow cytometry were used to determine the cell cycle and apoptosis in the breast cancer cells. The DNA histograms of MDA-MB-231 cells after 48 hours of treatment with Fc were shown in Fig. 3.24. It was reported that the apoptotic cells would undergo DNA fragmentation, and therefore its DNA content would be less than that of cells in G1 phase and generating a sub-G1 peak lying right before the G0/G1 peak (Zhang et al., 2006). In our results, sub-G1 peak could be seen representing the existence of apoptotic cells. More importantly, the sub-G1 peak content increased as we raise the dose of Fc added and a more than 10-fold elevation in sub-G1 peak compared with control was attained when 90 μg/ml Fc was used. This result further supports the argument that Fc could induce apoptosis in MDA-MB-231 cells. The presence of sub-G1 peak in Fc-treated MDA-MB-231 cells was supported by the results of DNA fragmentation (Fig. 3.25). After 48 hours of treatment with Fc, genomic DNA in MDA-MB-231 cells was extracted and electrophoresis was performed. Internucleosomal ladder pattern was found in Fc-treated MDA-MB-231 cells and the DNA fragmentation became more and more obvious when the dose was increased. Upon treatment with Fc, a decrease in the percentage of cells could be seen in G1, S and G2/M phase of cell cycle, without accumulation of cells in a single phase. Therefore, we can say that Fc inhibited the MDA-MB-231 cell growth by inducing

apoptosis rather than arresting the cell cycle progression.

The DNA histograms of MCF-7 cells that have been treated with Fc for 48 hours were shown in Fig. 3.24. In contrast to the results of MDA-MB-231 cells, no sub-G1 peak could be seen at all concentrations tested. This result was justified by the DNA fragmentation assay. After 48 hours of treatment with Fc, no DNA ladder was observed in gel electrophoresis at a concentration of 80 μg/ml. However, changes in the proportion of the cells in various phases of cell cycle could be observed. Upon treatment with Fc, the cell population staying in the G1 phase increased in a dose-dependent manner. Seventeen % elevation of cells in G1 phase was reached at 60 μg/ml compared to that of control. Meanwhile, the percentage of cells that could be found in S phase and G2/M phase decreased. After the treatment with Fc, MCF-7 cells could no longer progress from G1 phase to the later phase of cell cycle and therefore cell accumulation in G1 phase was found. Therefore, it is likely that the suppressive effect of Fc on the growth of MCF-7 cells was through the arrestment of cells in G1 phase and stopped any further proliferation.

Since the previous results like PS externalization, mitochondrial membrane depolarization and DNA fragmentation suggested that Fc may induce apoptosis in MDA-MB-231 cells, the apoptotic machinery activated was characterized through the use of Western blotting analysis. It was found that Fc could reduce the Bcl-2

expression dose-dependently in MDA-MB-231 cells after 48 hours of treatment (Fig. 3.27). Bcl-2 is recognized as an anti-apoptotic member in Bcl-2 family, reduction of Bcl-2 would favor the activation of the apoptotic pathway. Accompanying the reduction in Bcl-2 expression, the amount of procaspase-9 was also found to be lowered in a dose-dependent manner after treatment with Fc. The decrease in procaspase-9 level was justified by investigating the cellular level of caspase-9. It was found that the amount of capase-9 was elevated upon the Fc treatment. This implies that procaspase-9 was activated and cleaved into its active form caspase-9. Caspase-9 plays a crucial role in the apoptotic pathway, acting as an initiator of the caspase cascade downstream (Berger et al., 2006). We also detected the activation of one of the major executioners of apoptosis caspase-3. A dose-dependent reduction in the level of procapase-3 was observed after treating with Fc. As an inactive zymogen, procaspase-3 reduction hinted the possibility of its activation into caspase-3 through enzymatic cleavage. In addition, obvious reduction in the level of intact PARP in the Fc-treated MDA-MB-231 cells could be observed in a dose-dependent fashion. Such reduction in intact PARP level is likely to be caused by its cleavage. PARP is an abundant nuclear protein that is involved in the DNA-base-excision-repair system (Hong et al., 2004). Cleavage of PARP is an identical event in apoptosis which was viewed as a marker of DNA damage and was proposed to be associated with the

preventing the access of DNA repair enzymes to the fragmented chromatin (Yu et al., 2002).

Elevated level of Bcl-2 expression was found in 80 % of breast cancer patients (Krajewski et al., 1999). Members of the Bcl-2 family were associated with the regulation of the integrity of the outer mitochondrial membrane (Harris and Thompson, 2000) while Bcl-2 is viewed as an survival signal and would prevent the release of some caspase activators from mitochondria (Mignotte and Vayssiere, 1998). Bcl-2 reduction seen in our results may disturb the balance between pro-apoptotic proteins and anti-apoptotic proteins and free the pro-apoptotic proteins from inhibition such as Bax and Bak, leading to mitochondrial membrane depolarization and the release of cytochrome c (Manion and Hockenbery, 2003). Cytochrome c plays a pivotal role in the mitochondrial-dependent apoptotic cell death since its release would trigger the formation of apoptosome from Apaf-1, ATP and procaspase-9 (Baliga and Kumar, 2003) and leads to the allosteric activation of caspase-9. Activated caspase-9 then propagates caspase cascade through the proteolytic activation of the caspase-3 (Taylor et al., 2008). Since the level of procaspase-3 was lowered in our results meanwhile DNA fragmentation could be observed. Therefore, it is very likely that the decrease in procaspase-3 level was due to the enzymatic activation to activated caspase-3. Once activated, caspase-3 could cleave ICAD and

dissociate the ICAD:CAD complex, thereby letting the CAD to cleave the chromosomal DNA (Nagata, 2000) and led to the internucleosomal DNA ladder seen in gel electrophoresis and sub-G1 peak in cell cycle analysis.

On the other hand, G0/G1 phase cell cycle arrest could be seen in Fc treated MCF-7 cells. Therefore, we have investigated some proteins responsible in regulating the cell cycle progression from G1 to S phase through Western analysis. Our results indicated dose-dependent reduction in the amount of cyclin D_1 and cdk 2 in MCF-7 cells after the treatment with Fc for 48 hours. The replication of eukaryotic cell is governed by a tightly controlled programme. One of the most important decision making takes place in G1 phase, before the cell commits itself to the replication of its DNA in the S phase. There exists a so called G1 restriction point that the cell decides whether to proceed to the replication programme or not. Signals from extracellular matrix, growth factor or alarm signal generated by DNA damage would decide whether the cell would proceed to the next phase of cell cycle (Miele, 2003). Both cyclin D_1 and cdk 2 play a part in the cell cycle restriction check point in the G1 to S phase transition. The transition from G1 to S phase requires the sequential phosphorylating action of cyclin D / cdk 4 or cyclin D / cdk 6 and then by cyclin E / cdk 2 complex on a retinoblastoma protein Rb during late G1 phase (Herwig and Strauss, 1997). Originally, the binding of Rb inhibits E2F-dependent transcription.

probably by the action of Rb / E2F / HDAC (histone deacetylases) complex which inhibits transcription at E2F-dependent promoters by acetylating histones and limiting promoter access through chromatin remodeling (Zhang et al., 2000). Phosphorylated Rb would dissociate from E2F transcription factor and allow its activation on the transcription of genes indispensable for G1 to S phase progression, like DNA polymerase, cyclin E and thymidine kinase. As seen in our results, the down regulated expression of cdk 2 would hinder the phosphorylation of Rb protein which is needed for the transition from G1 to S phase. Since the activation of cdk 4 / cdk 6 requires the binding of their partner cyclin D_1, reduced cyclin D_1 would also lead to the decreased phosphorylation of Rb protein. As a result, Fc treated MCF-7 cells failed to pass the G1 restriction point, progression from G1 to S phase was blocked and therefore arrested and accumulated in G1 phase. This justified the results in cell cycle analysis in which elevation of cells in G1 phase could be observed in a dose-dependent fashion.

We have studied the effect of Fc on the effect on the proliferation of ER positive MCF-7 cells and ER negative MDA-MB-231 cells. The results of MTT assay indicated that MCF-7 cells were more responsive to Fc with an IC_{50} of 42 μg/ml while the IC_{50} of MDA-MB-231 cells was 65 μg/ml. Apart from the responsiveness, the mechanism of growth inhibition of Fc on these two cell lines is also different from

each other. Fc could induce caspase-dependent apoptosis in MDA-MB-231 cells by activating the intrinsic pathway. Reduction in Bcl-2 level, depolarization of mitochondrial membrane and activation of caspase-9 and caspase-3 could be seen. These result in the apoptotic features like PS externalization and DNA fragmentation. On the other hand, Fc induced G1 phase cell cycle arrest in MCF-7 cells, by reducing the expression levels of cyclin D_1 and cdk 2, therefore blocking the G1 to S phase transition. Typical features of apoptosis like PS externalization and DNA fragmentation were absent from the Fc treated MCF-7 cells. It has been reported that MCF-7 cells were resistant to apoptosis, because of the lack of caspase-3 due to the deletion of *casp-3* gene in this cell line (Essmann et al., 2004). Meanwhile, other groups have reported that MCF-7 cells could still exhibit apoptosis through the sequential activation of caspase-9, caspase-7 and caspase-6 despite the absence of caspase-3 (Simstein et al., 2003). However, the induction of apoptosis in MCF-7 cells with such pathway was usually accompanied by the observation of DNA fragmentation (Mc Gee et al., 2002;Nigam et al., 2008) or PS externalization (Mooney et al., 2002). Therefore, whether Fc would induce apoptosis in MCF-7 cells still remains elusive. However, we are confident to say that the anti-proliferative effect of Fc on MCF-7 cells was at least partly contributed by the G0/G1 cell cycle arrest.

In contrast to the results of MCF-7 cells, no cell cycle arrest was induced by Fc in MDA-MB-231 cells. This difference in response in the two cell lines hinted the possibility of involvement of ER in the cell cycle arrest action of Fc on MCF-7 cells. As an ER-expressing cell line, the proliferation of MCF-7 cells is dependent on the estrogen stimulation on the ER. Previously, there are reports about the anti-estrogen treatment or removal of estrogen source could lead to the G1 arrestment (Sutherland et al., 1983). It has been demonstrated that treatment of ER positive cell line with anti-estrogen ICI 182780 would lead to the reduction in level of cdk 2 and cyclin D_1 and cause growth arrest (Bindels et al., 2002). In addition, recent studies suggested that cyclin D_1 itself could activate the ER, and this activation cannot be inhibited by the action of anti-estrogen. Instead, a direct binding of cyclin D_1 to the hormone binding domain of the ER would lead to the activation of ER and enhanced the transcription of ERE-responsive genes in a cdk-independent fashion (Zwijsen et al., 1997). Therefore, the reduction of expression level of cyclin D_1 after treatment with Fc may also take a part in suppressive effect of Fc on MCF-7 cells through the mediation of ER.

Our results demonstrated that Acanthopanax exhibited anti-proliferative effect on MCF-7, MDA-MB-231 and Hep G2 cells, which has not been reported previously. One of the fractions Fc generated from Acanthopanax induced apoptosis in

MDA-MB-231 cells and cell cycle arrest in MCF-7 cells. However, we did not have clues about the components or active compound responsible for the anti-tumor activity in Fc. Therefore, further purification and isolation of active compound in Fc would definitely help solving the mist of anti-tumor effect of Acanthopanax on breast cancer cells. Studies on the chemical compositions of Acanthopanax suggested that lignans, saponins, sterols, flavonoids and polysaccharides could be found in Acanthopanax. Compounds including syringin, stearic acid, sesamin, liriodendrin, rutin, isofraxidin, chlorogenic acid, β-sitosterol, daucosterol, syringaresinol and 2,6-dimethoxy-p-benzoquinone have been isolated from Acanthopanax previously (Li et al., 2006;Zhou et al., 2007). Sesamin, stearic acid and chlorogenic acid has previously been reported to induce apoptosis in other tumor cell lines. However, other compounds such as the saponins can still be the potential active constituents in the anti-tumor effect of Acanthopanax.

Moreover, *in vivo* study of the anti-tumor activity would further confirm the findings in this study. Although Fc exhibited anti-tumor activity in the *in vitro* environment, such activities could be abrogated by low absorption or high metabolic degradation rate in *in vivo* settings. In addition, the *in vivo* experiments would also help answering the question of how selective is the effect of Fc and would Fc bring severe side effects to cancer patients. Although the effect of Fc on normal human cells

like Hs68 cells or PBMC is weaker compared to that on tumor cells, we cannot exclude the possibility that Fc may affect the proliferation of other normal human tissues that are not tested in this study. In short, *in vivo* studies would further confirm the anti-tumor activities of Acanthopanax extracts.

Chapter 5 Conclusion

The aqueous extract of Acanthopanax exhibited anti-proliferative effect on tumor cell lines MCF-7, MDA-MB-231 and Hep G2 and selectivity could be seen on the anti-proliferative activity of Acanthopanax when we compared the efficacy in inhibiting cancer cells and normal fibroblast Hs68 cells. The breast cancer cell lines were more responsive to the suppressive effect of Acanthopanax. After partition and column chromatography, it was found that one of the sub-fractions (Fc) in ethyl-acetate extract could inhibit the proliferation of MCF-7 and MDA-MB-231 cells with IC_{50} of 42 μg/ml and 65 μg/ml respectively. Fc inhibited the proliferation of human breast cancer cells more effectively than inhibiting the proliferation of human normal fibroblast Hs68 cells and PBMC. By flow cytometric analysis and Western blotting, it was found that Fc could induce G1 phase cell cycle arrest in MCF-7 cells through the reduction of cyclin D_1 and cdk 2 expression. On the other hand, caspase-dependent apoptosis was induced by Fc in MDA-MB-231 cells. Typical apoptotic hallmarks such as phosphatidylserine externalization, mitochondrial membrane depolarization and DNA fragmentation could be observed in Fc treated MDA-MB-231 cells. The expression of Bcl-2, procaspase-9, procaspase-3 and PARP was decreased while expression of caspase-9 was increased. Although Acanthopanax extract demonstrated anti-tumor effect in *in vitro* situation, animal study is still needed

to further verify the anti-tumor effect of Fc under *in vivo* condition in the future. Besides, further purification of sub-fraction Fc would help identifying the active component(s) responsible for the anti-tumor effect in Acanthopanax.

Reference List

Acehan D, Jiang X, Morgan DG, Heuser JE, Wang X, Akey CW (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell 9:423-432.

Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res 48:589-601.

Ashkenazi A (2002) Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer 2:420-430.

Baliga B, Kumar S (2003) Apaf-1/cytochrome c apoptosome: an essential initiator of caspase activation or just a sideshow? Cell Death Differ 10:16-18.

Baxa DM, Luo X, Yoshimura FK (2005) Genistein induces apoptosis in T lymphoma cells via mitochondrial damage. Nutr Cancer 51:93-101.

Berger AB, Witte MD, Denault JB, Sadaghiani AM, Sexton KM, Salvesen GS, Bogyo M (2006) Identification of early intermediates of caspase activation using selective inhibitors and activity-based probes. Mol Cell 23:509-521.

Bergh J, Jonsson PE, Glimelius B, Nygren P (2001) A systematic overview of chemotherapy effects in breast cancer. Acta Oncol 40:253-281.

Bernardi P, Scorrano L, Colonna R, Petronilli V, Di LF (1999) Mitochondria and cell death. Mechanistic aspects and methodological issues. Eur J Biochem 264:687-701.

Bindels EM, Lallemand F, Balkenende A, Verwoerd D, Michalides R (2002) Involvement of G1/S cyclins in estrogen-independent proliferation of estrogen receptor-positive breast cancer cells. Oncogene 21:8158-8165.

Boon-Niermeijer EK, van den BA, Wikman G, Wiegant FA (2000) Phyto-adaptogens protect against environmental stress-induced death of embryos from the freshwater snail Lymnaea stagnalis. Phytomedicine 7:389-399.

Bratton SB, Walker G, Srinivasula SM, Sun XM, Butterworth M, Alnemri ES, Cohen GM (2001) Recruitment, activation and retention of caspases-9 and -3 by Apaf-1 apoptosome and associated XIAP complexes. EMBO J 20:998-1009.

Bu Y, Jin ZH, Park SY, Baek S, Rho S, Ha N, Park SK, Kim H (2005) Siberian ginseng reduces infarct volume in transient focal cerebral ischaemia in Sprague-Dawley rats. Phytother Res 19:167-169.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X (1999) Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 15:269-290.

Chaturvedi V, Qin JZ, Denning MF, Choubey D, Diaz MO, Nickoloff BJ (1999) Apoptosis in proliferating, senescent, and immortalized keratinocytes. J Biol Chem 274:23358-23367.

Chen LX, Liao JZ, Guo WQ (1995) [Effects of Astragalus membranaceus on left ventricular function and oxygen free radical in acute myocardial infarction patients and mechanism of its cardiotonic action]. Zhongguo Zhong Xi Yi Jie He Za Zhi 15:141-143.

Cheng XD, Hou CH, Zhang XJ, Xie HY, Zhou WY, Yang L, Zhang SB, Qian RL (2004) Effects of Huangqi (Hex) on inducing cell differentiation and cell death in K562 and HEL cells. Acta Biochim Biophys Sin (Shanghai) 36:211-217.

Chiu LC, Ho TS, Wong EY, Ooi VE (2006) Ethyl acetate extract of Patrinia scabiosaefolia downregulates anti-apoptotic Bcl-2/Bcl-X(L) expression, and induces apoptosis in human breast carcinoma MCF-7 cells independent of caspase-9 activation. J Ethnopharmacol 105:263-268.

Cho WC, Leung KN (2007a) In vitro and in vivo immunomodulating and immunorestorative effects of Astragalus membranaceus. J Ethnopharmacol 113:132-141.

Cho WC, Leung KN (2007b) In vitro and in vivo anti-tumor effects of Astragalus membranaceus. Cancer Lett 252:43-54.

Cui FJ, Li Y, Xu YY, Liu ZQ, Huang DM, Zhang ZC, Tao WY (2007) Induction of apoptosis in SGC-7901 cells by polysaccharide-peptide GFPS1b from the cultured mycelia of Grifola frondosa GF9801. Toxicol In Vitro 21:417-427.

Daugas E, Susin SA, Zamzami N, Ferri KF, Irinopoulou T, Larochette N, Prevost MC, Leber B, Andrews D, Penninger J, Kroemer G (2000) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J 14:729-739.

Deyama T, Nishibe S, Nakazawa Y (2001) Constituents and pharmacological effects of Eucommia and Siberian ginseng. Acta Pharmacol Sin 22:1057-1070.

Duan P, Wang ZM (2002) [Clinical study on effect of Astragalus in efficacy enhancing and toxicity reducing of chemotherapy in patients of malignant tumor]. Zhongguo Zhong Xi Yi Jie He Za Zhi 22:515-517.

Earnshaw WC, Martins LM, Kaufmann SH (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383-424.

Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL (2001) DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. J Cell Biol 152:483-490.

Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, Ghayur T, Wong WW, Kamen R, Weichselbaum R, . (1995) Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. EMBO J 14:6148-6156.

Essmann F, Engels IH, Totzke G, Schulze-Osthoff K, Janicke RU (2004) Apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 and cell cycle control but caused by the lack of caspase-3 and a caffeine-inhibitable event. Cancer Res 64:7065-7072.

Evan GI, Vousden KH (2001) Proliferation, cell cycle and apoptosis in cancer. Nature 411:342-348.

Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA, Henson PM (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405:85-90.

Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 10:76-100.

Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA, Bratton DL (2000) Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem 275:23065-23073.

Fujikawa T, Kanada N, Shimada A, Ogata M, Suzuki I, Hayashi I, Nakashima K (2005a) Effect of sesamin in Acanthopanax senticosus HARMS on behavioral dysfunction in rotenone-induced parkinsonian rats. Biol Pharm Bull 28:169-172.

Fujikawa T, Miguchi S, Kanada N, Nakai N, Ogata M, Suzuki I, Nakashima K (2005b) Acanthopanax senticosus Harms as a prophylactic for MPTP-induced Parkinson's disease in rats. J Ethnopharmacol 97:375-381.

Fujikawa T, Soya H, Hibasami H, Kawashima H, Takeda H, Nishibe S, Nakashima K (2002) Effect of Acanthopanax senticosus Harms on biogenic monoamine levels in the rat brain. Phytother Res 16:474-478.

Fujikawa T, Yamaguchi A, Morita I, Takeda H, Nishibe S (1996) Protective effects of Acanthopanax senticosus Harms from Hokkaido and its components on gastric ulcer in restrained cold water stressed rats. Biol Pharm Bull 19:1227-1230.

Gewies A (2003) Introduction of Apoptosis, ApoReview, 1-26

Gordaliza M (2007) Natural products as leads to anticancer drugs. Clin Transl Oncol 9:767-776.

Grude P, Conti F, Mennecier D, Louvel A, Houssin D, Weill B, Calmus Y (2002) MDR1 gene expression in hepatocellular carcinoma and the peritumoral liver of patients with and without cirrhosis. Cancer Lett 186:107-113.

Han J (1988) Traditional Chinese medicine and the search for new antineoplastic drugs. J Ethnopharmacol 24:1-17.

Han SB, Yoon YD, Ahn HJ, Lee HS, Lee CW, Yoon WK, Park SK, Kim HM (2003) Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of Acanthopanax senticosus. Int Immunopharmacol 3:1301-1312.

Harris MH, Thompson CB (2000) The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 7:1182-1191.

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. Science 246:629-634.

Hengartner MO (2001) Apoptosis: corralling the corpses. Cell 104:325-328.

Herwig S, Strauss M (1997) The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. Eur J Biochem 246:581-601.

Hibasami H, Fujikawa T, Takeda H, Nishibe S, Satoh T, Fujisawa T, Nakashima K (2000a) Induction of apoptosis by Acanthopanax senticosus HARMS and its component, sesamin in human stomach cancer KATO III cells. Oncol Rep 7:1213-1216.

Hibasami H, Fujikawa T, Takeda H, Nishibe S, Satoh T, Fujisawa T, Nakashima K

(2000b) Induction of apoptosis by Acanthopanax senticosus HARMS and its component, sesamin in human stomach cancer KATO III cells. Oncol Rep 7:1213-1216.

Hishida I, Nanba H, Kuroda H (1988) Antitumor activity exhibited by orally administered extract from fruit body of Grifola frondosa (maitake). Chem Pharm Bull (Tokyo) 36:1819-1827.

Holdenrieder S, Stieber P (2004) Apoptotic markers in cancer. Clin Biochem 37:605-617.

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci 25:259-264.

Howell A, Wardley AM (2005) Overview of the impact of conventional systemic therapies on breast cancer. Endocr Relat Cancer 12 Suppl 1:S9-S16.

Hsieh TC, Wijeratne EK, Liang JY, Gunatilaka AL, Wu JM (2005) Differential control of growth, cell cycle progression, and expression of NF-kappaB in human breast cancer cells MCF-7, MCF-10A, and MDA-MB-231 by ponicidin and oridonin, diterpenoids from the chinese herb Rabdosia rubescens. Biochem Biophys Res Commun 337:224-231.

Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299-308.

Hsu H, Xiong J, Goeddel DV (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81:495-504.

Huang DB, Ran RZ, Yu ZF (2005) [Effect of Acanthopanax senticosus injection on the activities of human tumor necrosis factor and natural killer cell in blood in the patients with lung cancer]. Zhongguo Zhong Yao Za Zhi 30:621-624.

Inoue A, Kodama N, Nanba H (2002) Effect of maitake (Grifola frondosa) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. Biol Pharm Bull 25:536-540.

Ishizaki Y, Cheng L, Mudge AW, Raff MC (1995) Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. Mol Biol Cell 6:1443-1458.

Jang MH, Shin MC, Kim YJ, Kim CJ, Chung JH, Seo JC, Kim EH, Kim KY, Lee CY,

Kim KM (2003) Protective effect of Acanthopanax senticosus against ethanol-induced apoptosis of human neuroblastoma cell line SK-N-MC. Am J Chin Med 31:379-388.

Johnson PJ (2002) Hepatocellular carcinoma: is current therapy really altering outcome? Gut 51:459-462.

Jung HJ, Park HJ, Kim RG, Shin KM, Ha J, Choi JW, Kim HJ, Lee YS, Lee KT (2003) In vivo anti-inflammatory and antinociceptive effects of liriodendrin isolated from the stem bark of Acanthopanax senticosus. Planta Med 69:610-616.

Kastan MB, Bartek J (2004) Cell-cycle checkpoints and cancer. Nature 432:316-323.

Kataoka T (2005) The caspase-8 modulator c-FLIP. Crit Rev Immunol 25:31-58.

Kaufmann SH, Earnshaw WC (2000) Induction of apoptosis by cancer chemotherapy. Exp Cell Res 256:42-49.

Kodama N, Asakawa A, Inui A, Masuda Y, Nanba H (2005a) Enhancement of cytotoxicity of NK cells by D-Fraction, a polysaccharide from Grifola frondosa. Oncol Rep 13:497-502.

Kodama N, Komuta K, Sakai N, Nanba H (2002) Effects of D-Fraction, a polysaccharide from Grifola frondosa on tumor growth involve activation of NK cells. Biol Pharm Bull 25:1647-1650.

Kodama N, Murata Y, Asakawa A, Inui A, Hayashi M, Sakai N, Nanba H (2005b) Maitake D-Fraction enhances antitumor effects and reduces immunosuppression by mitomycin-C in tumor-bearing mice. Nutrition 21:624-629.

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997) Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science 278:294-298.

Krajewski S, Krajewska M, Turner BC, Pratt C, Howard B, Zapata JM, Frenkel V, Robertson S, Ionov Y, Yamamoto H, Perucho M, Takayama S, Reed JC (1999) Prognostic significance of apoptosis regulators in breast cancer. Endocr Relat Cancer 6:29-40.

Krammer PH (2000) CD95's deadly mission in the immune system. Nature 407:789-795.

Kucharczak J, Simmons MJ, Fan Y, Gelinas C (2003) To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 22:8961-8982.

Lee KH (1999) Novel antitumor agents from higher plants. Med Res Rev 19:569-596.

Lee KY, Jeon YJ (2005) Macrophage activation by polysaccharide isolated from Astragalus membranaceus. Int Immunopharmacol 5:1225-1233.

Lee S, Son D, Ryu J, Lee YS, Jung SH, Kang J, Lee SY, Kim HS, Shin KH (2004a) Anti-oxidant activities of Acanthopanax senticosus stems and their lignan components. Arch Pharm Res 27:106-110.

Lee S, Son D, Ryu J, Lee YS, Jung SH, Kang J, Lee SY, Kim HS, Shin KH (2004b) Anti-oxidant activities of Acanthopanax senticosus stems and their lignan components. Arch Pharm Res 27:106-110.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479-489.

Li Q, Jia Y, Xu L, Wang X, Shen Z, Liu Y, Bi K (2006) Simultaneous determination of protocatechuic acid, syringin, chlorogenic acid, caffeic acid, liriodendrin and isofraxidin in Acanthopanax senticosus Harms by HPLC-DAD. Biol Pharm Bull 29:532-534.

Lin JT, Liu WH (2006) o-Orsellinaldehyde from the submerged culture of the edible mushroom Grifola frondosa exhibits selective cytotoxic effect against Hep 3B cells through apoptosis. J Agric Food Chem 54:7564-7569.

Lin QY, Jin LJ, Cao ZH, Lu YN, Xue HY, Xu YP (2008a) Acanthopanax senticosus suppresses reactive oxygen species production by mouse peritoneal macrophages in vitro and in vivo. Phytother Res 22:740-745.

Lin QY, Jin LJ, Cao ZH, Xu YP (2008b) Inhibition of inducible nitric oxide synthase by Acanthopanax senticosus extract in RAW264.7 macrophages. J Ethnopharmacol.

Lin QY, Jin LJ, Ma YS, Shi M, Xu YP (2007) Acanthopanax senticosus inhibits nitric oxide production in murine macrophages in vitro and in vivo. Phytother Res 21:879-883.

Liu TP, Lee CS, Liou SS, Liu IM, Cheng JT (2005) Improvement of insulin resistance

by Acanthopanax senticosus root in fructose-rich chow-fed rats. Clin Exp Pharmacol Physiol 32:649-654.

Loeffler M, Kroemer G (2000) The mitochondrion in cell death control: certainties and incognita. Exp Cell Res 256:19-26.

Lopez-Gonzalez JS, Prado-Garcia H, guilar-Cazares D, Molina-Guarneros JA, Morales-Fuentes J, Mandoki JJ (2004) Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. Lung Cancer 43:275-283.

Lu Q, Lin X, Feng J, Zhao X, Gallagher R, Lee MY, Chiao JW, Liu D (2008) Phenylhexyl isothiocyanate has dual function as histone deacetylase inhibitor and hypomethylating agent and can inhibit myeloma cell growth by targeting critical pathways. J Hematol Oncol 1:6.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94:481-490.

Manion MK, Hockenbery DM (2003) Targeting BCL-2-related proteins in cancer therapy. Cancer Biol Ther 2:S105-S114.

Masuda Y, Murata Y, Hayashi M, Nanba H (2008) Inhibitory effect of MD-Fraction on tumor metastasis: involvement of NK cell activation and suppression of intercellular adhesion molecule (ICAM)-1 expression in lung vascular endothelial cells. Biol Pharm Bull 31:1104-1108.

Mc Gee MM, Hyland E, Campiani G, Ramunno A, Nacci V, Zisterer DM (2002) Caspase-3 is not essential for DNA fragmentation in MCF-7 cells during apoptosis induced by the pyrrolo-1,5-benzoxazepine, PBOX-6. FEBS Lett 515:66-70.

McCulloch M, See C, Shu XJ, Broffman M, Kramer A, Fan WY, Gao J, Lieb W, Shieh K, Colford JM, Jr. (2006) Astragalus-based Chinese herbs and platinum-based chemotherapy for advanced non-small-cell lung cancer: meta-analysis of randomized trials. J Clin Oncol 24:419-430.

Meier P, Finch A, Evan G (2000) Apoptosis in development. Nature 407:796-801.

Meng RD, McDonald ER, III, Sheikh MS, Fornace AJ, Jr., El-Deiry WS (2000) The TRAIL decoy receptor TRUNDD (DcR2, TRAIL-R4) is induced by adenovirus-p53 overexpression and can delay TRAIL-, p53-, and KILLER/DR5-dependent colon

cancer apoptosis. Mol Ther 1:130-144.

Mesner PW, Jr., Budihardjo II, Kaufmann SH (1997) Chemotherapy-induced apoptosis. Adv Pharmacol 41:461-499.

Mignotte B, Vayssiere JL (1998) Mitochondria and apoptosis. Eur J Biochem 252:1-15.

Mooney LM, Al-Sakkaf KA, Brown BL, Dobson PR (2002) Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells. Br J Cancer 87:909-917.

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13:261-291.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55-63.

Mukherjee AK, Basu S, Sarkar N, Ghosh AC (2001) Advances in cancer therapy with plant based natural products. Curr Med Chem 8:1467-1486.

Muller M, Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH, Galle PR (1997) Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest 99:403-413.

Nagata S (2000) Apoptotic DNA fragmentation. Exp Cell Res 256:12-18.

Naismith JH, Sprang SR (1998) Modularity in the TNF-receptor family. Trends Biochem Sci 23:74-79.

Newmeyer DD, Ferguson-Miller S (2003) Mitochondria: releasing power for life and unleashing the machineries of death. Cell 112:481-490.

Nigam M, Ranjan V, Srivastava S, Sharma R, Balapure AK (2008) Centchroman induces G0/G1 arrest and caspase-dependent apoptosis involving mitochondrial membrane depolarization in MCF-7 and MDA MB-231 human breast cancer cells. Life Sci 82:577-590.

Nishibe S, Kinoshita H, Takeda H, Okano G (1990) Phenolic compounds from stem bark of Acanthopanax senticosus and their pharmacological effect in chronic swimming stressed rats. Chem Pharm Bull (Tokyo) 38:1763-1765.

Nowak AK, Chow PK, Findlay M (2004) Systemic therapy for advanced

hepatocellular carcinoma: a review. Eur J Cancer 40:1474-1484.

Oberlies NH, Kroll DJ (2004) Camptothecin and taxol: historic achievements in natural products research. J Nat Prod 67:129-135.

Omata M, Yoshida H (2004) Prevention and treatment of hepatocellular carcinoma. Liver Transpl 10:S111-S114.

Park SH, Lee SG, Kang SK, Chung SH (2006) Acanthopanax senticosus reverses fatty liver disease and hyperglycemia in ob/ob mice. Arch Pharm Res 29:768-776.

Perera NM, Gui GP (2003) Multi-ethnic differences in breast cancer: current concepts and future directions. Int J Cancer 106:463-467.

Perl M, Chung CS, Ayala A (2005) Apoptosis. Crit Care Med 33:S526-S529.

Rhim YT, Kim H, Yoon SJ, Kim SS, Chang HK, Lee TH, Lee HH, Shin MC, Shin MS, Kim CJ (2007) Effect of Acanthopanax senticosus on 5-hydroxytryptamine synthesis and tryptophan hydroxylase expression in the dorsal raphe of exercised rats. J Ethnopharmacol 114:38-43.

Rock E, DeMichele A (2003) Nutritional approaches to late toxicities of adjuvant chemotherapy in breast cancer survivors. J Nutr 133:3785S-3793S.

Ruchaud S, Korfali N, Villa P, Kottke TJ, Dingwall C, Kaufmann SH, Earnshaw WC (2002) Caspase-6 gene disruption reveals a requirement for lamin A cleavage in apoptotic chromatin condensation. EMBO J 21:1967-1977.

Sakon M, Umeshita K, Nagano H, Eguchi H, Kishimoto S, Miyamoto A, Ohshima S, Dono K, Nakamori S, Gotoh M, Monden M (2000) Clinical significance of hepatic resection in hepatocellular carcinoma: analysis by disease-free survival curves. Arch Surg 135:1456-1459.

Saraste A, Pulkki K (2000) Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 45:528-537.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17:1675-1687.

Schafer P, Scholz SR, Gimadutdinow O, Cymerman IA, Bujnicki JM, Ruiz-Carrillo A, Pingoud A, Meiss G (2004) Structural and functional characterization of

mitochondrial EndoG, a sugar non-specific nuclease which plays an important role during apoptosis. J Mol Biol 338:217-228.

Schmitt CA, Lowe SW (2001) Bcl-2 mediates chemoresistance in matched pairs of primary E(mu)-myc lymphomas in vivo. Blood Cells Mol Dis 27:206-216.

Shan BE, Li QX, Liang WJ (2004) [Experimental study on anti-tumor effects of cortex Acanthopanacis senticosus in vivo and in vitro]. Zhongguo Zhong Xi Yi Jie He Za Zhi 24:55-58.

Shao BM, Xu W, Dai H, Tu P, Li Z, Gao XM (2004) A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a Chinese medicinal herb. Biochem Biophys Res Commun 320:1103-1111.

Sherr CJ (1996) Cancer cell cycles. Science 274:1672-1677.

Simstein R, Burow M, Parker A, Weldon C, Beckman B (2003) Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. Exp Biol Med (Maywood) 228:995-1003.

Singh B, Bhat TK, Singh B (2003) Potential therapeutic applications of some antinutritional plant secondary metabolites. J Agric Food Chem 51:5579-5597.

Sreenivasan Y, Raghavendra PB, Manna SK (2006) Oleandrin-mediated expression of Fas potentiates apoptosis in tumor cells. J Clin Immunol 26:308-322.

Sui DY, Lu ZZ, Ma LN, Fan ZG (1994) [Effects of the leaves of Acanthopanax senticosus (Rupr. et Maxim.) Harms. on myocardial infarct size in acute ischemic dogs]. Zhongguo Zhong Yao Za Zhi 19:746-7, 764.

Sun A, Chia JS, Chiang CP, Hsuen SP, Du JL, Wu CW, Wang WB (2005) The chinese herbal medicine Tien-Hsien liquid inhibits cell growth and induces apoptosis in a wide variety of human cancer cells. J Altern Complement Med 11:245-256.

Sutherland RL, Green MD, Hall RE, Reddel RR, Taylor IW (1983) Tamoxifen induces accumulation of MCF 7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle. Eur J Cancer Clin Oncol 19:615-621.

Suzuki I, Itani T, Ohno N, Oikawa S, Sato K, Miyazaki T, Yadomae T (1984) Antitumor activity of a polysaccharide fraction extracted from cultured fruiting bodies of Grifola frondosa. J Pharmacobiodyn 7:492-500.

Taylor RC, Cullen SP, Martin SJ (2008) Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9:231-241.

Tin MM, Cho CH, Chan K, James AE, Ko JK (2007) Astragalus saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft. Carcinogenesis 28:1347-1355.

Tong L, Huang TY, Li JL (1994) [Effects of plant polysaccharides on cell proliferation and cell membrane contents of sialic acid, phospholipid and cholesterol in S 180 and K 562 cells]. Zhongguo Zhong Xi Yi Jie He Za Zhi 14:482-484.

Tsujimoto Y, Shimizu S (2000) VDAC regulation by the Bcl-2 family of proteins. Cell Death Differ 7:1174-1181.

Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 102:43-53.

Wang GL, Li SY, Chen MK (2007) Studies on fatty acids and fatty acid esters of Radix Astragali by column chromatography separated way. Journal of Gansu Normal Colleges 12: 42-45

Wang WG, Wu Q, Hu BK, Bao DW, Zhao YL (2002) Comparative study on determination method of residual protained in Grifolan. Edible fungi of China 22: 27-30

Wang Y, Qian XJ, Hadley HR, Lau BH (1992) Phytochemicals potentiate interleukin-2 generated lymphokine-activated killer cell cytotoxicity against murine renal cell carcinoma. Mol Biother 4:143-146.

Weil M, Jacobson MD, Coles HS, Davies TJ, Gardner RL, Raff KD, Raff MC (1996) Constitutive expression of the machinery for programmed cell death. J Cell Biol 133:1053-1059.

Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81:323-330.

Wu LC, Fan NC, Lin MH, Chu IR, Huang SJ, Hu CY, Han SY (2008) Anti-inflammatory effect of spilanthol from Spilanthes acmella on murine macrophage by down-regulating LPS-induced inflammatory mediators. J Agric Food Chem 56:2341-2349.

Wyllie AH (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555-556.

Xi M, Hai C, Tang H, Chen M, Fang K, Liang X (2008) Antioxidant and antiglycation properties of total saponins extracted from traditional Chinese medicine used to treat diabetes mellitus. Phytother Res 22:228-237.

Yan X, Shen H, Jiang H, Zhang C, Hu D, Wang J, Wu X (2008) External Qi of Yan Xin Qigong induces G2/M arrest and apoptosis of androgen-independent prostate cancer cells by inhibiting Akt and NF-kappa B pathways. Mol Cell Biochem 310:227-234.

Yao J, Jiang Z, Duan W, Huang J, Zhang L, Hu L, He L, Li F, Xiao Y, Shu B, Liu C (2008) Involvement of mitochondrial pathway in triptolide-induced cytotoxicity in human normal liver L-02 cells. Biol Pharm Bull 31:592-597.

Yao Z, Duan S, Hou D, Heese K, Wu M (2007) Death effector domain DEDa, a self-cleaved product of caspase-8/Mch5, translocates to the nucleus by binding to ERK1/2 and upregulates procaspase-8 expression via a p53-dependent mechanism. EMBO J 26:1068-1080.

Yoon TJ, Yoo YC, Lee SW, Shin KS, Choi WH, Hwang SH, Ha ES, Jo SK, Kim SH, Park WM (2004) Anti-metastatic activity of Acanthopanax senticosus extract and its possible immunological mechanism of action. J Ethnopharmacol 93:247-253.

Yoshida H, Yoshida H, Shiina S, Omata M (2005) Early liver cancer: concepts, diagnosis, and management. Int J Clin Oncol 10:384-390.

Yoshida Y, Wang MQ, Liu JN, Shan BE, Yamashita U (1997) Immunomodulating activity of Chinese medicinal herbs and Oldenlandia diffusa in particular. Int J Immunopharmacol 19:359-370.

Yu SW, Wang H, Poitras MF, Coombs C, Bowers WJ, Federoff HJ, Poirier GG, Dawson TM, Dawson VL (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259-263.

Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, Harbour JW, Dean DC (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101:79-89.

Zhang M, Chiu LC, Cheung PC, Ooi VE (2006) Growth-inhibitory effects of a

beta-glucan from the mycelium of Poria cocos on human breast carcinoma MCF-7 cells: cell-cycle arrest and apoptosis induction. Oncol Rep 15:637-643.

Zhang ZJ, Yu J, Li Q (2002) Study on the Fingerprints of Ciwujia Crude Drugs by HPLC/ UV. Chinese Wild Plant Resources 21: 43-45

Zhou X, Zheng C, Huang J, You T (2007) Identification of herb Acanthopanax senticosus (Rupr. Et Maxim.) harms by capillary electrophoresis with electrochemical detection. Anal Sci 23:705-711.

Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90:405-413.

Zou YH, Liu XM (2003) [Effect of astragalus injection combined with chemotherapy on quality of life in patients with advanced non-small cell lung cancer]. Zhongguo Zhong Xi Yi Jie He Za Zhi 23:733-735.

Zwijsen RM, Wientjens E, Klompmaker R, van der SJ, Bernards R, Michalides RJ (1997) CDK-independent activation of estrogen receptor by cyclin D1. Cell 88:405-415.