**THE HONG KONG POLYTECHNIC UNIVERSITY**

**THE DEPARTMENT OF APPLIED BIOLOGY AND CHEMICAL TECHNOLOGY**

# Fingerprint Analysis and Pharmacological Study on the Chinese Medicines: Radix Paeoniae Alba and Radix Puerariae Lobatae

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**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of**

**MASTER OF PHILOSOPHY**

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# CERTIFICATE OF ORIGINALITY

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CHEUNG Chui-yee

# ABSTRACT

Abstract of the thesis entitled

**"Fingerprint Analysis and Pharmacological Study on the Chinese Medicines: Radix Paeoniae Alba and Radix Puerariae Lobatae"**

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Traditional Chinese medicine (TCM) is getting more and more popular nowadays in the whole world for improving health condition of human beings as well as preventing and healing disease. The efficacies of TCMs can be reflected from their complicated multi-components nature. Chromatographic separation techniques used in analysis for TCM chemical components together with bioassays on studying of biological activities of these TCMs are desirable in order to find out the relationship between these chemical components and their corresponding efficacy. This relationship is useful to provide more scientific information for assessing the quality of TCMs.

The solvent used for sample extraction largely determines the chemical composition of the TCM extracts obtained and its biological activity. Radix Paeoniae Alba (P. Alba) was investigated in this study to explore the effect of extraction solvent on the inhibitory hepatoma cells growth. Five extracts of P.

Alba named PR0, PR25, PR50, PR75 and PR100 as prepared by a mixture of two solvents, were found to produce variation of the components in chemical profiles as detected by High Performance Liquid Chromatography with Diode Array Detector (HPLC-DAD). And they exhibited different antiproliferation activities on HepG2 cells but no obvious activity on Hep3B cells was found. Then we tried to link the HPLC-DAD chromatographic fingerprints of these extracts with antiproliferation activity on HepG2 cells.

After finishing the above step through investigation of the pharmacological effect of P. Alba extracts, finding out the bioactive component or fraction of P. Alba that is responsible for the biological activity is the next one. The most potent PR100 among the five extracts was utilized to search for which bioactive component(s) or fraction(s) is/are responsible for HepG2 cells. One identified component and one fraction were found to have more effective growth inhibitory effect compared with PR100 itself.

Another herb investigated in this study was Radix Puerariae Lobatae (P. Lobatae. Most studies reported that P. Lobatae is a rich source of isoflavonoids components that are very effective in cardiovascular diseases. This disease is mainly attributed to antioxidant activity of isoflavonoids. In this part, the optimized condition of extraction of P. Lobatae was established by the use of the uniform design having five variable parameters. They were solvents, solvent volume, temperature, extraction time and number of repeats of extraction. The condition was optimized on the contents of isoflavonoids detected by HPLC-DAD in line with the results of one antioxidant test which was 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay. The results found that 180ml

of 100% methanol in two times of 75 min at 50℃ was the best condition for P. Lobatae. This study provides a model system for setting up the quality assessment based on the identified chemical ingredients and their bioactivities of a TCM.

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# TABLES OF CONTENTS

# LIST OF ABBREVIATIONS AND SYMBOLS

## Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| AF | Albiflorin |
| ALC | Analytical high performance liquid chromatography |
| CRM | Chemometric Resolution Method |
| DA | Daidzin |
| DE | Daidzein |
| DOX | Doxorubicin |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| EtOH | Ethanol |
| GE | Genistein |
| GG | Gegen |
| GN | Genistin |
| HP | Hewlett Packard |
| HPLC-DAD | High Performance Liquid Chromatography - Diode Array Detector |
| IC | Inhibitory Concentration |
| LC-MS | Liquid Chromatography – Mass Spectroscopy |

| | |
|---|---|
| MeOH | Methanol |
| MTS | (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| OD | Optical Density |
| PAC | Paclitaxel |
| PF | Paeoniflorin |
| PHPLC | Preparative High Performance Liquid Chromatography |
| ppm | part per million |
| PR | *Paeoniae Radix* |
| PU | Puerarin |
| $R^2$ | Coefficient of determination |
| RT | Retention Time |
| SD | Standard Deviation |
| TCHM | Traditional Chinese Herbal Medicine |
| TCM | Traditional Chinese Medicine |
| UD | Uniform Design |
| UV | Ultra-violet |

## Symbols

| | |
|---|---|
| $A_P$ and $A_A$ | Cross section area of column |
| $d_P$ and $d_A$ | Diameter of column |
| $L_P$ and $L_A$ | Length of column |
| $V_P$ and $V_A$ | Volumetric flow rate of column |
| $X_P$ and $X_A$ | Injection amount of column |
| λmax | Wavelength with Maximum UV Absorbance |

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF APPENDICES

# LIST OF CHEMICALS AND THEIR SOURCES

| | |
|---|---|
| Acetic acid | Universe Chemicals Trading Company, Ltd (Hong Kong), HPLC grade |
| Acetonitrile | Universe Chemicals Trading Company, Ltd (Hong Kong), HPLC grade |
| Albiflorin | Boppard, with 99% purity |
| Benzoic acid | Universe Chemicals Trading Company, Ltd (Hong Kong) |
| Daidzein | International Laboratory, with 99% purity |
| Daidzin | International Laboratory, with 99% purity |
| Double deionized water | Purified from Milli-Q water system |
| Doxorubicin | Sigma-Aldrich, with 98% purity |
| DPPH powder | Sigma |
| Ethanol | Universe Chemicals Trading Company, Ltd (Hong Kong), HPLC grade |
| Genistein | International Laboratory, with 99% purity |
| Genistin | International Laboratory, with 99% purity |
| Methanol | Universe Chemicals Trading Company, Ltd (Hong Kong), HPLC grade |
| Paclitaxel | International Laboratory, with 99% purity |
| Paeoniflorin | Boppard, with 99% purity |

| | |
|---|---|
| Paeonol | Boppard, with 99% purity |
| Phosphoric acid | Analytical-reagent grade |
| Potassium dihydrogen phosphate | Wako Pure Chemical Industries, Ltd (Japan) |
| Puerarin | International Laboratory, with 99% purity |

# Chapter 1: Introduction

Traditional Chinese medicine (TCM) is a unique medical system practiced in China and the Far East for thousands of years [1]. It is a remarkable alternative medicine that has played a significant role in the therapy field. Especially in oriental countries such as China, Korea, and Japan, TCMs have been widely used in clinical treatment for thousands of years and have gained increasable popularity worldwide, even in European and North American countries [2]. In 1985, the World Health Organization estimated that about 80% of the world's population relied on traditional medicines including TCMs for their primary health care needs [1]. In these days, more than 500 species of medicinal plants were recorded in the Chinese Pharmacopoeia and the different active components present in TCMs have significant effects on peoples health.

The remarkable increased popularity in uses of TCMs is attributed to that TCM can effectively control the state of illness and can improve the resistance of patient who suffer from some severe diseases which are difficult to cure by modern medicine. In addition, it is worth to mention that some kinds of Chinese herbal medicines have many distinct functions on therapy of certain disease such as rheumatism which cannot be replaced by other medicines [2]. Therefore more and more people are using TCMs to achieve best health condition and to prevent disease condition than ever before.

With the recent worldwide recognition of TCM as potential drug resources, quality assessment of TCMs is important and essential in order to provide scientific based information for customers and patients. It has been widely recognized that it is much more difficult to ensure quality control for TCM products than for synthetic drugs and hence the quality control of TCMs is not an

easy job. There are mainly two reasons. Firstly, the TCMs are indeed very complicated systems because a TCM is composed of at least hundreds of chemical components but only a few compounds are responsible for the beneficial effects. Analyzing such complex system is a challenge to analytical researchers. Secondly, in contrast to western medicine, the fundamental pharmacological activity of TCM comes from synergetic effect of a combination of active ingredients and this make the problem more difficult to deal with [3]. In order to analyze such complicated TCM system , the State Food and Drug Administration (SFDA) of China proposed to use fingerprint as a quality measure of an herbal extract or preparation [4]. The fingerprint can be obtained by using many chromatographic and electrophoresis techniques including thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas-chromatography (GC) and capillary electrophoresis (CE). Besides, some on-line hyphenated techniques such as liquid chromatography-mass spectroscopy (LC-MS), HPLC with diode-array detector (DAD) and CE-MS have been developed and strongly recommended for quality control of TCM in recent years [2, 5, 6].

The fingerprint of a TCM obtained from the chromatographic techniques mentioned above is pattern of the TCM extract that contains more chemical information of known and unknown constituents. Some of these detectable components have pharmacologically active or chemically characteristics even if they are not all identified. Therefore it provides more unique feature for identification of the herbs, herbal extracts or preparation concerned. This pattern approach has been proved to be an effective methodology for the assessment of TCM.

A good quality control of a TCM is not only achieved by examination of chemical compositions, the efficacy of the TCM also plays a very important role. To our knowledge, the chemical profile provides useful information that links to the biological activities of the TCM and hence the evident of efficacy should be also considered. The biological activities can be assessed through the use of a range of established bioassays. Therefore, combination of analytical chromatographic techniques and a range of bioassays should play an important role to improve the TCM quality control of TCMs as more significant information on both chemical constituents and therapeutic activities of a single TCM herb are put together. In this study, the objective is to apply fingerprint analysis techniques to selected Chinese medicines (CM) based products with known therapeutic effects with combination of chromatographic technique and bioassays as well as others. Two herbs, Radix Paeoniae Alba and Radix Puerariae Lobatae, were chosen to investigate their chemical constituents and biological activities by using the chromatographic technique and bioassays respectively in this study.

The analytical techniques and bioassays used for the quality assessment of TCMs is the main focus in this study. In Chapter 2, the analytical chromatographic technique HPLC-DAD for examination of chemical constituents and preparative HPLC (PHLC) for isolation and fractionation of TCM will be briefly introduced first. Afterwards, a bioassay, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(3-sulfophenyl)-2 H-tetrazolium, inner salt] MTS targeted for examining the cell growth proliferation will be discussed. Finally, the antioxidant assay, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay will be also mentioned.

Radix Paeoniae Alba (P. Alba) is one of the most important natural medicines, and is prescribed in various Chinese medicinal preparations as an anodyne, sedative antispasmodic and astringent [7]. Very recently, the extract of P. Alba was showed to have a marked antiproliferative effect on two human hepatoma cells HepG2 and Hep3B. Its mechanism underlying anticancer growth activities by P. Alba extracts was elucidated clearly [8]. Of course, these anticancer activities of P. Alba extract are totally depended by their chemical profiles. However, the chemical composition of P. Alba extract varies a lot in the extraction solvents used and hence the activities change to different extent. Two solvents in different ratio were utilized in this work as extraction solvents to prepare five extracts of P. Alba, named as PR0, PR25, PR50, PR75 and PR100, that were found to exhibit different growth inhibitory activities on HepG2 and Hep3B cells in MTS bioassay. By using the HPLC-DAD, the chromatographic fingerprint of these five extracts were obtained and used to explain the results obtained in MTS bioassay. Moreover, we attempted to establish the relationship between the fingerprint and the efficacy of P. Alba extracts. Details of investigation are given in Chapter 3.

Followed by investigation of the therapeutic effect of P. Alba extracts, the next step is to find out the bioactive components that are responsible for the anticancer activities in Chapter 4. P. Alba extract is composed of many chemicals that work synergistically to produce its specific activity. Therefore, not only a single component is considered as potential bioactive component, but also a group of components should be studied. In Chapter 4, three identified components were examined by MTS bioassay to see whether they are responsible for anticancer activity. In addition, the most potent PR100 extract

against HepG2 cells was fractionated into six fractions by PHPLC and then their antiproliferative activities were also investigated. Moreover, TCM become popular being used clinically as complementary and alternative medicine especially in cancer chemotherapy. So many studies were carried to investigate chemosentivity of TCM with drugs. Here an effective fraction in P. Alba extract was combined with two other drugs separately, paclitaxel (PAC) and doxorubicin (DOX) in order to see any enhancement in anticancer activity of this fraction is observed. The aim of this work is to provide modern information to both patient and doctor for reference and warning from the results obtained.

Another herb to be investigated is Radix Pueraria Lobatae (P. Lobatae) which is the roots of *Pueraria lobata* (Wild) Ohwi. It was officially included in Chinese Pharmacopoeia (CP) till 2000 edition under the same name ‘Gegen (GG)”[9, 10]. It is popular in treating cardiovascular diseases such as hypertension, myocardinal infarction and arrhythmia [10, 11, 12]. P. Lobatae was found to have a rich content of isoflavonoids that were inferred to be responsible for its antioxidant activity and hence give rise to the unique therapeutic effects in treating cardiovascular diseases [10, 13]. Therefore it is very important to find an optimal extraction condition to prepare P. Lobatae extract that not only give the highest content of isoflavonoids but also give the strongest antioxidant activities.

In Chapter 5, we attempted to establish the optimal extraction condition of P. Lobatae with the help of uniform experimental design technique which is an effective method to provide more information about the system under investigation after only carrying out a few experiments. Here, the uniform design was utilized to study twelve groups of P. Lobatae extracts obtained from

variations in five factors that were solvents, extraction time, number of repeat of extraction, solvent volume and temperature. The optimal extraction condition of P. Lobatae was finally found according to not only the chemical results of concentrations of identified isoflavonoids from their chromatographic fingerprints obtained by HPLC-DAD, but also the biological results of antioxidant activities by the antioxidants assay DPPH. This study is different from the conventional approach for finding the optimal condition of P. Lobatae based on the contents of isoflavonoids only.

To summarize, the combination of hyphenated chromatographic and bioassays were applied successfully in this research project. The results are found to be useful and lead us to have high confidence for quality control of TCM including P. Alba and P. Lobatae.

# Chapter 2

# Review on Chromatographic Fingerprint techniques and Biological Screening Methodology for studying Chinese Herbal Medicine

## 2.1 Introduction

Zhang pointed out that despite TCM existence, continued usage over many centuries, and its popularity and extensive use during the last decades, traditional medicine has not been officially recognized in most countries. Consequently, education, training and research in this area have not been accorded due attention and support. The quantity and quality of the safety and efficacy data on traditional medicine are far from sufficient to meet the criteria needed to support its utilization worldwide. The main reasons for the short of research data are due to not only to health care policies, but also to a lack of adequate or accepted research methodology for assessment traditional medicine and scientific evidences [15].

TCM is a very complicated multi-component system in which the components interact with one other in unknown ways. Most importantly, the change in chemical composition of TCM could significantly affect the efficacy of a TCM and so influence the health of the patient. As a result, quality control of the complex TCM system is essential and a challenging job to scientists.

As the chemical constituents in TCMs determine the efficacy to great extent, so the quality control of TCMs is carried out not only on the qualitative and quantitative of their chemical compositions but also its efficacy. In these days, chromatography has been developed into a very powerful analytical separation tool that makes more detailed analysis of the multi-component TCM system possible. The common chromatographic techniques include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and

gas chromatography (GC). They are employed in different ways, depending largely on the need of analysis. Together with the availability of more advanced detectors such as diode array detector (DAD), evaporate light scattering detector (ELSD) and mass spectrometry (MS), we can obtain additional spectral information such as ultra-violet (UV) spectra and mass spectral that are useful for qualitative and quantitative analysis of TCMs. After the chemical components are examined by these analytical chromatography techniques, the active components or fractions can be found via biological screening and isolation or fractionation are needed for further characterization and investigation. Preparative HPLC (PHPLC), which is a large scale application, can be employed for isolation task. These chromatography techniques and their applications for determination of chemical characterization of the TCMs are introduced in this chapter.

Since the composition of TCM varies a lot, evidence of efficacy should also be considered. The efficacy of TCM can be examined through the use of a range of bioassays. In this work, two biological activities were studied. One is cell proliferation on a specific kind of cancer cell line and another one is anti-oxidant activity. For the investigation of cell proliferation of cancer cell line, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt) (MTS) bioassay, trypan blue staining, flow cytometry analysis, ATP-Lite and DNA fragmentation are commonly utilized. In this study, MTS bioassay [16, 17] was utilized and so it is introduced briefly in this chapter. For the measurement of anti-oxidant capacity in TCMs, ferric reducing anti-oxidant power (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical generating assay and oxygen radical absorption capacity (ORAC) are widely used. Only DPPH assay was utilized in this work and therefore just this one is

discussed in detail.

## 2.2 Chromatographic techniques for analysis of TCMs

Analysis of pharmaceutical preparations by chromatographic method can be traced back to at least the 1920s. By 1955, descending and ascending paper chromatography was described in the United States Pharmacopoeia [18] and now in the Chinese Herbal Pharmacopoeia [19] for the identification of herbal medicines and their commercial products. Afterward, high-performance liquid chromatographic (HPLC) and gas chromatographic (GC) were also introduced. At present, these chromatographic techniques are not only utilized for analysis of TCM, but also are introduced for isolation and fractionation of TCM samples. For example, PHPLC was used for isolation in large scale. The chromatographic methods have clearly become the analytical methods of choice, with over 800 cited [20]. In this study, the HPLC and PHPLC were utilized. A brief introduction of these two chromatographic methods and their application in analysis of TCMs will be discussed in the follow sections.

### 2.2.1 High-performance liquid chromatography

Over the past decades, high-performance liquid chromatography (HPLC) with powerful separation ability has been widely applied for herbal medicine analysis [2]. It is primarily used for the separation of high molecular weight, non-volatile or and polar compounds in three modes, i.e. normal phase, reverse-phase and gel-permeation chromatography through suitable optimization procedures involving the composition of mobile phase, pH, and analytical columns [2].

These modes are provided to analyze different kinds of samples. Among these, reverse-phase (RP) chromatography is the most widely used one. Interfaced with detector such as diode-array detector (DAD), mass spectroscopy (MS) and evaporated-scattering light detector (ELSD), additional information including chromatograms and spectra is provided for characterization and authentication. Because of its versatility, high reproducibility, ease of automation and good linear range, a vast range of applications has been reported using this technique, especially in the area of pharmaceutical and drugs analysis [5]. Significant increase in the number of HPLC application to analysis of TCMs can be also reflected by China Pharmacopoeia 2005 edition.

Many literatures reported the use of HPLC in quality assessment and control of TCMs and their preparations. HPLC-MS was employed to identify 25 ginsenosides that are both neutral and acidic ginsenosides from P. ginseng [21] and HPLC-MS-MS was utilized to develop a rapid screening and structural assignment of isomeric saponins of P. ginseng [22]. HPLC technique was also applied to analyze the toxic compounds from TCMs samples. HPLC-MS has also been used to detect aristolochic acids isolated from *Aristolochia manshuriensis* [23] and the highly toxic lignan called podophyllotoxic was found in the anti-cancer herbs *Podophyllum emodi* by HPLC-MS-MS [24].

### 2.2.2 Preparative high-performance liquid chromatography

Liquid phase chromatography (LC) has been developed successfully as a powerful analytical technique. Modification of LC for the large scale was also attempted. Preparative high pressure liquid chromatography (PHPLC) was

originally introduced in the 1980s [25]. Both analytical techniques and large scale applications have been continuously developed by chemists and chemical engineer respectively. The main task of analytical liquid chromatography (ALC) is to separate individual components of a mixture for qualitative and quantitative determination; while PHPLC is utilized to isolate the pure components out for subsequent applications such as structural studies, bioassays, pharmacological tests, reference substances and standards for quantitative determinations.

The features of the equipment used in ALC and PHPLC are also different. It is very important to decide the scale of the preparative when choosing which equipment. If the compounds of interest needed are only in micrograms, the ALC system is sufficient. Otherwise, the PHPLC system is essential. The working devices within a PHPLC system include sample injection system, pumps, columns, detector, solvent recycler and fraction collector. Furthermore, ancillaries such as rotary evaporators, freeze-driers, purification system may be needed.

In the process of scaling up preparative chromatography, the chromatograms of initial analytical scale runs were the starting point of the scale-up procedure. It is very important and should not be omitted. The basic idea behind scale-up is to preserve the quality of the separation achieved at small scale [26, 27]. Maintaining resolution and throughput in chromatographic separations during scale-up can be difficult unless the performance of the packed beds at the larger scale is comparable to those of the smaller scale [28]. The flow and the injection

amount are then easily scaled up by calculation.

## 2.3 Bioassays for study biological activities

### 2.3.1 Bioassays for studying cell proliferation in cancer cell line

Cancer is the second most common cause of death over the world but probably the first most feared disease since it is inevitably associated with death in most people's minds [29]. In the past decades, many TCMs are also complemented in the cancer therapy because they are found to have growth inhibitory activity on associated cancer cell lines. For example, the lipophilic extract of *Hypericum perfratum* excerts significant cytotoxic activity against urinary bladder tumor cells [30], the acetone extract of *Angelica sinensis* inhibits proliferation of human cancer cells [31], the water extract of herbal preparation called Bu-Zhong-Yi-Ui-Tang inhibited the proliferation of human hepatoma cell lines [32] and others. Many bioassays has been developed for investigation of cell proliferation of cancer cell line. The most simple one is (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt) (MTS) assay [16, 33]. It belongs to reaction methods between the reagent and the cells of interest. The following section will give a concise introduction on this topic.

#### 2.3.1.1 MTS bioassay

(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2

H-tetrazolium, inner salt) MTS bioassay is a colormetric assay system which measures the reduction of a yellow tetrazolium component (MTS) into a soluble purple formazan product by the mitochondria of viable cells. Figure 2.1 shows the structures of MTS tetrazolium salt and its formazan product.

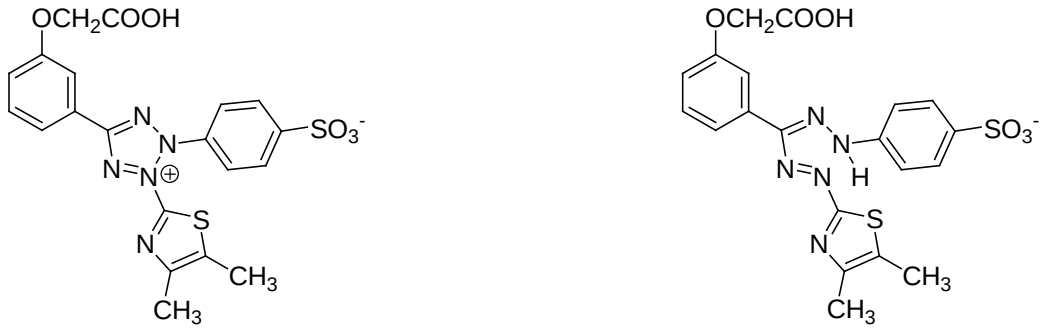



Figure 2.1 Structures of MTS tetrazolium salt and its formazan product.

Optical density (OD) of cells treated with medium and OD of cells treated with sample are measured using a microplate reader at a wavelength of 595nm. The growth inhibitory is calculated by the following equation [33].

$$\text{Growth inhibitory} = \frac{\text{OD of cells treated with medium} - \text{OD of cells treated with sample}}{\text{OD of cells treated with medium}} \qquad \text{[Eq. 2.1]}$$

The antiproliferation curve is established by plotting the growth inhibitory against a serial concentration of sample. When compared the antiproliferation activities among different samples, the IC50 value (the inhibitory concentration at which 50% of cell is inhibited to grow) is commonly used and is determined

from the growth inhibitory curve.

MTS bioassay is capable to give an accurate, straightforward quantification of change in proliferation and also it can be adapted to high-throughput screening. Therefore, MTS bioassay is very popular and widely utilized in study cell proliferation of TCMs extracts. For example, MTS bioassay was employed as growth inhibition assay for anaylsis of *Gleditsia sinensis* fruit extract in chronic and acute myelogenous leukemia [16] and cytotoxic activity of cantharidin isolated from *Mylabris caraganae* was carried out by MTS bioassay [17].

### 2.3.2 Assay for study of anti-oxidant activity.

There has been intense interest recently among the public and the media in the possibility of the increase intake of dietary antioxidants to protect against chronic diseases, including cardiovascular and cerebrovascular diseases. Most herbal medicines and dietary foods are found rich in anti-oxidant components. For example, the *Ganoderma lucidum* is rich of terpenes components [34] and the Radix Puerariae Lobatae is rich of isoflavonoid components [35] that are shown to have anti-oxidant activity.

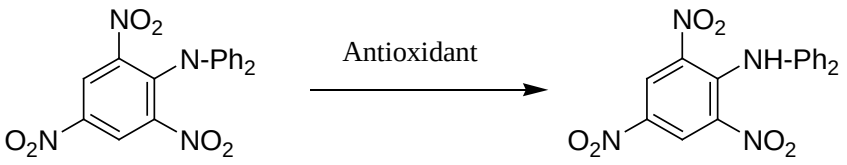
Anti-oxidants are substances that, when present at low concentration, compared with those of an oxidizable substrate, significantly prevent or delay a pro-oxidant-initiated oxidation of the substrate. A pro-oxidant is a toxic substance that can cause oxidative damage to lipids, proteins and nucleic acid (e.g. DNA), resulting in various pathological events or diseases. Example of pro-oxdiants includes reactive oxygen and nitrogen species (ROS and RNS).

ROS include superoxide ($O_2^{-\cdot}$), hydroxyl ($OH^{\cdot}$), peroxyl radical ($ROO^{\cdot}$) and hydrogen peroxide ($H_2O_2$) while RNS include nitric oxide ($NO^{\cdot}$) and nitrogen dioxide ($NO_2^{\cdot}$). And the anti-oxidant capacity is defined as the ability of a compound to reduce pro-oxidants [36].

All the methods developed for measuring total anti-oxidant capacity of a biological sample involve oxidants or oxidizing agents that accept electrons from reductants, which are often treated as the anti-oxidants being measured. On the basis of the oxidants used, these methods can be divided into two groups: one using oxidants that are not necessarily pro-oxidant such as FRAP and ORAC assays, and the other using oxidants that are pro-oxidants such as DPPH assay [36]. In this study, DPPH was utilized for examining the antioxidant activities of samples. This two anti-oxidant assay will be discussed briefly as follow.

### 2.3.2.1 2, 2-diphenyl-1-picrylhydrazyl radical assay

2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) assay is a free radical scavenging test that is based on the measurement of the scavenging ability of anti-oxidants towards the stable free radical DPPH [36, 37, 38]. DPPH is reduced by donation of hydrogen atom or other radicals from anti-oxidant to the corresponding hydrazine and the reaction is depicted in Figure 2.2.




**DPPH** **DPPHH**

Detected in 515nm

Figure 2.2 The reaction in DPPH assay

The concentration of DPPH radical is monitored by the absorption at 515 nm. Therefore the antioxidant capacity is measured by decrease in absorbance at this wavelength. DPPH• signal intensity is being related inversely to anti-oxidant concentration and to the reaction time. The percentage of Free Radical Scavenging Capacity (SR %) of the antioxidant is determined by the following equation

$$SR\ \% = (1 - \frac{A_{sample} - A_{original}}{A_{control}}) \times 100\% \qquad \text{[Eq. 2.2]}$$

where $A_{sample}$, $A_{original}$ and $A_{control}$ represent the absorbance of sample in DPPH• radical solution, sample in methanol solution and DPPH• solution respectively. The $A_{original}$ of herbal sample or standard in methanol was also measured in order to eliminate the color interference of the herbal sample. Then the graph of SR% against the concentration of sample is plotted and the scavenging activity at constant concentration is used for comparison. In this assay, DPPH • serves as both the oxidizable substrate and the reaction indicator molecule. Besides, this assay is carried in a direct and simple way. In addition, the free radicals, are involved in the process of lipid peroxidation and so are considered to play a

fundamental role in several chronic disease. The radicals can also reduce the DPPH• into its hydrazine [39]. The anti-oxidant activity reflected from this assay is similar to anti-oxidant in the body. Therefore, it is widely use in investigation of anti-oxidant capacity in TCMs. For example, anti-oxidant activity f *Myristica malabarica* extracts and their constituents [37] and the scavenging capacity of methanol extract of *Tillandsia streptocarpa* Baker were tested by DPPH assay [38].

# Chapter 3

# Chemical and biological studies of Radix Paeoniae Alba extracts on human hepatoma cancer cell lines

## 3.1 Introduction

Herbal, or “botanical”, medicines, recorded in Chinese Pharmacopoeia had been prescribed in many diseases for long time, and began to be matched by increasing scientific attention recently [40]. Due to the different components in a herb may have synergistic activities or buffering toxic effects, mixtures or extracts of herbs might have more therapeutic or preventive activity than as a single compound [40, 41]. Several studies have demonstrated that extracts from several herbal medicines or mixtures had an anticancer potential in vitro or in vivo. Hu et al demonstrated that an alcohol extract of *Ganderma lucidum* could induce apoptosis in MCF-7 human breast cancer cells [42]. Kao et al reported that a water extract of Bu-Zhong-Yi-Qi-Tang (mixture of then herbs) could apoptosis in hepatoma cells [32].

Radix Paeoniae Alba (P. Alba) is the root of traditional Chinese Herb named *Paeonia lactiflora* Pallas and it is a crude drug used in many transitional prescriptions such as Shao-yao-tang (Penoy combination) in China and Japan. Study showed that P. Alba extract exhibited a marked direct cytotoxic effect to both HepG2 and Hep3B human hepatoma cells (IC50 are 4.6 mg/ml and 4.2 mg/ml respectively). And the mechanism underlying anticancer growth activities was clear and elucidated. The cytotoxicity of P. Alba extract to the cells is through activation of the cell death program, apoptosis, evidenced by induction of internucleosomal DNA fragmentation and chromatin condensation appearance, and accumulation of sub-G1 phase of cell cycle profile [8].

The TCM is composed of a very complicated multi-component system in which

the components interact with each other in unknown ways. These components of a TCM even a same herb are varied greatly not only in cultivation conditions including temperature and soil characteristics and manufacturing processes including extraction and so the quality of TCMs are influenced. In this study, different ratios of solvent A and B, that were commonly employed to study saponins class of P. Alba extracts, were utilized in sample extraction and therefore five extracts of P. Alba were obtained. The growth inhibitory activities of these five extracts on human hepatoma cell lines were investigated by MTS bioassay first. Afterward, the chemical compositions of these extracts were determined by their corresponding chromatographic fingerprint that was obtained by HPLC-DAD. Finally we attempted to find the relationship between the fingerprint and the efficacy in order to provide science base for assessment of P. Alba extracts.

## 3.2 Background of Radix Paeoniae Alba

Radix Paeoniae *A*lba (P. Alba), also known as Bai Shao (白芍), is the dried root of traditional Chinese Herbs named *Paeonia lactiflora* Pallas (P.albiflora Pall.) or *Paeonia veitchii* Lynch [43, 44, 45, 46] that is shown in the Figure 3.1. It is taken into “Chinese Drug Monographs and Analysis”.

Figure 3.1 The dried root of *Paeonia lactiflora* Pallas

P. Alba belongs to the Ranunculacease (毛茛科) family [45, 46]. It always appears in white color with smooth surface and cut into thin slices. Figure 3.2 shows its appearance.

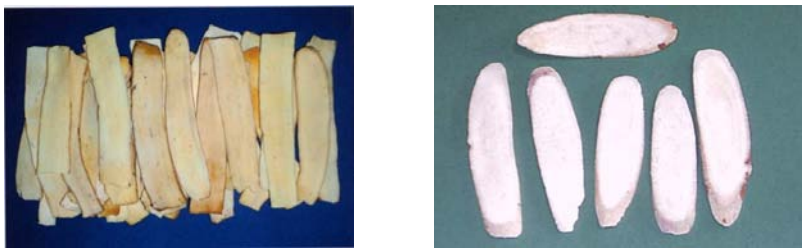


Figure 3.2 The appearances of Radix Paeoniae Alba

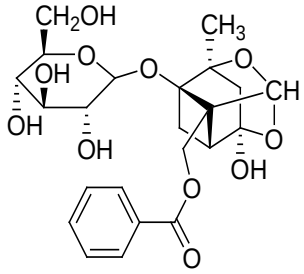
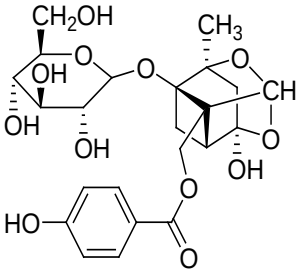
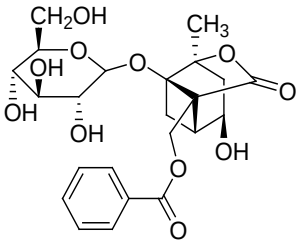
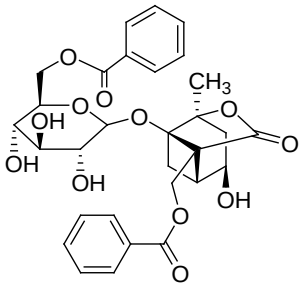
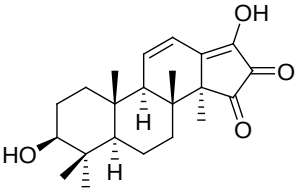
P. Alba is collected in summer and autumn [7] and its origin is mainly Chekiang, Anhwei, Szechwan, and other provinces. Its taste is bitter and sour while its nature is slightly cold. The channels entered are liver and spleen [45]. The functions of P. Alba is to clear heat [45], nourish the blood [44, 45], adjust menstruation [45, 47], repress Liver Yang, relieve abdominal spasmodic and alleviate pain [45] according to the literature of traditional Chinese medicine (TCM). It is indicated for uterine bleeding, menstrual irregularities, lower blood pressure, pain in the chest, abdomen and hypochondrium. That's why P. Alba is always prescribed in Chinese medicinal preparations as an anodyne, sedative and antispasmodic. The common dosage of P. Alba is about 5-10g to alleviate pain by calming the Liver.

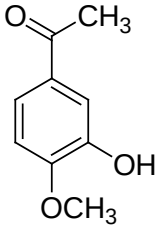
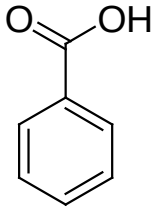
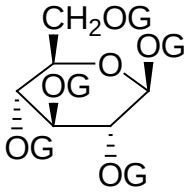
## 3.3 Literature review on chemical study and biological study of P. Alba

### 3.3.1 Chemical composition

Protein, polysaccharides, monoterpenes, monoterpene glycoside and volatile oils are the main ingredients in P. Alba while the bioactive chemical components are found mainly in the classes of monoterpene and monoterpene glycoside [7, 43, 44, 47]. Several bioactive chemical components in these two classes were reported [48, 49]. They are paeoniflorin (PF), albiflorin (AF), oxypaeoniflorin (OPF), benzoylalbiflorin (BAF), paeonol (PN), benzoic acid (BA), pentagalloyglucose (PG) and palbinone (PB). PF and AF are used as marker to evaluate the quality of P. Alba [50, 51]. The molecular masses and structures of bioactive components are given in Table 3.1. However, the supply of commercial chemical standards is always limited, so five of these nine chemical components were studied to see whether or not they have inhibitory effect on both HepG2 and Hep3B. These five chemical components are C1 (temporily name as it is to keep confidential), AF, PF, BA and PN.

Table 3.1: Molecular masses and structures of bioactive components of P. Alba [1,3,6]

| Paeoniflorin, $C_{23}H_{28}O_{11}$ | Oxypaeoniflorin $C_{23}H_{28}O_{12}$ | Albiflorin, $C_{23}H_{28}O_{11}$ | Benzoylalbiflorin, $C_{30}H_{32}O_{12}$ | Palbinone $C_{22}H_{30}O_4$ |
|---|---|---|---|---|
|  |  |  |  |  |

| Paeonol $C_9H_{10}O_3$ | Benzoic Acid $C_7H_6O_2$ | Pentagalloylglucose. $C_{41}H_{32}O_{26}$ |
|---|---|---|
|  |  |  G = galloyl |

### 3.3.2 Pharmaceutical activity of bioactive components

These bioactive components have their own pharmacological activities and are listed in Table 3.2.

Table 3.2. Pharmaceutical activities of bioactive components in P. Alba

| No. | Name of Component | Molecular Mass | Pharmaceutical activties |
|---|---|---|---|
| 1 | Paeoniflorin (PF) | $C_{23}H_{28}O_{11}$ | Anti-coagulant, neuromuscular blocking, immunoregulating, cognition-enchancing, anti-hyperglycemic,depressant, antispasmodic [47, 50, 52], acute toxicity of this is very low [52], anti-inflammatory effect |
| 2 | Oxypaeoniflorin(OPF) | $C_{23}H_{28}O_{12}$ | Potent radical scavenging effect[8] |
| 3 | Albiflorin(AF) | $C_{23}H_{28}O_{11}$ | Relatively weak inhibitory effects on DNA cleavage [49, 50], inhibition the EEG power spectrum |
| 4 | Benzoylalbiflorin(BAF) | $C_{30}H_{32}O_{12}$ | Inhibited blood platelet coagulation, inhitory effects on plasminogen and plasmin [52] |
| 5 | Paeonol(PN) | $C_9H_{10}O_3$ | Inhibit the growth of Escherichia coli and Bacillus subtilis, inhibit blood platelet aggregation, anti-inflammatory actions, acute toxicity is low [52] |
| 6 | Benzoic acid(BA) | $C_7H_6O_2$ | Antioxdiant |
| 7 | Pentagalloylglucose (PG) | $C_{41}H_{32}O_{26}$ | Cholesterol lowering, treatment of gastric and peptic ulcers , capable of perturbing the cell cycle of the human breast cancer cell line MCF-7 [53] |
| 8 | Palbinone(PB) | $C_{22}H_{30}O_4$ | Strong inhibitory activity on the reduced form of NADPH-linked 3α-HSD of rat liver cytosol and significant inhibitory activity on human monocyte |

| | | | |
|---|---|---|---|
| | | | interleukin-1β [48, 54] |

### 3.3.3 Chemical studies of Radix Paeoniae Alba

Many investigations in chemical characterization of constituents of P. Alba by different chromatographic and spectroscopic techniques have been reported. The volatile constituents of P. Alba have been studied by GC-MS [55]. HPLC-MS methods using reversed-phase column were also reported to determine the saponins class of P. Alba [7]. In addition, the chromatographic conditions of HPLC-DAD with well separation in analysis of saponin components of P. Alba were developed [43] and therefore these conditions were chosen in chemical study of P. Alba extracts in this study.

### 3.3.4 Biological studies of Radix Paeoniae Alba

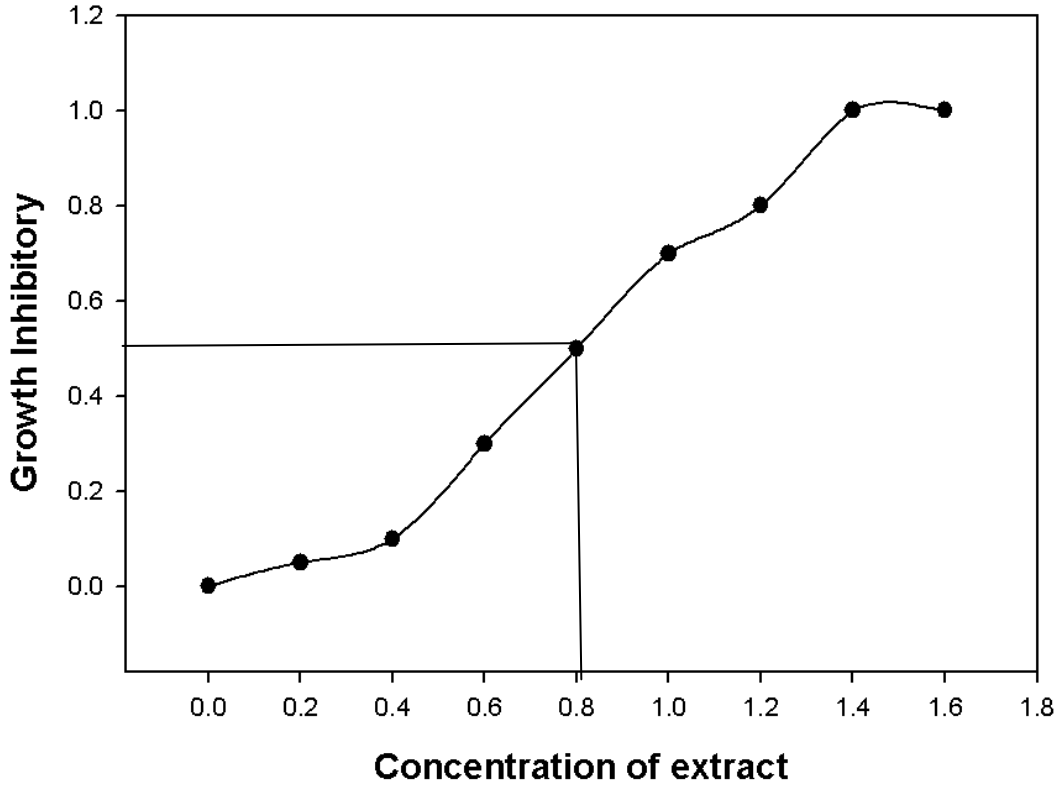
The pharmacokinetic study of paeoniflorin in mice after oral administration of P. Alba extract has been studied [56]. The molecular mechanism of the effect of P. Alba on human hepatoma cell lines HepG2 and Hep3B was evaluated by using DNA fragmentation and grow cytometry bioassays [8]. In addition, the anti-cancer activities of pentagalloyglucose on human breast cancer cells was studied by trypan blue staining, ATP-Lite and flow cytometry bioassay [53]. Furthermore, the anti-oxidant activities of P. Alba extract and some components of P. Alba were studied by DPPH assay [57, 58].

## 3.4 Methods of investigation

The aims of this work were to investigate that different ratio of more polar solvent A and less polar solvent B were employed to give five extracts of P. Alba, named as PR0, PR25, PR50, PR75 and PR100, and then their activities on HepG2 and Hep3B cells were examined. In addition, the five extracts of P. Alba were studied by HPLC-DAD chromatographic technique to obtain their corresponding chromatographic fingerprints through which the changes in chemical composition of these extracts were observed. Finally, the relationship was found according to the results of change of chemical composition in the five extracts of P. Alba shown in the chromatographic profile together with the results of bioassay on cell proliferation on these cell lines.

The triterpene saponins of five extracts of P. Alba was obtained by different ratio of solvent A and solvent B. Then the cell proliferations of these extracts were investigated by MTS bioassays. In the MTS bioassay, the optical density (O.D.) of cell treated with medium only and extract were measured at 595nm. The growth inhibitory was expressed in the following equation [59]. The cell proliferation curve was obtained by plotting the growth inhibitory against a series of concentration. The cell proliferation of P. Alba extracts was expressed in term of IC50 value. Figure 3.3 demonstrated how to find the IC50 value from the antiproliferation curve.

Figure 3.3 The graph illustrating how IC50 of an extract can be found from the plot of growth inhibitory activity against the concentration of the extract




At the same time, the chromatographic fingerprints of triterpene saponins in the five extracts were obtained by the hypentnated technique HPLC-DAD. The retention time and UV spectra of peaks in chromatograms profile were used to compare with that of standards of C1, AF, PF, BA and PN for qualitative analysis. The quantitative analysis of identified triterpene saponins components in P. Alba extracts was carried by using the calibration curves of standards available. Finally combination of the results of MTS bioassay and chromatographic fingerprints of P. Alba extracts, the efficacy on anti-cancer activities and change in chemical compositions were related to each other.

## 3.5 Experimental

### 3.5.1 Herbal sample

Radix Paeoniae Alba, the dried roots of *Paeonia lactiflora* Pallas, was purchased from the famous local shop of Tung Fong Hung Medicine Company (Hong Kong, China).

### 3.5.2 Sample extraction and standards preparation

The sample of pulverized and dried P. Alba roots (1:10 w/v) was extracted by refluxing with different ratio of solvent A (more polar) and B (less polar) for 30 minutes according to Table 3.3. The names of the two solvents are not mentioned in this thesis for confidential purpose

Table 3.3: Different ratio of solvent A and B used for sample extraction of P. Alba

| Name of extracts | PR0 | PR25 | PR50 | PR75 | PR100 |
|---|---|---|---|---|---|
| **Solvent A** | 0 | 25 | 50 | 75 | 100 |
| **Solvent B** | 100 | 75 | 50 | 25 | 0 |

The filtrate was collected by filtration through using 0.45μm pre-cut membrane and the solvent was removed by rotatory evaporator. Then the filtrate was dissolved into fixed volume of desired solvent and then lyophilized by freeze dryer to yield the dry powders. The dry powders were kept in 4℃ and dark place when not in use. The concentration used in the experiment was based on the dry

weight of extract (mg/ml).

The five standards were prepared with different concentrations from 20 ppm to 100 ppm.

### 3.5.3 Chromatographic conditions of analytical LC

Aglient 1100 series HPLC apparatus, equipped with a quaternary solvent delivery system, an auto-sampler and UV detector, was utilized. Chromatographic runs were carried out by a Thermo ODS Hypersil column (250 X 4.6mm, 5.0μm) at a room temperature. The injection volume was 20μl and the flow rate was 1.0ml/min. The running time was 95 minute and the detection wavelength was set at 230 nm. The mobile phase consisted of (A) 50mM $KH_2PO_4$ and ACN ( 95:5) at pH 3 which was adjusted by addition of $H_3PO_4$ and (B) DDI and ACN (1:4) using a gradient elution as listed in Table 3.4.

Table 3.4 The elution gradient of mobile phase in HPLC-DAD measurement

| Time (min) | A / % | B / % |
|---|---|---|
| ***0-10*** | ***100-90*** | ***0-10*** |
| ***10-15*** | ***90*** | ***10*** |
| ***15-40*** | ***90-77*** | ***10-23*** |
| ***40-45*** | ***77-67*** | ***23-33*** |
| ***45-70*** | ***67-17*** | ***33-83*** |
| ***70-80*** | ***17-0*** | ***83-100*** |
| ***80-95*** | ***0-100*** | ***100-0*** |

### 3.5.4 Cell Culture on HepG2, Hep3B and WRL-68

HepG2 and WRL-68 cells were maintained and propagated in 90% RPMI-1640 medium while Hep3B cells was maintained in Dulbecco modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in the 75cm$^2$ tissue culture flask at 37℃ in 5% $CO_2$ humidified incubator. When the cells were grown to 95% confluency, they were rinsed with phosphate-buffered saline (PBS) for three times. The cultured cells were then detached by trypsinization and pelleted through centrifugation at 2500rpm for 15mins. The cells were resuspended in corresponding medium to obtain a cell density of 1 x $10^4$ cells/ 0.1ml for seeding in 96-well microplates.

### 3.5.5 Cell Proliferation on Radix Paeoniae Alba extracts by MTS bioassay

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(3-sulfophenyl)-2 H-tetrazolium, inner salt] MTS bioassay was performed. The cells were seeded in a 96-well flat-bottomed plate on day 1. On day 2, different concentration of P. Alba extracts were added and the cells were incubated for a further 1-5 days at 37℃ and 5% carbon dioxide. For incubation time of 1-5 days, the cells were washed with PBS buffer solution. Afterwards, 20 μl of MTS solution (MTS dissolved in PBS solution in 1:9 v/v) was added and the plate was allowed to incubate for further 2 hours at 37℃ in incubator. Optical density (OD) was recorded at 490 nm using microplate reader (Model550, Bio-Rad). Cells without any drug treatment were served as a control of 100% survival. Results shown in the next section are the average values of three independent experiments

performed in triplicate and are presented in form of the means ± standard deviation (S.D.).

## 3.6 Results and Discussion

### 3.6.1 Cell proliferation of Radix Paeoniae Alba extracts on three human hepatoma cell lines

Cell proliferation activities of cancer cells are dependent of time- and dose-manner of extracts. Firstly, to assess the effect of PR100 on the inhibition of both HepG2 and Hep3B cell proliferation, the cells were treated with 2mg/ml PR100, incubated for day one to day five. The results are given in Figure 3.4.

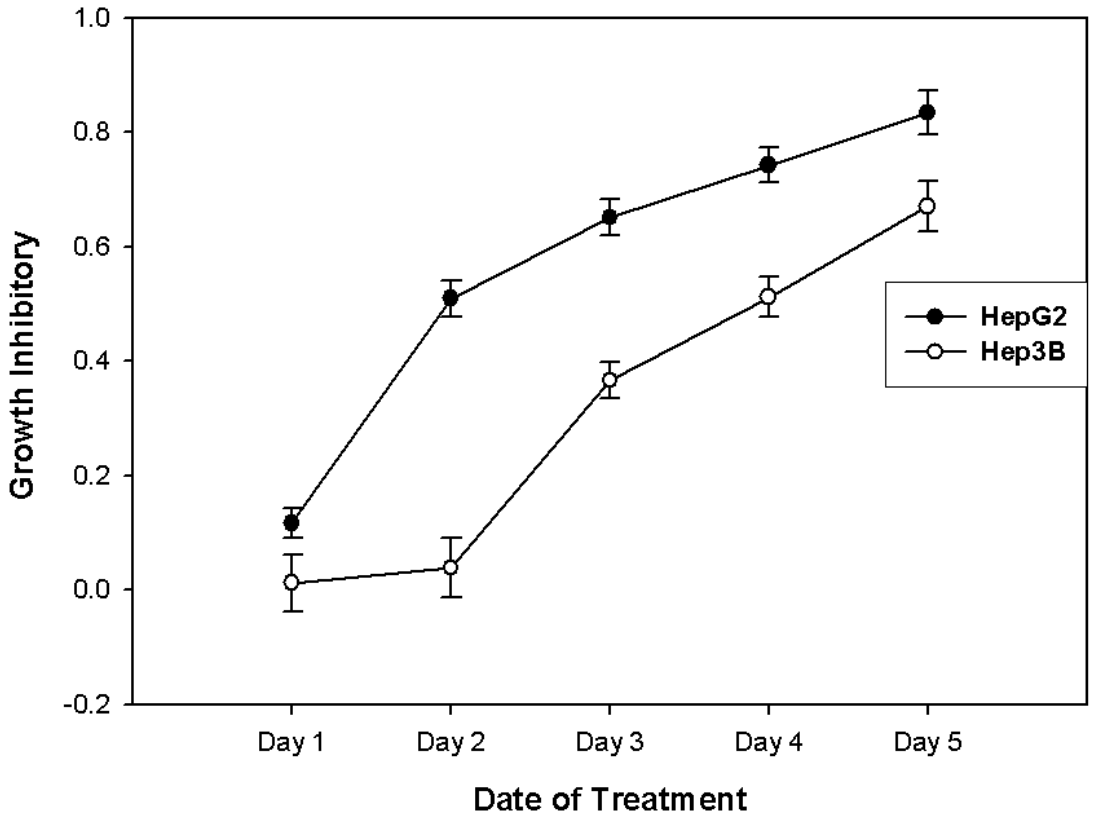



Figure 3.4 Effects of 2 mg/ml of PR100 on cell proliferation of HepG2 and Hep3B human hepatoma cancer cells from day one to day five.

Figure 3.4 shows that the growth inhibitory activities on HepG2 and Hep3B cells were showed time-dependently from day one to day five by PR100. Both HepG2 and Hep3B cells were significantly inhibited from day one to day three with reduction 0.65±0.03 and 0.38±0.04 respectively. Afterwards, the inhibitory effects of HepG2 and Hep3B cells changed to lesser extent (just further reduced by 0.18±0.03 and 0.28±0.04 respectively). Therefore, three days of treatment was chosen in the following experiments.

After the investigation of the time-dependent manner of P. Alba extracts on HepG2 and Hep3B cells, the dose-dependent manner of P. Alba extracts on these two cell lines were examined in this part. The results of experiments on determining antiproliferation activities of PR0, PR25, PR50, PR75 and PR100 extracts on HepG2 and Hep3B cells, after three days at dosages from 0.2mg/ml to 5.0mg/mll were depicted in Figure 3.5 and Figure 3.6.

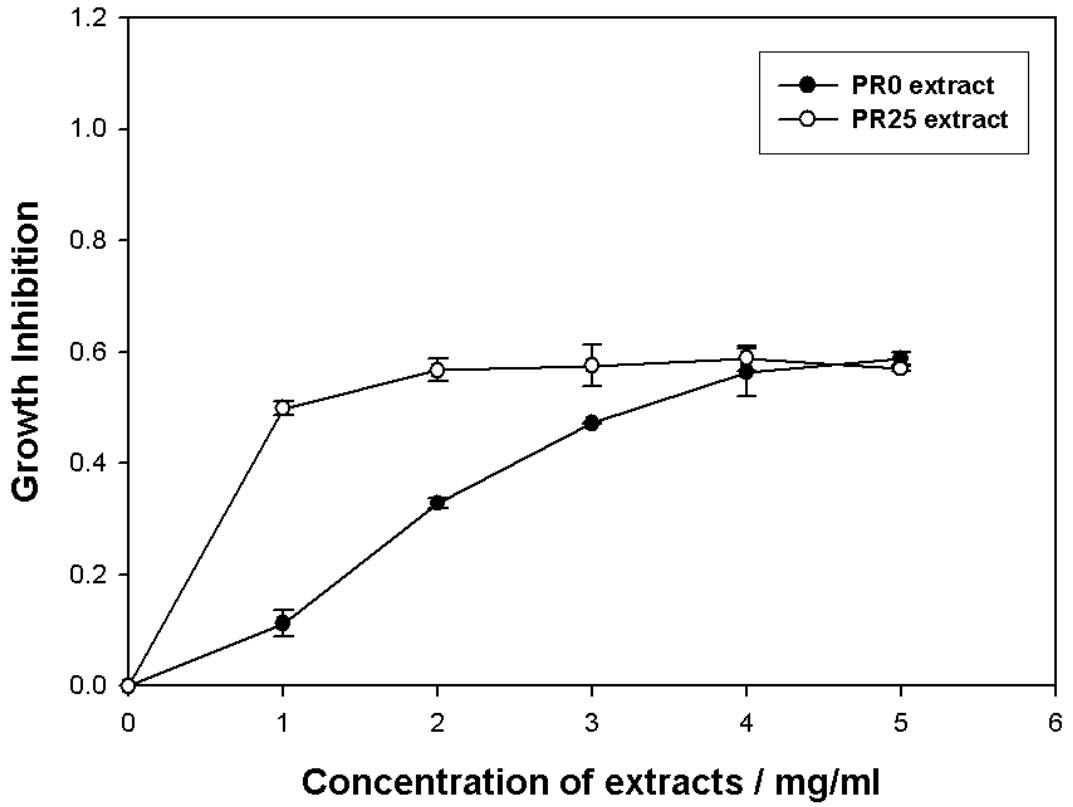



Figure 3.5 Growth inhibition effect of PR0 and PR25 (in the concentration range of 1mg/ml – 5mg/ml) on HepG2 cells

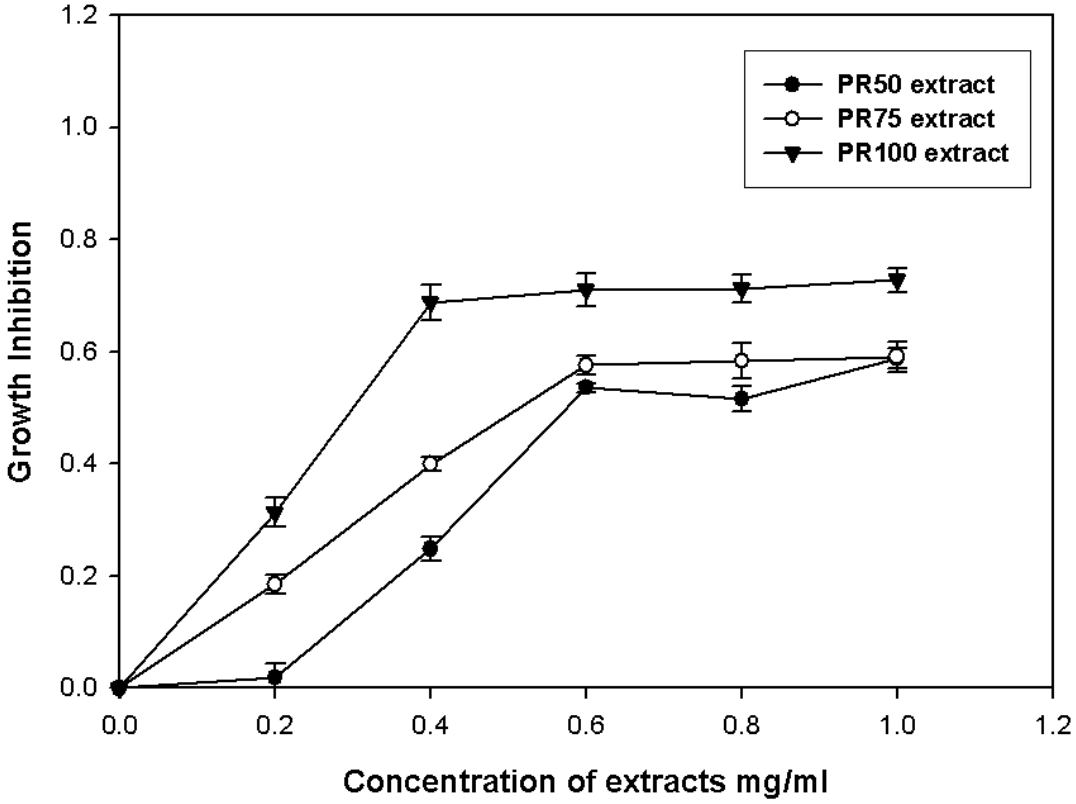



Figure 3.6 Growth inhibition effect of PR50, PR75 and PR100 (in the concentration range of 0.2mg/ml – 1.0mg/ml) on HepG2 cells

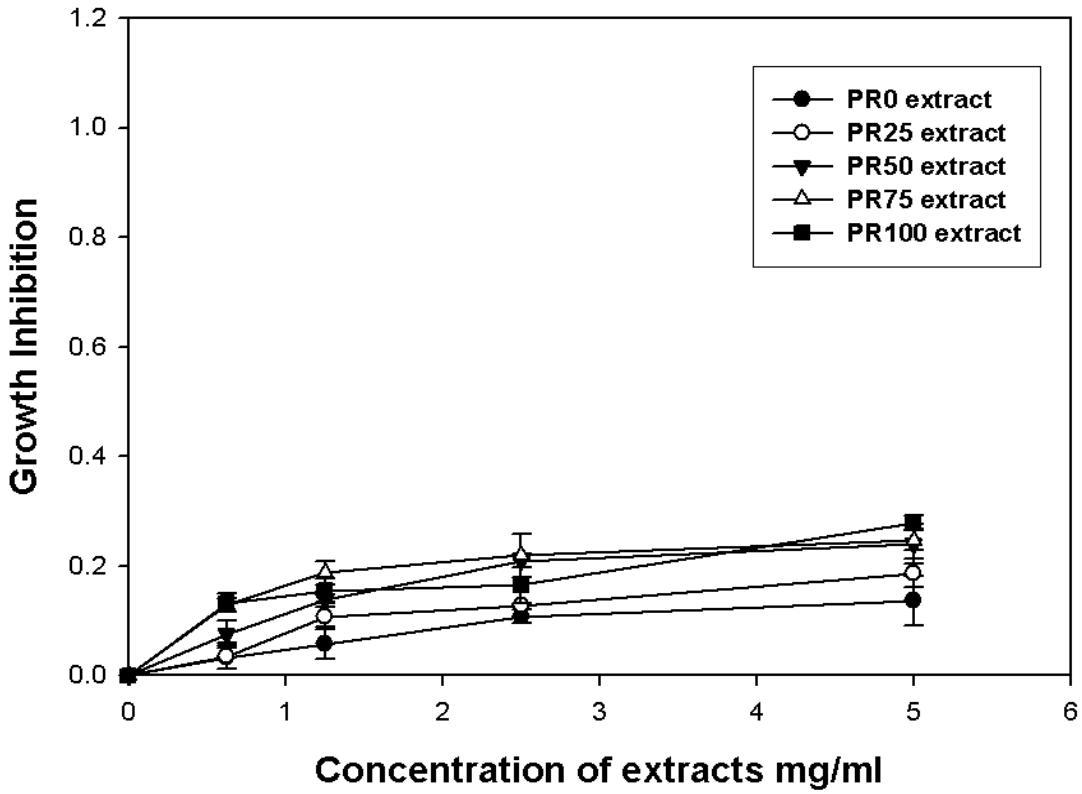



Figure 3.7 Growth inhibition effects of the five extracts of P. Alba (in the concentration range of 0.625mg/ml – 5.0mg/ml) on Hep3B cells

It can be seen from these figures that the five extracts dose-dependent inhibitory effects on the proliferation of HepG2 cell line. The IC50 values of these P. Alba extracts at three day were determined from the curves in Figure 3.5 and 3.6 and are summarized in the Table 3.5.

Table 3.5 *IC50 values of the five extracts of P. Alba on HepG2 cells

| **Extracts** | **PR0** | **PR25** | **PR50** | **PR75** | **PR100** |
|---|---|---|---|---|---|
| **IC50 value / mg/ml** | 3.33±0.42 | 1.01±0.04 | 0.56±0.01 | 0.50±0.03 | 0.33±0.01 |

* Results given are the average values of three independent experiments performed in triplicate and were presented in form of the means ± standard deviation (S.D.)

Table 3.5 shows that the five extracts of P. Alba have different cell proliferation activities on HepG2 cells. The IC50 of PR100 is the lowest among all. This means that PR100 was the most potent extract against HepG2 cells. Besides, the growth inhibitory of PR100 was greater than those of the other four extracts at 1mg/ml concentration. In addition, the antiproliferation effect of each P. Alba extract keeps lowering at higher concentration because the curves become flat at higher concentration. Furthermore, it is interesting to note that the IC50 values decrease from PR0 to PR100 (Table 3.5). This trend tells us that higher ratio of solvent A used for P. Alba extraction can get more active ingredients from P. Alba and gives greater growth inhibitory effect on HepG2 cells. This indicates that the use of different solvent influence significantly the biological activities of extracts.

On the other hand, the growth inhibitory effect of the five extracts of P. Alba on Hep3B cells is depicted in Figure 3.7. No IC50 values were found for all P. Alba extracts even at the concentration of 5mg/ml. The five extracts did not give significant growth inhibitory effect on the Hep3B cells. PR0 shows almost no such effect while only about 11% of Hep3B cells was inhibited by PR25. PR100 exhibited a comparable high % on inhibiting Hep3B cells, just about 28% that of Hep3B cells. Therefore PR100 have the greatest inhibitory effect on Hep3B cells among all but the effect is still not significant compared to HepG2 cells. Table 3.6 shows how many percentages of Hep3B cells were inhibited at the concentration 5mg/ml of the five extracts.

Table 3.6 The Percentage of inhibited Hep3B cells by P. Alba extracts at 5mg/ml

| **Extracts** | **PR0** | **PR25** | **PR50** | **PR75** | **PR100** |
|---|---|---|---|---|---|
| ***Percentage of Hep3B inhibited** | 0 | 11±3.1 | 24±1.6 | 25±2.7 | 28±3.3 |

* Results given are the average values of three independent experiments performed in triplicate and were presented in form of the means ± standard deviation (S.D.)

Compared with the results on HepG2 cells, the growth inhibitory effects of P. Alba extracts (in the concentration range of 0.2-5mg/ml) on HepG2 cells were greater and more obvious than that on Hep3B cells. This means that P. Alba extracts exhibited stronger growth inhibitory on HepG2 cells than that on Hep3B cells. In addition, among the five extracts of P. Alba, PR100 gives the greatest inhibitory effect on the growth of both HepG2 and Hep3B cells. Therefore it is the most potent extract against the liver cancer according to the results obtained.

PR100 has been found to have the greatest inhibitory activity on HepG2 and Hep3B cells. Yet, it is necessary to examine the cell proliferation of the extracts on normal liver cells. WRL-68, which is normal ovary cell line but has normal liver cell characterization, was used to examine the cell proliferation of P. Alba extracts. WRL-68 cells were treated with PR100 in the concentration range of 0.2-1.0mg/ml under three day treatment. Figure 3.8 shows the anti-proliferation effect of PR100 on WRL-68 cells. PR100 exhibited growth inhibitory activity on WRL-68 cells to lesser extent. It just inhibited about 35% of WRL-68 cells at 1mg/ml of PR100. At the concentration of 0.3mg/ml of PR100, which was near the IC50 value of PR100 on HepG2 cells, just about 20% of WRL-68 cells was inhibited. Therefore, it seems safe to use this dosage of PR100 to treat the patients with liver cancer based on these preliminary results. But more work such

as animal tests needed to be done before carrying out clinical trial.

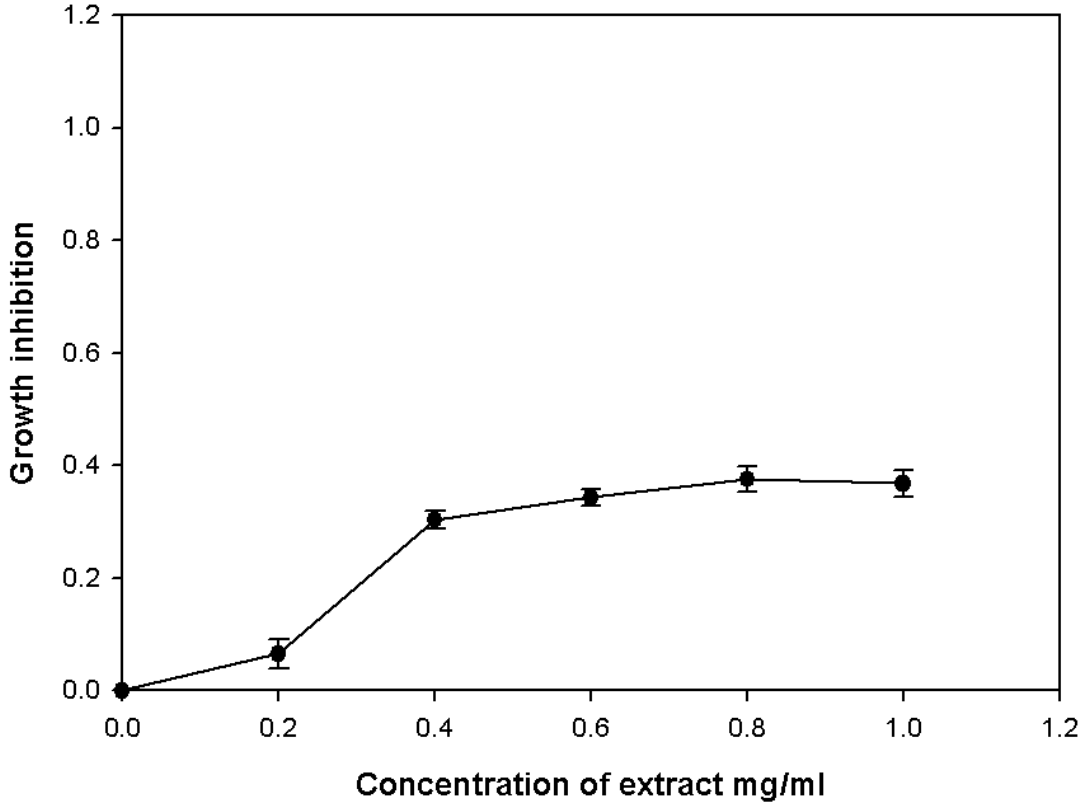



Figure 3.8 Growth inhibition effect of PR100 (in the concentration range of 0.2-1.0mg/ml) on WRL-68 cells.

## 3.6.2 Chemical analysis of the five extracts of Radix Paeoniae Alba

The five extracts of P. Alba were prepared by using the procedure mentioned in Section 3.5.3. The dry extracts were obtained and their percentage yield is given in Table 3.7.

Table 3.7 The % yield of the five extracts of P. Alba

| Extracts | PR0 | PR25 | PR50 | PR75 | PR100 |
|---|---|---|---|---|---|
| **% Yield *** | 7.30±4.97 | 15.35±5.50 | 12.92±3.46 | 7.14±2.92 | 3.86±3.19 |

*** mean ± relative standard derivation**

For qualitative and quantitative analysis of the five extracts, the HPLC-DAD was utilized. The chromatography conditions used (Table 3.4) to determine saponins components in P. Alba extracts were based on reference [43]. Well-separation, good peak shape and high reproducibly were obtained. Here these chromatographic conditions were employed throughout this study.

For qualitative analysis of the five extracts, the five standards, C1, AF, PF, BA and PN are commercially available and therefore they were used for quality control of P. Alba. In getting chromatograms of these standards, 200ppm of each standard was firstly injected into HPLC-DAD according to the chromatographic conditions (Table 3.4). Figure 3.9 and 3.10 give the chromatograms of the five standards mixture at 230nm and their UV-spectra respectively. The retention times of these standards obtained from the chromatograms profile and their UV spectra were used for qualitative analysis of the PR extracts.

Figure 3.9 Chromatogram of the 200ppm standards mixture at 230nm

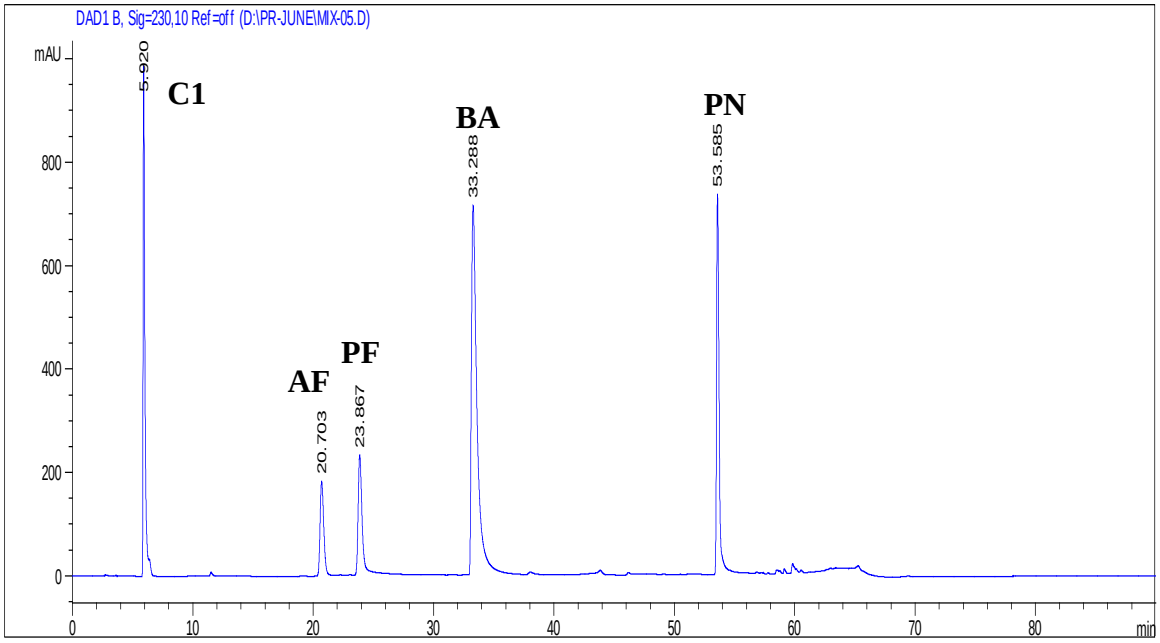
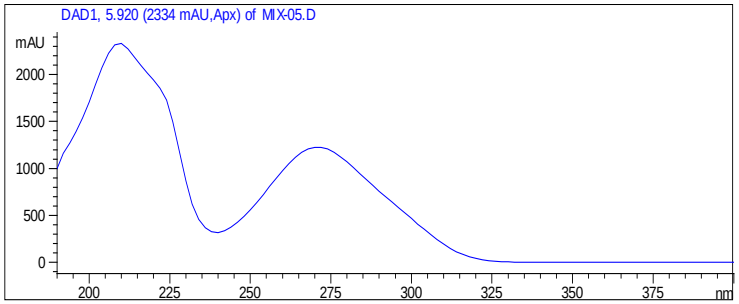
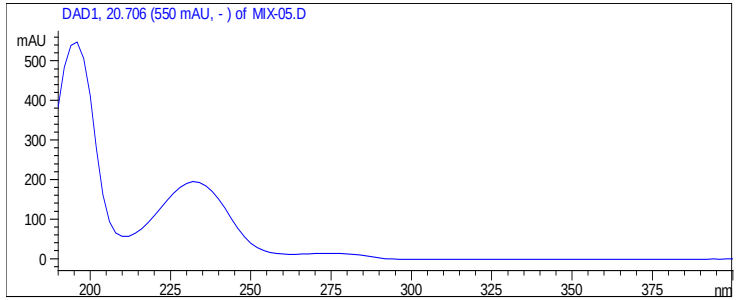
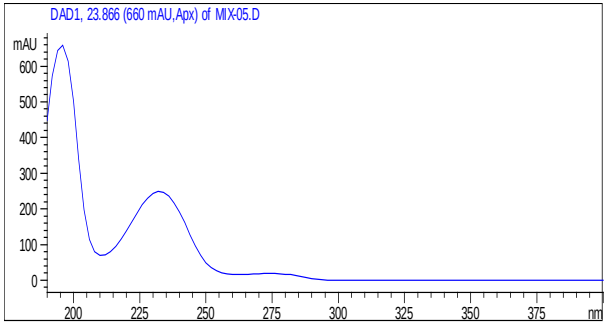
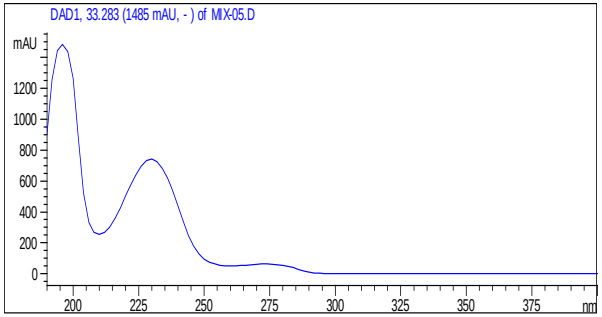
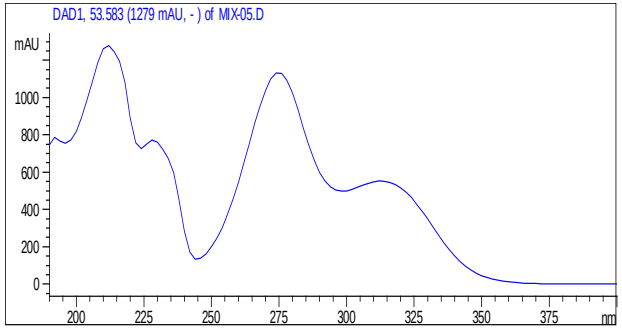


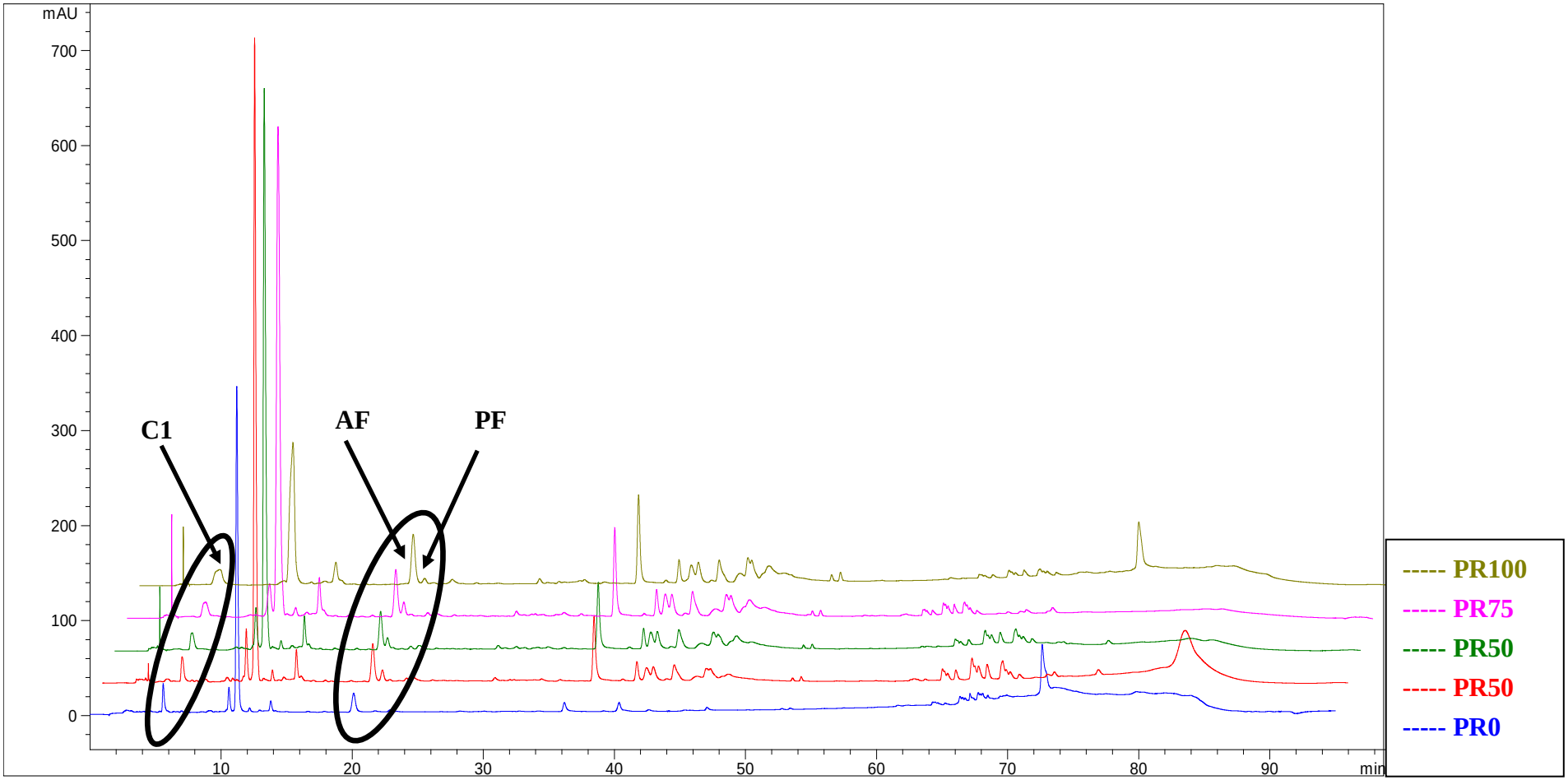
Figure 3.10 UV spectra and retention times of the five standards

| Name of Components | C1 | Albiflorin (AF) |
|---|---|---|
| Retention time / min | 5.920 | 20.103 |
| UV spectra |  |  |

| Paeoniflorin (PF) | Benzoic acid (BA) | Paeonol (PN) |
|---|---|---|
| 23.861 | 33.286 | 53.586 |
|  |  |  |

The chemical compositions in the five extracts of P. Alba were examined by HPLC-DAD using the same chromatographic conditions as given in Table 3.4. 5mg/ml of each extract was injected. Figure 3.11 shows the overlay chromatographic fingerprints of all the five extracts at 230nm. Individual chromatographic fingerprints were provided in the Appendix 3.1. It can be seen from the Figure 3.11 that the chromatographic patterns of the five extracts are different in the number of peaks and the peak areas of the components. Obviously, the number and the peak areas of UV-active components observed in the chromatogram of PR0 are less than those of the other four extracts. With the help of the retention properties and UV spectra of the five standards, three peaks in all these chromatogram fingerprints were identified. They are C1, AF and PF. Of course, their concentrations vary in these five extracts.

Figure 3.11 The overlay chromatographic fingerprints of the five extracts of P. Alba in 5mg/ml at 230nm



Following qualitative analysis, the amounts of C1, AF and PF in these five extracts of P. Alba were determined from the calibration plots of these standards at 230nm. Concentrations of these standards in the linear range were injected into HPLC-DAD and their calibration plots were thus obtained (see Appendix 3.2). Their regression equations of these standard curves and correlation coefficients ($r^2$) are listed in Table 3.8. In addition, the concentrations of these three components identified in the five PR extracted were determined by using the calibration curves and the results are listed in Table 3.9.

Table 3.8 Linear range, regression equations and correlation coefficients of the standards C1, AF and PF

| **Compound** | **Linear range / ppm** | **Regression equations*** | **Correlation coefficients ($r^2$)** |
|---|---|---|---|
| C1 | 1-100 | y=49.557x – 22.910 | 0.9998 |
| AF | 5-300 | y=30.415x – 1.017 | 0.9997 |
| PF | 1-100 | y= 24.974 – 42.552 | 0.9990 |

***y : Peak area; x: actual concentration (ppm)**

Table 3.9 The concentrations of GA, AF and PF found in the five extracts of P. Alba

| | **The concentration of identified components / ppm** | | |
|---|---|---|---|
| **P. Alba extract** | **C1** | **Albiflorin (AF)** | **Paeoniflorin (PF)** |
| **PR0** | 6.56±0.11 | 11.90±1.00 | 3.23±0.14 |
| **PR25** | 8.05±0.11 | 22.39±0.20 | 3.56±0.13 |
| **PR50** | 7.56±0.08 | 23.05±0.36 | 3.82±0.27 |
| **PR75** | 9.57±0.07 | 30.54±0.15 | 6.31±0.25 |
| **PR100** | 14.12±0.23 | 34.68±0.50 | 6.85±0.05 |

- Results shown are the average values from three independent experiments and are presented in

the form of the means ± standard deviation (S.D.)

It is interesting to note that the concentrations of C1, AF and PF in the five extracts are different. The amounts of these three saponins components vary with the extraction solvent especially the polarity of the solvent used. Their chromatographic peaks increase from PR0 to PR100. It is probably due to the fact that the polarities of these components are very close to that of solvent A and therefore they were extracted in greater amount compared to the other one. This leads to these components having the highest amount in PR100. Compared with PR0 and PR100, the concentrations of C1, AF and PF in PR100 are higher by 2-3 times than those in PR0. It may be due to the significant polarity change of the extraction solvents used and therefore the amounts of these three components change to a large extent in PR0 and PR100. However, in PR25 and PR50, the change in their concentrations is less. It is because the change in polarity of the extraction solvents used in these three extracts do not vary so great relatively and so their amounts of differ less significantly.

Other than these three identified components, unknown components were also found in the chromatographic fingerprint of the five extracts of P. Alba. However, no correct matching was found with the standards available, identification of these unknown components are a challenging job. Two observable peaks, namely peak 1 and peak 2 with retention times at about 11.5 and 36 minutes respectively, were observed in the chromatograms (Appendix 3.1). The saponins components with similarities in their spectra can be used to estimate the relative amounts of other unidentified saponins components in the extracts [60]. The UV spectra of peak 1 (Figure 3.12) is similar to that of AF while the UV spectra of peak 2

(Figure 3.12) is similar to that of C1. So AF and C1 were used here as external standards for component 1 and 2 corresponding to peak 1 and peak 2 respectively for quantitative purpose. Then the amounts of the corresponding component 1 and 2 in the five PR extracts were estimated by using the calibration plots of AF and C1 respectively and the results are given in Table 3.10.

Figure 3.12 UV spectra of peak 1 and peak 2 present in the five extracts of P. Alba

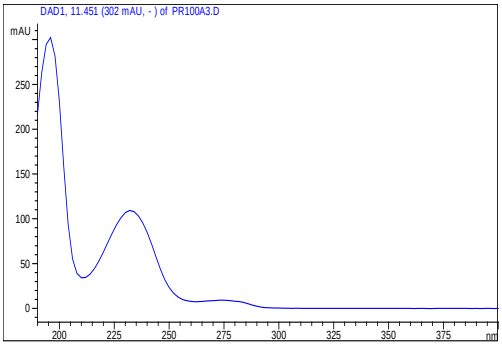
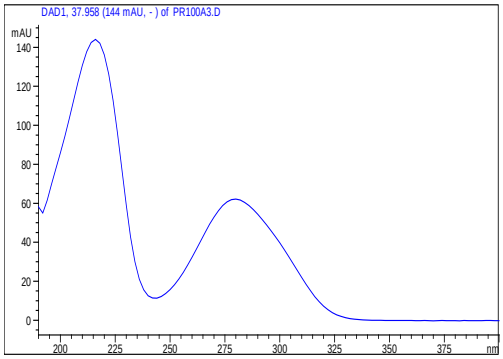
| **Name of peak** | **Peak 1** | **Peak 2** |
|---|---|---|
| **Retention time / mins** | **~11.5** | **~36** |
| **UV spectra** |   |   |

Table 3.10 The concentrations of component 1 and 2

| | **Concentration / ppm** | |
|---|---|---|
| **P. Alba extract** | **Component 1** | **Component 2** |
| **PR0** | 120.85±5.04 | 3.67±0.84 |
| **PR25** | 238.42±0.51 | 22.36±2.15 |
| **PR50** | 240.28±0.65 | 21.90±1.24 |
| **PR75** | 297.97±0.71 | 27.47±0.84 |
| **PR100** | 123.64±1.49 | 27.79±0.50 |

The concentrations of component 1 and 2 vary in different P. Alba extracts. The amount of component 1 increased from PR 0 to PR75 but deceased dramatically in PR100 while the concentration of component 2 increases significantly from PR0 to PR25 but increased to a lesser extent in PR25, PR50, PR75 and PR100. The use of external standards for quantitative analysis is not accurate because the absorptivities of the components are totally different. Therefore the external standards of AF and C1 employed in determination the amounts of components 1 and 2 in this study was just used for comparison but can not reflected their actual amounts of these components in the extracts of P. Alba.

Besides, many components eluted in the retention time at 40-50 minutes and 65-70 minutes were observed in the chromatographic fingerprints of the five extracts of P. Alba. These components vary among these extracts. The peaks of the components in these time ranges cannot be used for qualitative and quantitative determination because they overlap seriously with one other.

### 3.6.3 Relationship between the results of cell proliferation on human hepatoma cancer cell lines and chemical study

One of the main tasks in this study is to find out the relationship between the chromatographic fingerprint of a herb and its efficacy. From the chromatographic fingerprints of the five extracts as obtained by HPLC-DAD, three identified components, C1, AF and PF were found. Here we try to use these components to explain the MTS results first.

Compared with the MTS results of PR0 and PR100, the IC50 value of PR100 is

smaller by ten times than that of PR0. But the concentrations of individual components C1, AF and PF in PR100 are not higher than ten times those in PR0. As TCMs are complex mixtures containing many chemically constituents, these multiple constituents work together synergically to give the ultimate therapeutic effect of each TCM. So it is difficult to use the concentration of a single component to account for the biological activities of the extracts. Therefore, we tried to use variations of the contents of these components in the extracts of P. Alba to account for their MTS results.

A trend with decreasing IC50 value can be found in the MTS data from PR0 to PR100. The increase in the amounts of these three identified components from PR0 to PR100 may lead to the decrease in IC50 value of PR0 to PR100. Besides, the change in the MTS value is probably due to the increase in amount of unidentified component 2. Therefore the C1, AF PF and component 2 may be mainly responsible for the antiproliferation activity of the extracts of P. Alba with the information available. However, the amount of component 1 in PR0 and PR100 are very similar to, about 121 and 124 ppm respectively. So this component seems to have no relation with the difference in IC50 values between PR0 and PR100 extracts. Therefore, component 1 was not considered as responsible for anti-cancer activity on HepG2 cells in this work.

In fact, the whole chromatographic fingerprint of P. Alba extracts should be used to explain the biological activities of HepG2 cells. The peak areas of components within the retention time range of 36-42 minutes vary in the five extracts and they are probably contributed to the cell proliferation activities on HepG2 cells to certain extent. However, many constituents were eluted in the extracts but their

peaks were overlapped seriously so that the corresponding components of these overlapping peaks cannot be quantified easily. Hence, it is difficult to use these components to account for the MTS results on HepG2 cells.

HPLC-DAD was employed to determine the chemical compositions in the five extracts of P. Alba. The chemical composition detected by HPLC-DAD must be UV-active. Thus UV inactive chemical components were not detected. However, these UV-inactive components may contribute on the growth proliferation activities on HepG2 cells to certain extent. This is another problem in finding the relationship between the efficacy and the chemical compositions of the extracts of P. Alba. Liquid chromatography – mass spectrometry (LC-MS) is needed in further study.

## 3.7 Conclusion

Chinese herbs have been used to treat diseases including cancer. Several studies have demonstrated that extracts from several herbal medicines had anticancer potential [61].The P. Alba extract had been found to induce apoptosis in HepG2 and Hep3B hepatoma cells [8, 62]. As chemical components in the herbs extracts are largely determined by the extraction solvent. Pharmacological activities of the herbal extracts are also affected by this factor. In this study, the cell growth proliferation activities of the five extracts of P. Alba were determined from different ratios of two solvent A and B with different polarity. Their activities on HepG2 and Hep3B cells were examined by MTS bioassay and the MTS results indicated that these five extracts had growth inhibitory effect on these two cell lines depending on time and dosage. The growth inhibitory effect of PR0 on HepG2 and Hep3B cells were significant and obvious at three day and changed little after three day. In addition, all the five extracts of P. Alba (in the concentration range of 0.2-5mg/ml) have different growth inhibitory effects on HepG2 and Hep3B cells and the IC50 values for HepG2 cells by MTS bioassay show a decreasing trend from PR0 to PR100. This trend indicates that using more polar solvent A for extraction gives the extract with stronger effect. As a result, PR100 (with the IC50 value about 0.33mg/ml) was found to be the most potent extract among all against HepG2 cells.

The five extracts of P. Alba in this study gave no significant anti-proliferation effects on Hep3B cells. The most potent PR100 in 1mg/ml just inhibited about 28% Hep3B cells only. Besides, the growth inhibitory activity of PR100 on WRL-68 human normal ovary cells with normal liver cells characterization was

also investigated. Only 20% of WRL_68 cells were inhibited by about 0.3mg/ml of PR100. Therefore, it is probably safe to use PR100 at this dosage to cure patients with liver cancer. Of course, more works are needed to be done on the animal test before conclusion can be made.

Qualitative and quantitative analyses of the five extracts of P. Alba were carried by HPLC-DAD and their chromatographic fingerprints were obtained. From these fingerprints, three saponins C1, albiflorin (AF) and paeoniflorin (PF) were identified and their concentrations in the five extracts were determined by using the calibration curves of the standards available. We found that the concentrations of C1, AF and PF increase from PR0 to PR100. This is probably due to the polarity of these three components were similar as that of solvent A. Therefore they were extracted out in higher quantity by solvent mixture containing more solvent A. Besides, two obvious and unidentified chromatographic peak 1 and peak 2 were also observed and the concentrations of their corresponding components 1 and 2 were estimated by using the external standards of AF and C1 respectively. Their concentrations vary in the five extracts.

Combined with the results of MTS bioassay and chromatographic fingerprints of the five extracts of P. Alba, we attempted to find the relationship between chemical composition and the pharmacological activity of the extracts. The trend with decreasing in IC50 values from PR0 to PR100 on HepG2 cells was related to increasing amount of C1, AF and PF. Besides, the increasing amount of component 2 from PR0 to PR100 may also be used to account partly for the observation. However, the relationship is still not established with high

confidence because some components with their chromatographic peaks with serious overlapping cannot be included for comparison.

Finally, the anticancer activities of the extracts of P. Alba on HepG2 cells seem to be related to the components of C1, AF, PF and component 2 in our study. But further investigation is required to examine whether these components have growth inhibitory effects on HepG2 cells or not. Besides, further examination on antiproliferation activities of other components present as observed in the chromatographic fingerprint are also needed to see whether they also contribute to the anticancer activity. Part of the investigation was carried out and reported in Chapter 4.

# Chapter 4

# Finding the bioactive component(s) and fraction(s) from the extract of Radix Paeoniae Alba effective for treating liver cancer and the chemosensitivity effects of the two western anti-cancer drugs to Radix Paeoniae Alba fraction

## 4.1 Introduction

Followed by investigation of the pharmacological effect of the traditional Chinese herbal medicine (TCHM), finding out the bioactive components that are responsible for the therapeutic effect is the next step. Unlike the single chemical entity of Western drugs that forms the basis of modern pharmacology and drug development, the paradigm of TCHM is typically viewed as a whole of the multi-compound, multi-ingredient system representing its activity. Selection of individual components for determining either efficacy or quality of the TCHM in conventional QC approach is contradict to the basic TCM working principles [63]. In addition, the TCM is complex mixture and usually contains hundreds of chemically different constituents that work synergistically to produce its specific efficacy activity. This leads to that the efficacy activity of a TCM should not always be attributed to a single constituents but a group of constituents [2].

In the previous Chapter 3, we reported that the extracts of P. Alba exhibited growth inhibitory activity on human hepatoma cancer cells HepG2. However, each extract of P. Alba is a complex mixture and most of the active components are not known to tell whether the inhibitory effect comes from a single component or more. In the literature, three components of P. Alba were identified and they were probably responsible for the antiproliferation activity on HepG2 cells. In this study, the activity of these three individual identified components on HepG2 cells was further investigated. As mentioned before that the biological activities of TCM cannot be accounted for a single active component. Hence, PR100, which gives the most potent extract against HepG2 cells, was fractionated in order to search for which fraction(s) with different groups of

compounds is good for this anti-proliferation effect.

Recently, the popularity of TCM being used clinically as complementary medicine or alternative medicine is increasing. On top of this increase in popularity of TCM in this kind of clinical applications, the co-administration of TCM and drugs (manufactured chemicals) has also increased remarkably. Especially in cancer chemotherapy, it is true that the incidence of co-administration of TCM and anticancer drugs is high. Interaction between herbs and drugs may increase or decrease the pharmacology or toxicological effects of either component. Yet, synergistic therapeutic effects may complicate the dosing of long-term medications. For example, herbs that are traditionally used to decrease glucose concentrations in diabetes could theoretically precipitate hypoglycemia if taken in combination with conventional drugs. In this study, the effective fraction of PR100 found above was combined with two western drugs, paclitaxel (PAC) and doxorubicin (DOX), that have active anti-proliferation on HepG2 cells [64], so as to investigate their effects on the sensitivity of the effective fraction to HepG2 cells. The aim of this work is to provide new knowledge to both patient and doctors to give warning and valuable information respectively from our results.

## 4.2 Methods of Investigation

### 4.2.1 Preparative high-performance liquid chromatography

Preparative high-performance liquid chromatography (PHPLC) was utilized in

this investigation to fractionate the extract of P. Alba mentioned in Chapter 3. The applications of PHPLC and analytical liquid chromatography (ALC) are different. The main task of ALC is to separate small amounts of a mixture of compounds into individual components for qualitative and quantitative analyses; while PHPLC is employed to isolate the specific pure substances in larger quantities for other studies.

The feasibility of PHPLC process is, of course, determined by trying to see how good ALC in separating the mixture. The main reason is that ALC is faster and uses less solvent and shorter time than that of PHPLC with a large size of preparative column. However, in practice, it is impossible to reproduce the exact ALC results on the larger scale PHPLC system. Nevertheless, the analytical results obtained can be of great help in optimizing PHPLC separation. In order to have a successful scale-up, it is desirable to maintain kinetic (particle size, pore size, temperature, mobile phase) and dynamic (bed height, flow velocity, packing density) equivalence between the chromatography columns used in both ALC and PHPLC [65].

The common approach is to hold the plate count constant upon scale-up and increase the feed volume and column volume proportionately. This approach was originally based on the assumption of linear adsorption [66]. In this case, the length and efficiency (i.e. plate numbers or heights equivalent to a theoretical plate respectively) are the same for different columns, then the elution profiles obtained should be identical. Equation 4.1 and 4.2 are the scale-up formulas of the volumetric flow rate and the amount injected [66, 67].

$$\frac{V_P}{V_A} = \frac{A_P^2}{A_A^2} = \frac{d_P^2}{d_A^2} \qquad \text{[Eq.4.1]}$$

$V_P$ and $V_A$ are the volumetric flow rates, $A_P$ and $A_A$ are the cross section areas, and $d_P$ and $d_A$ are the diameters of the PHPLC and ALC columns respectively.

$$\frac{X_P}{X_A} = \frac{V_P}{V_A} = \frac{d_P^2 L_P}{d_A^2 L_A} \qquad \text{[Eq.4.2]}$$

$X_P$ and $X_A$ are the injection amounts and $L_P$ and $L_A$ are the lengths of the PHPLC and ALC columns, respectively. We did utilize the scale-up PHPLC technique successfully in this way to isolate and fractionate of the TCM extract concerned in our work.

### 4.2.2 MTS bioassay

MTS bioassay was employed to study the antiproliferation activity of individual identified components and the fractions of PR100. And the sensitivities of the two drugs to the fraction of P. Alba on HepG2 cells were also investigated by MTS bioassay. Cells with a constant concentration of drug were served as a control of 100% survival in the study of chemosensitivity. Details of MTS bioassay was mentioned in Section 2.3.1.2 and Section 3.5.6 already.

## 4.3 Experimental

### 4.3.1 Herbal sample

Radix Paeoniae Alba, the dried roots of *Paeonia lactiflora* Pallas, was purchased from famous Tung Fong Hung Medicine Company (Hong Kong, China).

### 4.3.2 Sample extraction and standards preparation

PR100 was prepared by the sample of pulverized and dried roots of P. Alba (1:10 w/v) that was prepared by refluxing with different ratio of 100% solvent A. PR100 filtrate was collected by filtration by using 0.45μm pre-cut membrane and the solvent was removed to the half by rotatory evaporator. Then the filtrate was injected to PHPLC for fractionation.

The solvent of each fraction of P. Alba was removed by rotatory evaporator and then lyophilized by freeze dryer to yield the dry powder. The dry powder was kept in 4°C and dark place when not in use. The concentration of each fraction used in subsequent studies was based on the dry weight of the extract (mg/ml).

The stock solutions of C1, albiflorin (AF) and paeoniforin (PF) in 1000ppm were prepared and utilized to produce a serial concentration from 10ppm to 100ppm for investigating their proliferation activity on HepG2 cells.

### 4.3.3 Chromatographic conditions of PHPLC and ALC

The model and chromatographic conditions of the ALC used were mentioned in Section 3.5.4 while the mobile phase of ALC was given in Table 3.4.

In PHPLC, the extracts of P. Alba were analyzed by using a Hypersil ODS $C_{18}$ preparative column (5 μm, 21.2 x 250 mm) with HP 1100 preparative series HPLC Pump, preparative autosampler and diode-array detector. The flow rate was 21.6ml/min and the injection volume was 400μl that were calculated by Equation 3.1 and 3.2 respectively. The running time was 50 minutes and the detection wavelength was set at 230 nm. The solvent saver, Solvent Recycler Junior (Alltech, USA), was connected with the preparative system. The mobile phase of PHPLC is listed in Table 4.1 [7] and the fractions of P. Alba were collected with a Foxy® Jr. fraction collector (ISCO, Inc., USA).

Table 4.1 The elution gradient of mobile phase in PHPLC

| Time / min | ACN / % | Water / % |
|---|---|---|
| 0.01 | 3 | 97 |
| 2.00 | 3 | 97 |
| 12.00 | 18 | 82 |
| 16.00 | 18 | 82 |
| 20.00 | 22 | 78 |
| 25.00 | 32 | 68 |
| 35.00 | 72 | 28 |
| 50.00 | 72 | 28 |

### 4.3.4 Cell culture on HepG2 and WLR-68 cells

HepG2 and WRL-68 were maintained and propagated in 90% RPMI-1640

medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in the 75cm$^2$ tissue culture flask at 37°C in 5% $CO_2$ humidified incubator. The cells were grown to 95% confluency and then were rinsed with phosphate-buffered saline (PBS) for three times. The cultured cells were then detached by trypsinization and pelleted through centrifugation at 2500 rpm for 15min.They were resuspended in corresponding medium to obtain a cell density of 1 x $10^4$ cells/ 0.1ml for seeding in a 96-well microplates.

### 4.3.5 Cell proliferation activity by MTS bioassay

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(3-sulfophenyl)-2 H-tetrazolium, inner salt] MTS growth inhibition assay was performed. The cells were seeded in a 96-well flat-bottomed plate on day one. On day two, different concentrations of standards or fractions were added and cells were incubated for a further three day at 37°C and 5% carbon dioxide. Afterwards, cells were washed with PBS buffer solution before 20 μl of MTS solution (MTS dissolved in PBS solution in 1:9 v/v) was added and the plate was allowed to incubate for further 2 hour at 37°C in incubator. Optical density (OD) of cell treated with medium and OD of cells treated with samples were recorded at 490 nm using microplate reader (Model550, Bio-Rad). Cells treated with drug-free were served as a control of 100% survival.

In studying the chemosensitivity of PAC and DOX drugs to the fraction of P. Alba on HepG2 cells, the cells were treated by an assigned concentration of the drug and a serial concentration of the fraction. The cells treated with fixed concentration of drug only were served as control. Three independent

experiments performed in triplicate and averaged values are reported in form of the means ± standard deviation (S.D.).

## 4.4 Results and Discussion

### 4.4.1 Qualitative analysis of P. Alba fractions by PHPLC and ALC

There is limitation in using the mobile phase for PHPLC fractionation. The mobile phase (see Table 3.4) for analysis of the five extracts of P. Alba as mentioned in Chapter 3 cannot be chosen as the mobile phase in PHPLC because the salts of potassium dihydrogen phosphate employed in ALC could be precipitated out after removal of solvents by rotatory evaporator. The salts could affect the dry mass of the fraction obtained and hence affect the change in the masses of fractions weighed to cells for studying their antiproliferation activity. Therefore, another mobile phase for PHPLC was chosen. It was just composed of acetonitrile and water without salt involved [7]. The mobile phase for PHPLC is given in Table 4.1.

The analytical chromatographic fingerprint of PR100 with the solvent system given in Table 4.1 is depicted in Figure 4.1. PR100 was fractionated into six fractions (named as F1, F2, F3, F4, F5 and F6) according to the chromatographic profile shown in Figure 4.1 by PHPLC. As can be seen in Figure 4.1, the peak separation, resolution and the number of peaks are reduced compared with ALC fingerprint of the same extract (Appendix 3.1) acquired from the mobile phase as listed in Table 3.4. Therefore the same mobile phase as given in Table 3.4 was

employed in order to re-examine the chemical components of the fractions obtained. Please be noted that the ranges of the retention times of these six fractions are not provided here for confidential purpose. The percentage yields of the six fractions acquired from PHPLC is summarized in Table 4.2.

Figure 4.1 Analytical chromatographic fingerprint of PR100 obtained by ALC using the mobile phase given in Table 4.1

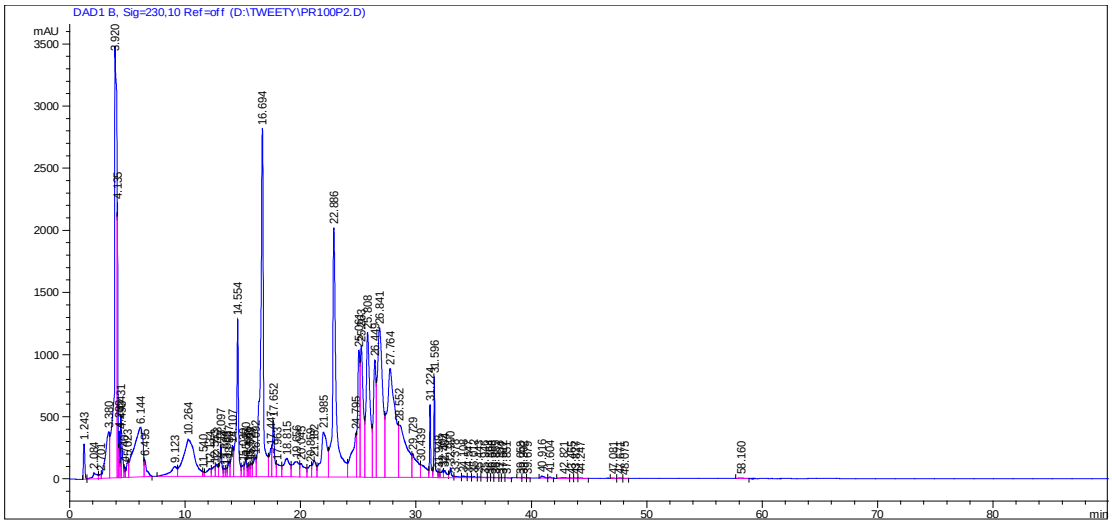



Table 4.2 Percentage yield of the six fractions of PR100

| | **PR fractions** | | | | | |
|---|---|---|---|---|---|---|
| | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** |
| % yield* | 1.545 | 0.057 | 0.019 | 0.077 | 0.150 | 0.132 |

** mg of fractions / g of raw herb*

## 4.4.2 Cell proliferation activity of identified components

Three components, C1, albiflorin (AF) and paeoniflorin (PF) were identified in the five extracts of P. Alba (see Chapter 3). Some or all of them should have antiproliferation activity on HepG2 cells. Now, the antiproliferation effects of

these components on HepG2 cells (in the concentration range from 10-100ug/ml) were investigated separately under three day treatment. Their activities are given in Figure 4.2 and Figure 4.3.

It can be seen from Figure 4.2 and Figure 4.3 that these three components exhibited different growth inhibitory effects on HepG2 cells. AF and PF in the concentration range of 12.5 to 100ug/ml in that covered the concentration of AF and PF in the extracts of P. Alba does not give the effect. Even the mixture of AF and PF (in 1:1 ratio) in the same concentration range did not suppress any HepG2 cells (see Appendix 4.2). On the other hand, the activity of C1 alone is more significant on HepG2 cells. The activity increases from 10 to 20ug/ml but no longer increases beyond 20ug/ml. The IC50 value of C1 was found to be 16.7±0.6ug/ml from the curve in Figure 4.3.

C1 has been reported to have anti-cancer growth effects in lung cancer cells, stomach cancer cells KATO III and colon adenocarcinoma COLO 205 cells. It was also reported that C1 can carry out the induction of apoptosis in several lung cancer cells involved caspase activation. Probably, C1 was recognized as naturally occurring anti-oxidant property but also act as prooxidants catalyzing DNA degradation in the presence of transition metal ions such as copper. DNA breakage is one of the pathways to induce the cell death so that C1 is considered as a potential active component against cancer. Therefore the antiproliferation activity of P. Alba extracts on HepG2 cells was, most likely attributable to the presence of C1. The anticancer pathway of C1 through DNA degradation in P. Alba extract is required and confirmed in further investigation.

The growth inhibitory activity of PR100 at 5mg/ml on HepG2 cells was found to be 0.7 which had not been shown in Figure 3.6 in Chapter 3. The concentration of C1 present in 5mg/ml of PR100 is about 14 ug/ml that is close to the IC50 of C1. Therefore it was possibly one of the chemical components that provide the anti-cancer activity on HepG2 cells at 5mg/ml of PR100. Interestingly, the IC50 value of PR100 on HepG2 cells found earlier (Chapter 3) was 0.33mg/ml which just about 1ug/ml of the value of C1 present. According to Figure 4.3, less than 20% of HepG2 cells were inhibited by concentration of C1 below 10ug/ml. Therefore this tells us that not only C1 but also other components play role in the activity on the HepG2 cells.

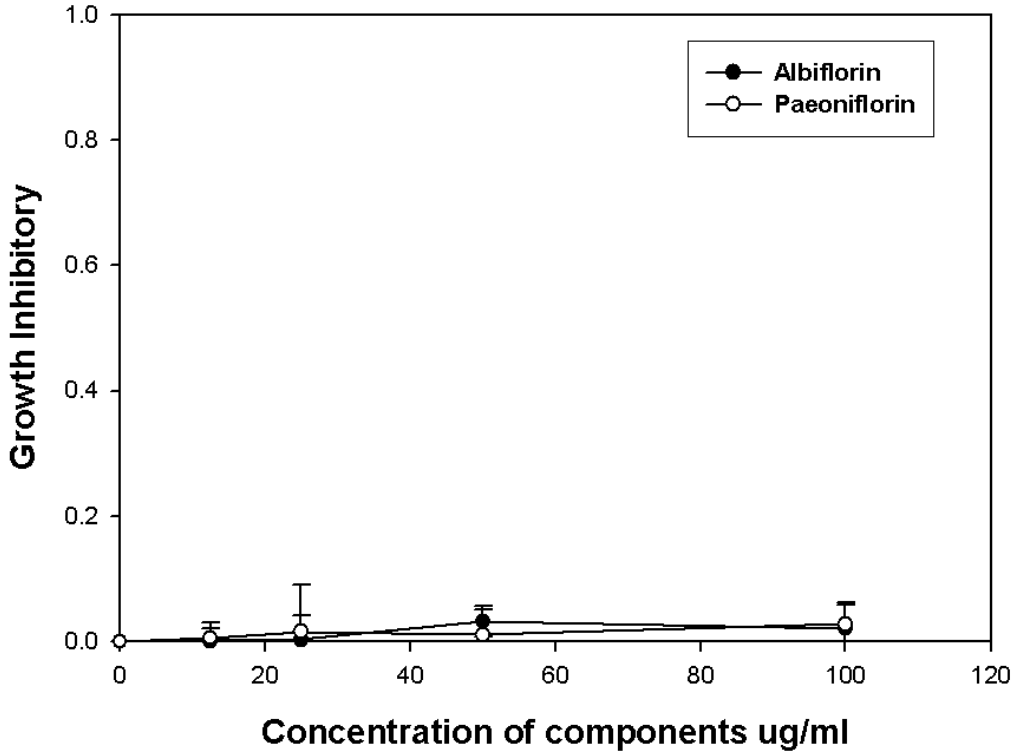



Figure 4.2 The antiproliferation effects of AF and PF (in concentration range of 12.5-100ug/ml) on HepG2 cells

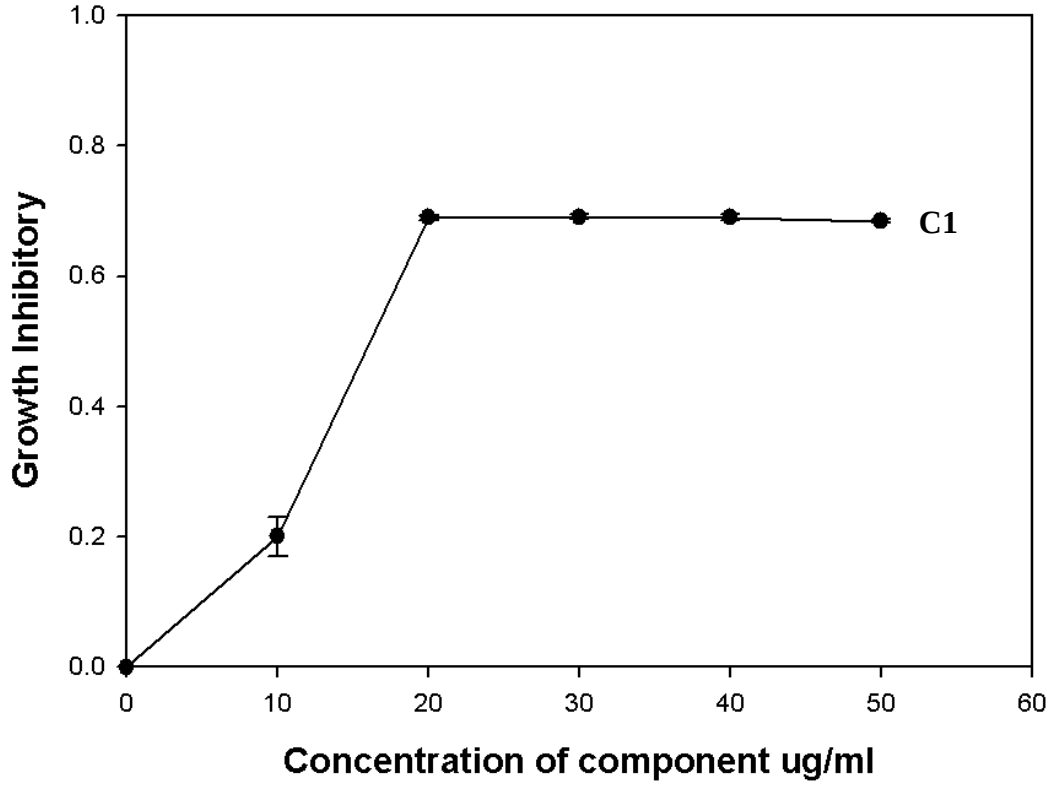



Figure 4.3 The antiproliferation effects of C1 (in concentration range of 10-50ug/ml) on HepG2 cells

### 4.4.3 Cell proliferation of Radix Paeoniae Alba fractions

Although the component C1 in P. Alba was found to have activity on HepG2 cells, yet the antiproliferation activity of P. Alba extracts cannot be attributed to just this single component but also to others in the fraction. PR100 was fractionated by PHPLC and six fractions (Table 4.2) were obtained in this study. These six fractions (F1, F2, F3, F4, F5 and F6) in the concentration range 0.2 – 1.0mg/ml were added to HepG2 and WRL-68 cells and their anti-cancer growth effects on these cells were assessed. The results are depicted in Figure 4.4 and 4.5 respectively.

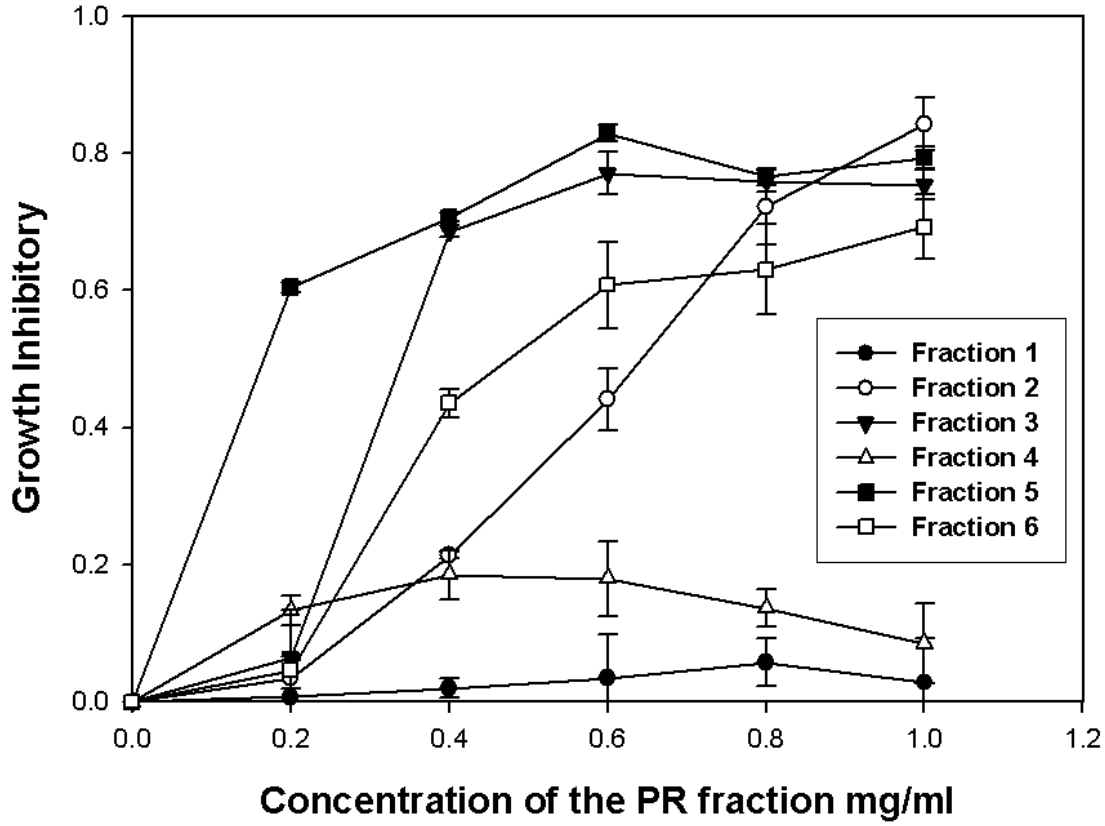



Figure 4.4 The antiproliferation effects of the six fractions (in concentration range of 0.2-1.0 ug/ml) on HepG2 cells

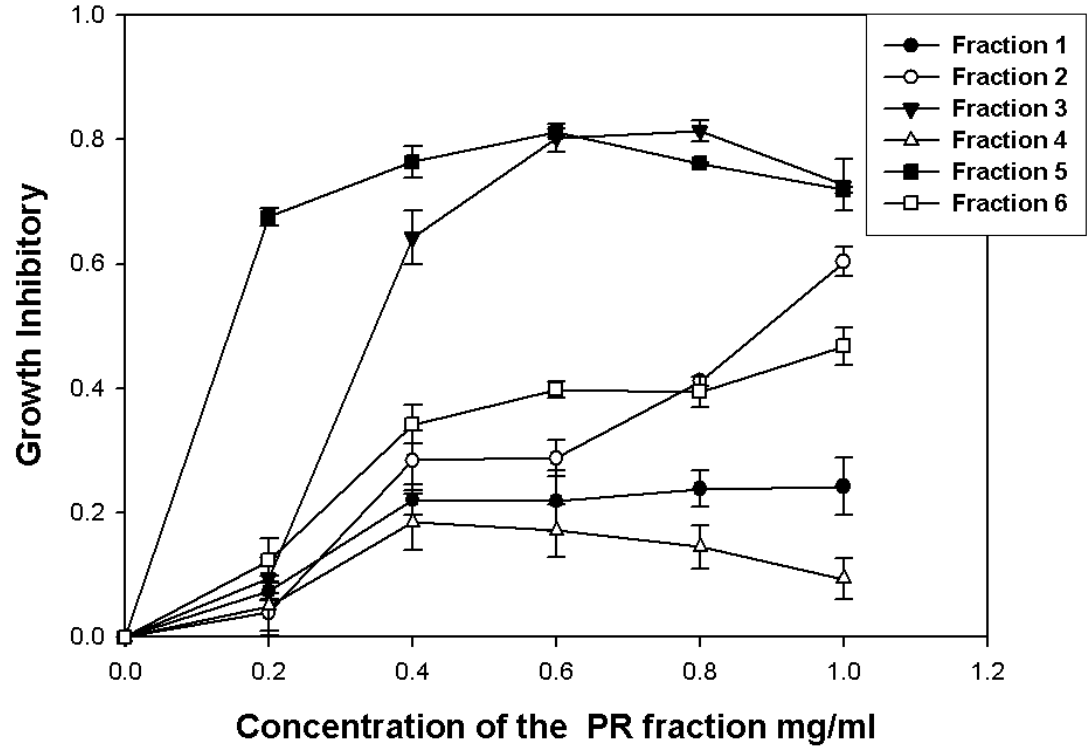



Figure 4.5 The antiproliferation effects of the six fractions (in concentration range of 0.2-1.0 ug/ml) on WRL-68 cells

Figure 4.4 shows that the six fractions (F1-F6) had different growth inhibitory effects on HepG2 cells. The IC50 values of these fractions are summarized in Table 4.3. F1 exhibits no significant activities on HepG2 cells even at the 1.0mg/ml. F4 performs better than F1 but still has just 20% of cells inhibited. So its effect on HepG2 cells is not obvious. The other fractions F2, F3, F5 and F6 exhibited obvious growth inhibitory effects in dose-dependent manner. To F3, F5 and F6, these effects increase significantly from 0.2 mg/ml to 0.6mg/ml, but change very little beyond 0.8mg/ml. The inhibitory activity of F2 increases dramatically from 0.4 to 0.8mg/ml and then increases less beyond 0.8mg/ml. Among the six fractions, the growth inhibitory effect of F5 is the most prominent one with the lowest IC50 value.

IC50 value of F3 is comparable to that of PR100 extract (about 0.33mg/ml, see Table 3.5) so that the F3 is probably responsible for anti-cancer growth effect of PR. Interestingly, the IC50 value of F5 was lower by about five times than those of PR100. This indicates that the inhibitory activity of F5 is stronger than that of PR100 itself. As P. Alba extract is composed of a mixture with more chemical components than those of F5, interaction between components present may be more significant. Of course, the interaction may affect the biological response of the whole mixture to be greater than, equal to, or less than the theoretical response value depending on whether the interactions are synergistic, additive, or, the other way round [68]. Since the components in other fractions that may have antagonistic interaction on the components of F5 were removed, the inhibitory effect of F5 is enhanced. As a result, F5 also contributes antiproliferation activity of PR100.

On the other hand, the results on growth inhibitory effects of the six fractions on WRL-68 cells were displayed in Figure 4.5 and their IC50 values are summarized in Table 4.3. The results are similar to those in HepG2 cells. Both F1 and F4 displayed no significant anticancer effects. Maximal of 20% of WRL-68 cells were inhibited by these two fractions in 0.2-1.0mg/ml. In addition, the antiproliferation activities of F2 and F6 exist but are still low while those of F3 and F5 exhibited significant effect on WRL-68 cells. More than 60% of the cells were inhibited by these two fractions at the concentration level above 0.4mg/ml.

Combined with the antiproliferation results of the six fractions on HepG2 and WRL-68 cells, it can be seen that the F2 and F3 exhibited effects on these two cell lines at comparable concentration. Although F2 and F3 can inhibit human liver cancer cells, they also have antiproliferation activity on human liver normal cells. This implies that both F2 and F3 may not be suitable for alternative medicine for treating liver cancer in daily life. On the other hand, although IC50 of F5 and F6 on HepG2 cells are two times smaller than that of WRL-68 cells, still one has no high confidence to say that they are safe to be used at the dosage at IC50 on HepG2 cells. Of course, further investigations such as animal tests are necessary for confirmation.

In this work, we found that the six fractions and PR100 itself gave significant difference on HepG2 and WRL-68 cells. PR100 alone did not have marked effect on WRL-68 cells. However, after PR100 was fractionated into six fractions, three of these fractions work well on WRL-68 cells. The synergistic enhancement not only acts in the anti-cancer effect on HepG2 cells, meanwhile it also gives rise to the effect on WRL-68 cells and hence more normal cells were inhibited. As can

be seen in Figure 4.4 and 4.5, the shapes of antiproliferation curve of F3 and F5 on HepG2 and WRL-68 are similar and so not only HepG2 cells but also WRL-68 cells were inhibited at the same time.

Table 4.3 *IC50 values (mg/ml) of six fractions based on HepG2 and WRL-68 cells

| | **PR fraction** | | | | | |
|---|---|---|---|---|---|---|
| | **F1** | **F2** | **F3** | **F4** | **F5** | **F6** |
| **IC50 on HepG2** | > 1 | 0.64±0.03 | 0.35±0.01 | > 1 | 0.06±0.01** | 0.53±0.07 |
| **IC50 on WRL-68** | > 1 | 0.90±0.05 | 0.36±0.01 | > 1 | 0.15±0.01** | > 1 |

* Results given are the average values of three experiments performed and were presented in form of the means ± standard deviation (S.D.)

** IC50 of Fraction 5 found from the Figure 4.4 and 4.5 were inaccurate so the IC50 value of this fraction on HepG2 and WRL-68 were determined from the curves in Appendix 4.1 with the scale of the concentration in a narrower range

Now we come to a question on that the IC50 of six fractions (Table 4.3) on HepG2 and WRL-68 cells could directly reflect their real anticancer activity of PR100 for comparison. As the relative concentrations of components within the fractions at IC50 are probably higher than their actual amounts present in PR100, the inhibitory effect of some containing the active components in F5 may be expected to be higher than that in PR100. Therefore the IC50 values of these fractions may be overestimated values. For comparison on the same ground of content of the fraction present in PR100, we recommended to adjust the IC50 of the fractions in order to make that the adjusted values equivalent to the concentration of the fraction in PR100 giving a better picture of their corresponding anticancer activities. Here, we suggested doing the adjustment of

IC50 of a fraction by dividing its IC50 value by the % mass of the fraction or corresponding % of peak area of the fraction in the same chromatogram. The reason on the use of % peak area for adjustments is that all UV-active components shown in HPLC fingerprint are involved so this way could be employed when the class of components is known from the literatures before investigation. However, we know that not only UV-active components but also UV-inactive components are possible to contribute the anticancer activities in the extract so that both kinds of components should be considered. This supports we use % mass for adjustment. Table 4.4 lists the adjusted IC50 of the six fractions on HepG2 and WRL-68 cells obtained and Appendix 4.3 gives the % of peak area at 230nm and % mass of the six fractions.

Table 4.4 Adjusted IC50 (mg/ml) of the six fractions on HepG2 and WRL-68 cells

| | **Adjusted IC50 by peak area** | | **Adjusted IC50 by mass** | |
|---|---|---|---|---|
| | **HepG2** | **WRL-68** | **HepG2** | **WRL-68** |
| **F1** | > 10 | > 10 | > 10 | > 10 |
| **F2** | > 10 | > 10 | > 10 | > 10 |
| **F3** | 9.24±0.21 | 9.51±0.25 | > 10 | > 10 |
| **F4** | > 10 | > 10 | > 10 | > 10 |
| **F5** | 0.14±0.01 | 0.37±0.02 | 0.72±0.01 | 1.93±0.08 |
| **F6** | 6.96±0.98 | > 10 | 8.01±1.00 | > 10 |

It can be seen from the adjusted IC50 values (Table 4.4), F5 has the smallest IC50 value and it gives the strongest growth inhibitory activity on both HepG2 and WRL-68 cells. This is the same as that before IC50 adjustment. The new

IC50 value of F5 adjusted by % peak area is two times smaller than the IC50 of PR100 (~0.33mg/ml) and therefore F5 can be regarded as the fraction mainly responsible in giving the activity in PR100. However, the adjusted IC50 of F5 obtained by % mass is larger than the IC50 of PR100 and so F5 may not be mainly responsible for the anticancer activity in PR100. Then which one adjustments or no adjustment is better to give the 'true' picture? It is hard to define but these two adjustments have their reasons.

The adjusted IC50 obtained by % mass is an all-round adjustment. Undoubtedly, the growth inhibitory activities may not be only contributed by UV-active components but also UV-inactive components that cannot be detected by HPLC-DAD instrument. Therefore the effect contributed by these inactive components is considered in this adjustment. It should be noted that only UV active components were detected with the use of HPLC-DAD, UV-inactive components may be active ingredient also. The UV-inactive components can be detected by other chromatographic techniques such as LC—MS. This kind of adjustment involved too many components in different classes and so it is difficult to trace which components give the activity within the fraction. In doing so, the IC50 can be adjusted by % peak area. This adjustment can narrow down the number of components present within a specific wavelength and so it is easy to determine which components contribute the activities within the fraction.

### 4.4.4 Cell proliferation of two anticancer drugs and the combined effect of F5 with these two drugs

It is quite common cancer patients in the Chinese communities may take herbal

medicines as complementary therapy at the same time with the cancer drugs used in the conventional treatments [69]. Hence we tried to investigate whether the anticancer drugs may affect the chemosensitivity of the liver cancer cells. Thus a preliminary investigation on PR fraction was carried out in this way on HepG2 cells.

Paclitaxel (PAC) and doxorubicin (DOX) were chosen in this work to study their chemosensitivity effect to the effective of P. Alba fraction on HepG2 cells. Firstly, the growth inhibitory effects of individual PAC and DOX on HepG2 cells were investigated so as to estimate their IC50 values (Appendix 4.3). The IC50 values of PAC and DOX were found to be 0.4ug/ml and 1.0ug/ml respectively. Then PAC and DOX at their IC50 concentration were added to a serial concentration of F5. The antiproliferation effects of DOX and PAC to F5 on HepG2 are displayed in Figure 4.6. In addition, the IC50 values of F5 with and without the presence of DOX or PAC are summarized in Table 4.5.

Figure 4.6 Antiproliferation effects of DOX and PAC to F5 on HepG2

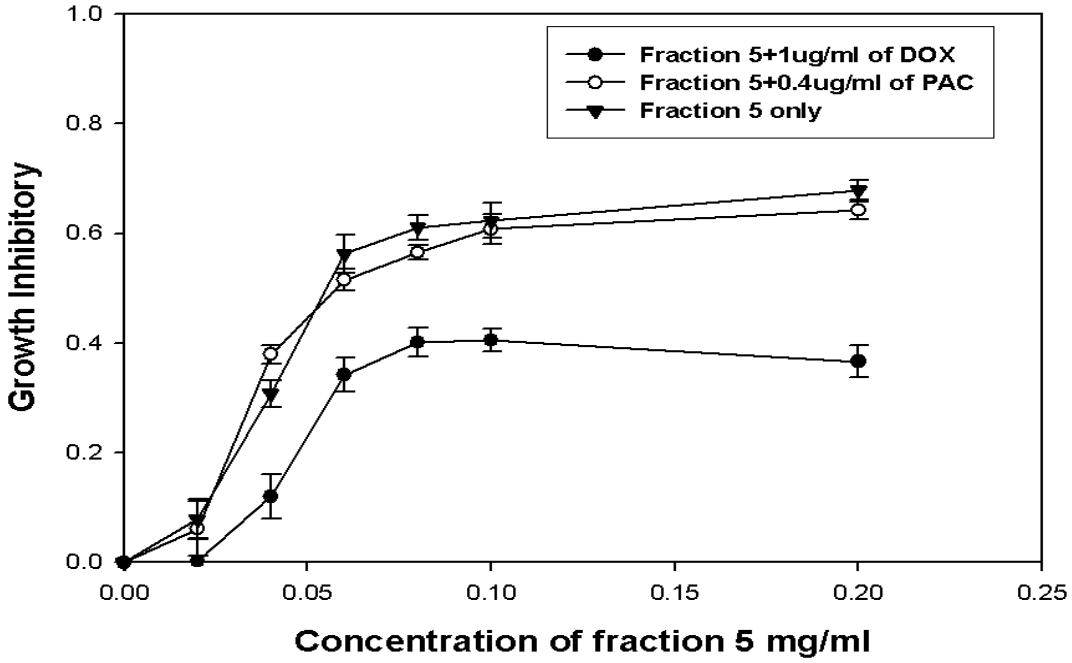



Table 4.5 The *IC50 values of F5 with addition of DOX and PAC on HepG2 cells

| | Mixture | | |
|---|---|---|---|
| | F5 only | F5 + 0.4ug/ml PAC | F5 + 1.0ug/ml DOX |
| IC50 values mg/ml | 0.06±0.01 | 0.06±0.01 | > 0.2 |

* Results given are the average values of three experiments performed and were presented in form of the means ± standard deviation (S.D.)

Figure 4.6 shows the differences in the sensitivity of DOX and PAC to F5 on HepG2 cells. The antiproliferation curves of F5 alone and F5 containing PAC overlapped with each other. Yet, the IC50 value is kept unchanged in F5 mixed with PAC (0.06mg/ml) compared with that in F5 alone. This implies that the growth inhibitory effect of F5 remained unchanged with or without PAC. Besides, it seems no synergistic interaction between components in F5 and PCA drug.

Therefore, the chemosensitivity effect of PAC had no significant effect to F5 on HepG2 cells.

On the other hand, the antiproliferation curve of F5 with DOX changes dramatically to that of the F5 alone (see Figure 4.6). And the IC50 of F5 was increased about three times than that of F5 with DOX. All these indicate that the growth inhibitory activity of F5 was reduced significantly by the concomitant of DOX. It is probably due to the antagonistic interaction existed in the components of F5 and DOX and hence DOX suppressed the growth inhibitory activity of components in F5. Therefore, DOX drug had adverse effects on F5 on HepG2 cells.

## 4.5 Conclusion

P. Alba extract was found to have growth inhibitory activity on HepG2 cells. However, this efficacy coming from a single component or more than one in the herb is still unknown. The aim of this study was to find the active component or fractions with components of P. Alba contributing this efficacy. One of P. Alba components C1 was found to have antiproliferation activity with IC50 value of 16.7±0.6ug/ml. So this component should be the one responsible for a part of the anticancer activity of extract. Our results in this study indicates that anticancer efficacy of PR100 cannot be attributed by C1 only but also other components as more work being done on the extract.

PR100 was fractionated into six fractions by PHPLC. The antiproliferation activities of these six fractions on HepG2 cells were assessed. We found that F1 and F4 had no effects on HepG2 cells while F2 and F6 suppressed the growth of HepG2 cells to some extent. The anticancer growth effect of F3 was comparable to that of PR100. Surprisingly, F5 was found to be more effective based on the results without adjustment. This may be due to that the components in other fractions that may have antagonistic interaction on the components in F5 were removed in the fractionation process and therefore the growth inhibitory activity of F5 was enhanced. Besides, the concentrations of the fractions at IC50 are not same as that of them in PR100 and thus IC50 values of the fractions cannot represent their activity in PR100. Therefore IC50 values of the fraction may need to be adjusted. We proposed to adjust their values by % peak area or % mass for comparison and the results are listed. Each of these two methods has its own advantages.

The co-administration of traditional herbal medicine and drugs has been a great concern especially in cancer chemotherapy in Chinese communities. Another objective of this work was to examine the chemosensitivity effects of two anticancer drugs paclitaxel (PAC) and doxorubicin (DOX) to F5 on HepG2 cells. Our results showed that the anticancer activity of F5 remained unchanged by the concomitant of PAC but decreased markedly with the presence of DOX. These valuable findings can be provided to both patients and doctors when P. Alba is co-administrated with PAC and DOX. Of course, more investigations on animals and human being are needed for confirmation.

# Chapter 5

# Optimization of extraction condition of Radix Puerariae Lobatae by Uniform Design

## 5.1 Introduction

Radix Puerariae Lobatae (P. Lobatae), one of commonly used Chinese herbs, was first described in the Chinese Materia Medica, *Shen Nong Ben Cao Jing*. It exerts sedative and antipyretic actions and is often used to treat influenza, wrist stiffness and headache. Moreover, RP and its medical preparation can also be used as clinical medicines to treat coronary heart disease, myocardial infraction, hypertension and anti-cancer.

Flavonoids including isoflavonoids have received considerable attention because of their beneficial effects as antioxidants in the prevention of human diseases such as cancer and cardiovascular diseases [70]. Pharmacological studies reveal that the active ingredients in P. Lobatae are isoflavonoids and its derivatives [11, 13, 71, 72]. Most studies reported that it is a rich source of isoflavonoids and therefore P. Lobatae is highly valued for its medicinal properties [12]. Five isoflavonoids components in P. Lobatae that include puerarin, daidzein, daidzin, genistein and genistin are often drawn attentions to scientist because they are believed to response for the major efficacies of P. Lobatae. The pharmacological activities of isoflavonoids are mainly attributed to their anti-oxidant activity [12, 35, 70, 71, 73, 74, 75]. Some studies revealed that the optimal condition for extraction of P. Lobatae was established just based on the one or two isoflavonoids components only. In fact, the biological activities are required to be considered seriously in establishing a good optimal condition for extraction of TCM because the efficacy is the most important part.

The conventional approach of optimization is that an experimental parameter is

changed while keeping the remaining conditions constant. But this approach is a time-consuming process. In addition, the optimization in extraction condition is always influenced by the interaction of different experimental variables. Therefore experimental designed-based approaches are usually recommended for optimization. Experimental design has been applied to obtain a product or process with desirable characteristics in an efficient way. Experimental designs and statistical tools for data analysis can provide more information about the system under investigation after doing only a few experiments systemically [76, 77]. Uniform Design (UD) is one of the fractional experimental designs and it was developed by Kai-tai Fang and Yuan Wang in 1980. The main idea of UD is to find a set of representative experimental points according to a quasi-Monte Carlo or number-theoretic method. The experimental points in a space are scattered uniformly in the domain[78]. It is always employed in the situation with different levels of many experimental factors and hence it is quite useful in many chemical and chemical engineering applications.

In this work, the first thing was to screen the sample extractions and extraction solvent. The concentration of puerarin and total concentration of isoflavonoids were designed as chemical responses. Here, the total isoflavonoids was defined as the total concentrations of the five major isoflavonoids puerarin, daidzein, daidzin, genistein and genistin in P. Lobatae. Besides, the results in antioxidant activities detected by 2,2-diphenyl-1-picrylhydrazyl (DPPH) was assigned as the biological responses in the screening step. Following by screening sample extractions and extraction solvent, the pseudo-level uniform experimental design technique was employed to set up twelve groups with variations in the five extraction factors with three or six levels. The contents of the five major

isoflavonoids in these twelve different extractions were utilized to assess the extraction efficiency of P. Lobatae. Moreover, DPPH assay was employed to evaluate the antioxidant activities in different extracts of P. Lobatae. Finally, the optimal conditions of extraction were determined for P. Lobatae according to the chemical responses and biological responses that are the results of concentrations of identified isoflavonoids in line with the results of antioxidant activity by DPPH assay.

## 5.2 Background of Radix Puerariae Lobatae

Radix Puerariae Lobata (P. Lobatae), referred to as *Ge Gen* (GG) in Chinese, is a commonly used Chinese herb drug material which is available in the form of dried roots of a wild leguminous creeper, *Pueraria lobata* (Wlid) Ohwi and *Pueraria thomsonii* Benth [11, 12, 72, 79]. Figure 5.1 shows the roots of the two legume plants, which was officially recorded in Chinese Pharmacopoeia (CP) till 2000 edition[9], Pharmacopoeia of the People's Republic of China (English Edition) 1997 [80] and Japanese Pharmacopaeia (English Edition) 1986 [81].

Figure 5.1 The dried roots of *Pueraria lobata* (Wild) Ohwi

P. Lobatae belongs to Fabaceae family and is collected in autumn and winter [11] and its origin is mainly from Hunan, Henan, Guangdong, Zhejiang and Sichuan. It always appears in pale brown with outer bark and with longitudinal wrinkles, rough, cut surface yellowish-white, and striations indistinct. The texture of P. Lobatae is pliable and strongly fibrous. It is odorless and the taste is slightly sweet and pungent [11].

The functions of P. Lobatae are to increase coronary artery blood, relieves fever, promote the production of body fluid, facilitate eruption and invigorate the spleen Yang to arrest diarrhea. It is indicated for fever, headache, stiff and painful nape in hypertension, measles, diarrhea, coronary heart disease, and alcohol addition angina pectoris. Therefore it is used as an anti-pyretic, antidiarrhetic, diaphoretic, antispasmodic and antimicrobic remedy. The channels entered are spleen and stomach [11, 12, 82].

## 5.3 Literature review on chemical study and biological study of Radix Puerariae Lobatae

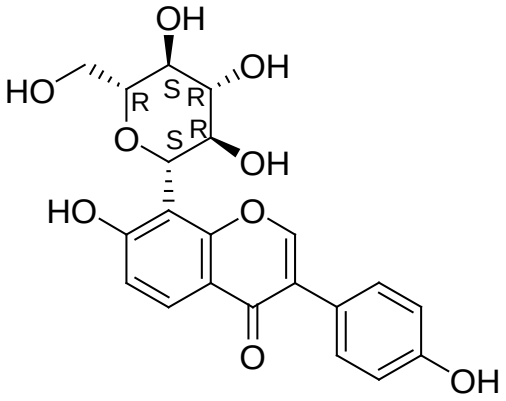
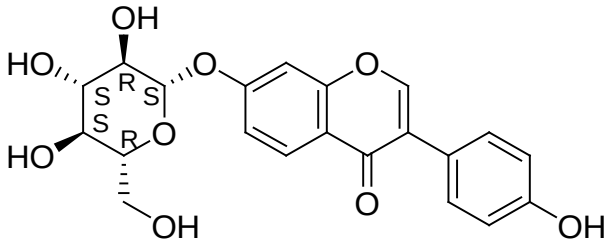
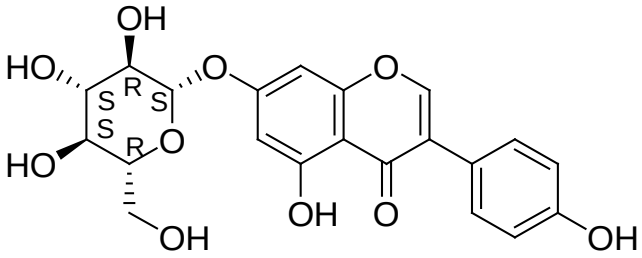
### 5.3.1 Chemical composition

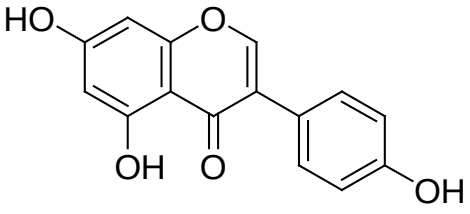
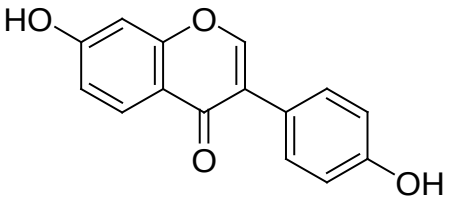
Isoflavonoids and its derivative are the major class of P. Lobatae. Many studies reported that P. Lobatae is a rich sources of isoflavonoids [12, 35, 73, 79] and its biological activities are mainly attributed to this class [11, 35, 72]. The major components of isoflavonoids and their derivatives are puerarin (PU), daidzein (DE), daidzin (DA) genistin (GN), genistein (GE), formononetin, 3’-hydropuerarin, 3’-methoxypuerarin, etc [12, 72, 82]. Among them, puerarin is

present in the greatest amount, higher than 2.4% in P. Lobatae sample [9, 13, 83]. In addition, the contents of isoflavonoids in P. Lobatae vary from 0.5% to 16% due to different sources. These high levels of isoflavonoids may account for larger biological activities [84]. Furthermore, puerarin and daidzein have been used as the marker compounds to characterize P. Lobatae isoflavonoids [71, 83]. Five of them, PU, DE, DA, GE and GN are commercially available so that these five isoflavonoids components in P. Lobatae sample were studied. Table 5.1 shows the chemical structures and molecular masses of these five major isoflavonoids components.

Other than isoflavonoids, P. Lobatae is also composed of sapogenins and saponins. The main components in these two classes include kudzusapogenol A, kudzusapogenol B, kudzusapogenol C, sophoradiol, cantoniensistriol, soyasapogenol A, soyasapogenol B, soyasaponins and other oleanene-type triterpene glycosides [11]. Furthermore, P. Lobatae also contains other compounds including β-sitosterin and its glyucoside, 6,7-dimethoxycoumarin, 5-methylhydantoin and arachinic acid [11].

Table 5.1 Chemical structures and molecular masses of the five major isoflavonoids components in P. Lobatae

| **Puerarin (PU) $C_{21}H_{20}O_9$ (MW=416)** | **Daidzin (DA) $C_{21}H_{20}O_9$ (MW=416)** | **Genistin (GN) $C_{21}H_{20}O_{10}$ (MW=432)** |
|---|---|---|
|  |  |  |

| **Genistein (GE) $C_{15}H_{10}O_5$ (MW=270)** | **Daidzein (DE) $C_{15}H_{10}O_4$ (MW=254)** |
|---|---|
|  |  |

### 5.3.2 Pharmaceutical activity of isoflavonoids compounds

Isoflavonoids components in P. Lobatae possess many important therapeutic activities, including antiproliferative effects on human cancer cell lines, inhibiting alcohol dehydrogenase and xanthine oxidase, anti-oxidant, cardiovascular diseases etc [35, 85]. All these components have their own pharmacological activities [86] and are listed in the following table.

Table 5.2 Pharmaceutical activities of the five isoflavonoids components in P. Lobatae

| Name of isoflavonoids | Pharmaceutical activities |
|---|---|
| **Puerarin** | Dilation coronary arteries[83, 86], inhibition of a variety of enzymes, antioxidant, anti-inflammatory activities [71], anti-hypertension [86], anti-oxidant activity [73] |
| **Daidzein** | Inhibition of a variety of enzymes, antioxidant, anti-inflammatory activities [71], antigiardial activity [85], anti-cancer protection [87], potential antidiabetic activities [75] |
| **Daidzin** | Treating alcoholism [73], enhancement of acetylcholinesterase activity in rat neuronal cell line PC12 cells by binding to the estrogen receptor [88] |
| **Genistin** | Inhibition of UV light-induced plasmid |

| | |
|---|---|
| | DNA damage, skin anticancer [89] |
| **Genistein** | Promising anti-cancer, inhibition of platelet aggregation, inhibitition leukotriene production [73], skin anticancer [89] |

### 5.3.3 Chemical studies of Radix Puerariae Lobatae

P. Lobatae is abundant of isoflavonoids that are believed to be responsible for its efficacies, therefore the detection and identification of isoflavonoids components from P. Lobatae is very important for quality control purpose. Many investigations in chemical characterization of isoflavonoids constituents of P. Lobatae by different chromatographic and spectroscopic techniques have been reported. The isoflavonoids in P. Lobatae samples can be characterized and detected by liquid chromatography coupled with negative and positive electrospray ionization (ESI) tandem mass spectrometry (MS-MS), and photodiode array detection (DAD) [12, 82]. Also, 12 isoflavonoids components of P. Lobatae were examined by high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) [72]. Besides, the concentration of puerarin and daidzein in P. Lobatae were determined by capillary electrophoresis and micellar electrokinetic capillary chromatography with electrochemical detection [71, 85]. Thin layer chromatographic technique was also used to analysis of isoflavonoids compounds, saponines and sapogenins is also reported [11]. Recently, high-performance thin-layer chromatographic technique was employed to examine the content of isoflavonoids for distinguishing different P. Lobatae samples [9].

### 5.3.4 Biological studies of Radix Puerariae Lobatae

The important therapeutic effects of P. Lobatae are cardiovascular diseases, anticancer and anti-alcohol [35, 79, 87, 90, 91, 92]. The first two disorder are attributed to antioxidant activities of isoflavonoids [35, 73, 79]. Therefore many investigations were carried out to study the antioxidant effects of P. Lobatae. It has been reported that the radical scavenging activities of P. Lobatae isoflavonoids by chemiluminescence study, reactive oxygen species (ROS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) [35, 93]. Supplementation of water extract of P. Lobatae was studied on the changes of antioxidant enzymes and lipid profile in ethanol-treated rats [94]. The antiproliferation effects of daidzein, genistein, genistin and puerarin were tested for cytotoxic effects on cancer cell lines of the human gastrointestinal origin [92] and the extract of P. Lobatae also exhibited a mild antiproliferation effect on the growth of human breast cancer cell line MCF-7 [87]. Furthermore, P. Lobatae has been found to have antidipsotropic (antialcohol abuse) activity [91]. Besides, the antidepressant effect of ethanol extract of P. Lobatae was tested in mice exposed to cerebral ischemia reperfusion [74].

## 5.4 Methods of investigation

Isoflavonoids components are the major chemical constituents of P. Lobatae and they also mainly determine the efficacies of P. Lobatae that contribute the anti-oxidant activities significantly. With the use of uniform design, the condition on sample extraction of P. Lobatae was explored based on two different types of response from chemical and biological screening studies. The former ones are the

concentration of puerarin and the total concentration of isoflavonoids, while the later one is obtained from the DPPH assay

### 5.4.1. Uniform design

Uniform design (UD) is a space filling design with experimental points distributing regularly in the domain. The principle in building the uniform design is to determine a set of representative experimental points based on the quasi-Monte Carlo or the number –theoretic method. Suppose that there are $\boldsymbol{s}$ experimental parameters and each has $\boldsymbol{q}$ different levels. Then the total number of factor level combination is $\boldsymbol{q}^{\boldsymbol{s}}$. However if $\boldsymbol{s}$ and $\boldsymbol{q}$ are large, the number of experiments needed will be too high to be carried out in practice. But, the number of experiments can be reduced significantly when uniform design is used.

The features of UD are especially addressed here. There are three merits in using UD. 1. Space filling: It is capable of producing samples with high representativeness in the experimental domain studied. 2. Robustness: It imposes no strong assumption on the model, and is against changes of model in a certain sense. 3. Multiple levels: It accommodates the largest possible number of levels for each factor among all experimental designs. A U-type design denoted by $U_n(q^s)$ is a design with n runs and s factors, each having q levels. Table 5.3 gives an uniform design $U_6(6^3)$ with six experiments for three factors at the six different levels.

Table 5.3 Uniform Design $U_6(6^3)$

| **Experiment** | **Factor** | | |
|---|---|---|---|
| | $\mathbf{X_1}$ | $\mathbf{X_2}$ | $\mathbf{X_3}$ |
| **1** | 2 | 4 | 6 |
| **2** | 3 | 6 | 2 |
| **3** | 6 | 5 | 4 |
| **4** | 1 | 2 | 3 |
| **5** | 5 | 3 | 1 |
| **6** | 4 | 4 | 5 |

If the level numbers in each experimental factor are different due to the nature of the factors or the limitation of the experimental environment, the mixed levels uniform design is employed in this situation. Therefore the notation of the design can be extended to the case of mixed levels, and denoted by $U_n$ ($q_1$ x …$q_i$…x $q_s$) with $q_i$ representing the number of levels of factors *i*.

Suppose three 8-levels factors and two 4-levels factors are considered in an experimental set-up. This $U_8(8^5)$ uniform design with eight experiments for five factors at eight levels is listed in Table 5.4 (left one). If the first three columns (X1, X2 and X3) of $U_8(8^5)$ are used for the three 8-levels factors, the original levels in columns X4 and X5 are merged by (1,2)⇒1, (3,4)⇒2, (5,6)⇒3 and (7,8)⇒4. In this way, the U-type design $U_8(8^3 \text{ x } 4^2)$ is generated (Right hand side of Table 5.4). This technique in uniform design is called pseudo-level technique. In this study, this technique was used because four 3-level factors and one 6-level factor were considered in extraction condition.

Table 5.4 Uniform Design $U_8(8^5)$ (left) and pseudo-level uniform design $U_8(8^3 \times 4^2)$ (right)

| **Experiment** | **Factor** | | | | | **Factor** | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $\mathbf{X_1}$ | $\mathbf{X_2}$ | $\mathbf{X_3}$ | $\mathbf{X_4}$ | $\mathbf{X_5}$ | $\mathbf{X_1}$ | $\mathbf{X_2}$ | $\mathbf{X_3}$ | $\mathbf{X_4}$ | $\mathbf{X_5}$ |
| **1** | 1 | 2 | 4 | 7 | 8 | 1 | 2 | 4 | 4 | 4 |
| **2** | 2 | 4 | 8 | 5 | 7 | 2 | 4 | 8 | 3 | 4 |
| **3** | 3 | 6 | 3 | 3 | 6 | 3 | 6 | 3 | 2 | 3 |
| **4** | 4 | 8 | 7 | 1 | 5 | 4 | 8 | 7 | 1 | 3 |
| **5** | 5 | 1 | 2 | 8 | 4 | 5 | 1 | 2 | 4 | 2 |
| **6** | 6 | 3 | 6 | 6 | 3 | 6 | 3 | 6 | 3 | 2 |
| **7** | 7 | 5 | 1 | 4 | 2 | 7 | 5 | 1 | 2 | 1 |
| **8** | 8 | 7 | 5 | 2 | 1 | 8 | 7 | 5 | 1 | 1 |

## 5.4.2 Antioxidant assay

### 5.4.2.1 2,2-diphenyl-1-picrylhydrazyl antioxidant assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was adopted in this study to examine the antioxidant activities of P. Lobatae. This assay is a free radical scavenging test. The mechanism of DPPH assay was mentioned in Section 2.3.2.2 in Chapter 2.

There are two ways for finding the scavenging power of extracts in DPPH literatures. The first one is that the scavenging activity in constant concentration at certain time is used for comparison. It is only utilized if the relationship of the scavenging activity and the serial concentrations of sample are kept linear and

hence the scavenging activity has not reached to plateau level in this concentration range. Here, this method was adapted. In contrast, when the linear relationship is not valid and the scavenging activity reach plateau level, the SR50 value was calculated and used in results comparison. SR50 is defined as the concentration of the extract at which 50% of DPPH radical is scavenged and it can be found in the plot of SR% against the concentration of the extract. This is another method in comparison with radical scavenging ability of different extracts.

## 5.5 Experimental

### 5.5.1 Herbal sample

Radix Puerariae Lobatae was collected from Sichuan in China and it is stored in humidifier.

### 5.5.2 Sample extraction and standards preparation

In the screening of sample extraction, 2g of P. Lobatae were extracted by reflux, ultrasonic and maceration with 60ml of the same solvent for 45mins to give three extracts named as GG-R, GG-U and GG-M respectively. In the screening of solvents, 2g of P. Lobatae were extracted by the same sample extraction with 60ml methanol, ethanol and water for 45mins to obtain three extract named as GG-MeOH, GG-EtOH and GG-water respectively.

After the screening process was completed, 2g of P. Lobatae were extracted

according to the methods listed in Table 5.5 and Table 5.6, and twelve extracts of P. Lobatae were obtained. For the re-extraction of P. Lobatae, the residue from the first extraction was filtered, and the same extracting conditions were applied. The filtrate was collected by filtration through using 0.45μm pre-cut membrane and the solvent was removed by rotatory evaporator. Then the filtrate was dissolved into fixed volume of desired solvent and then lyophilized by freeze dryer to yield the dry powders. The dry powders were kept in 4℃ and dark place when not in use. The concentration of these powders used in the experiment was based on the dry weight of extract (mg/ml). The five standards, PU, DE, DA, GE and GN were dissolved in methanol to give serial concentrations from 5ppm to 150ppm and three injections on HPLC were performed for each dilution. The concentrations of these five compounds in P. Lobatae samples were calculated according to the standards curve of these five standards.

Table 5.5 Levels of each factor in the extraction of P. Lobatae

| **Factor** | **Level** | | | | | |
|---|---|---|---|---|---|---|
| **Solvent*** | 1 (0%) | 2 (20%) | 3 (40%) | 4 (60%) | 5 (80%) | 6 (100%) |
| **Time / min** | 1 (45) | | 2 (60) | | 3 (75) | |
| **Number of extraction** | 1 (1) | | 2 (20) | | 3 (3) | |
| **Volume of solvent / ml** | 1 (60) | | 2 (120) | | 3 (180) | |
| **Temperature / ℃** | 1 (30) | | 2 (40) | | 3 (50) | |

* Solvent was mainly composed of methanol and DDI water. 100% is absolute methanol, 75% is 75% methanol and 25% DDI water, and so on.

Table 5.6 Pseudo-level uniform design to optimize the extraction of P. Lobatae

| Group | Factor in extraction of P. Lobatae | | | | |
|---|---|---|---|---|---|
| | **Solvent** | **Time** | **Number of extraction** | **Volume of solvent** | **Temperature** |
| **1** | 6 | 3 | 2 | 3 | 3 |
| **2** | 3 | 3 | 3 | 2 | 1 |
| **3** | 5 | 1 | 2 | 3 | 1 |
| **4** | 2 | 1 | 2 | 1 | 1 |
| **5** | 4 | 3 | 1 | 2 | 1 |
| **6** | 3 | 1 | 1 | 2 | 3 |
| **7** | 2 | 2 | 3 | 3 | 2 |
| **8** | 4 | 1 | 3 | 1 | 3 |
| **9** | 5 | 2 | 1 | 1 | 2 |
| **10** | 1 | 2 | 1 | 3 | 2 |
| **11** | 6 | 2 | 3 | 1 | 2 |
| **12** | 1 | 3 | 2 | 1 | 3 |

### 5.5.3 Chromatographic conditions of HPLC

Agilent 1100 series HPLC apparatus, equipped with a quaternary solvent delivery system, an auto-sampler and UV detector, was utilized. Chromatographic runs were carried out by a Thermo ODS Hypersil column (250 X 4.6mm, 5.0μm) at room temperature. The injection volume was 10μl and the flow rate was 0.6ml/min. The running time was 50 minutes and the detection wavelength was set at 254 nm. The mobile phase consisted of (A) acetonitrile and

(B) 0.3% acetic acid in DDI water using a gradient elution as listed in Table 5.7.

Table 5.7 The elution gradient of mobile phase in HPLC-DAD measurement

| Time (min) | A / % | B / % |
|---|---|---|
| ***0-30*** | *0-25* | *100-75* |
| ***30-35*** | *25-45* | *75-55* |
| ***35-40*** | *45-95* | *55-5* |
| ***40-45*** | *95* | *5* |
| ***45-50*** | *67-17* | *33-83* |

## 5.5.4 DPPH assay

The scavenging effects of the phenolic compounds towards the stable free radical DPPH• were measured. Briefly, 0.025mg/ml of DPPH• solution was prepared by dissolving the radical in methanol. In the measurement, 1.95ml DPPH• solution and 50μl sample solution were mixed well in the cuvette and the absorbance of the mixture solution was recorded spectrophotometrically at 515nm at each minute until the change of absorbance was less than 0.003 per minute. The reaction mixture was covered and left in the dark as possible at room temperature. The free radical scavenging radical capacity (SR) was calculated by equation 2.4 (see Chapter 2). And the SRmax, defined as maximum SR value at the concentration of 5mg/ml of P. Lobatae samples at 10mins, was chosen in results comparison. The DPPH assay of each sample was carried out in triplicate.

## 5.6 Results and Discussion

### 5.6.1 Qualitative and quantitative analysis of puerarin and total isoflavonoids

In this section, we report the concentration of puerarin and five major isoflavonoids (puerarin PU, daidzin DA, genistin GN, daidzein DE and genistein GE) as total isoflavonoids were investigated qualitatively and quantitatively by HPLC-DAD based on the chromatographic conditions given in Table 5.7. Figure 5.2 depicts the HPLC chromatogram of these five standards with concentration 100ppm each at $\lambda$=254nm. The UV-spectra and retention times of these standards for qualitative analysis are shown in Figure 5.3 and Table 5.8 lists the regression equations of their calibration curves and correlation coefficients ($r^2$) for quantitative analysis. The HPLC calibration curves of these five standards are shown in Appendix 5.1. It can be seen from Table 5.8 that the HPLC based calibration curves of PU, DA, GN, DE and GE exhibit good linearity in concentration range under study.

Figure 5.2 Chromatogram of the 100ppm standards mixture at 254nm

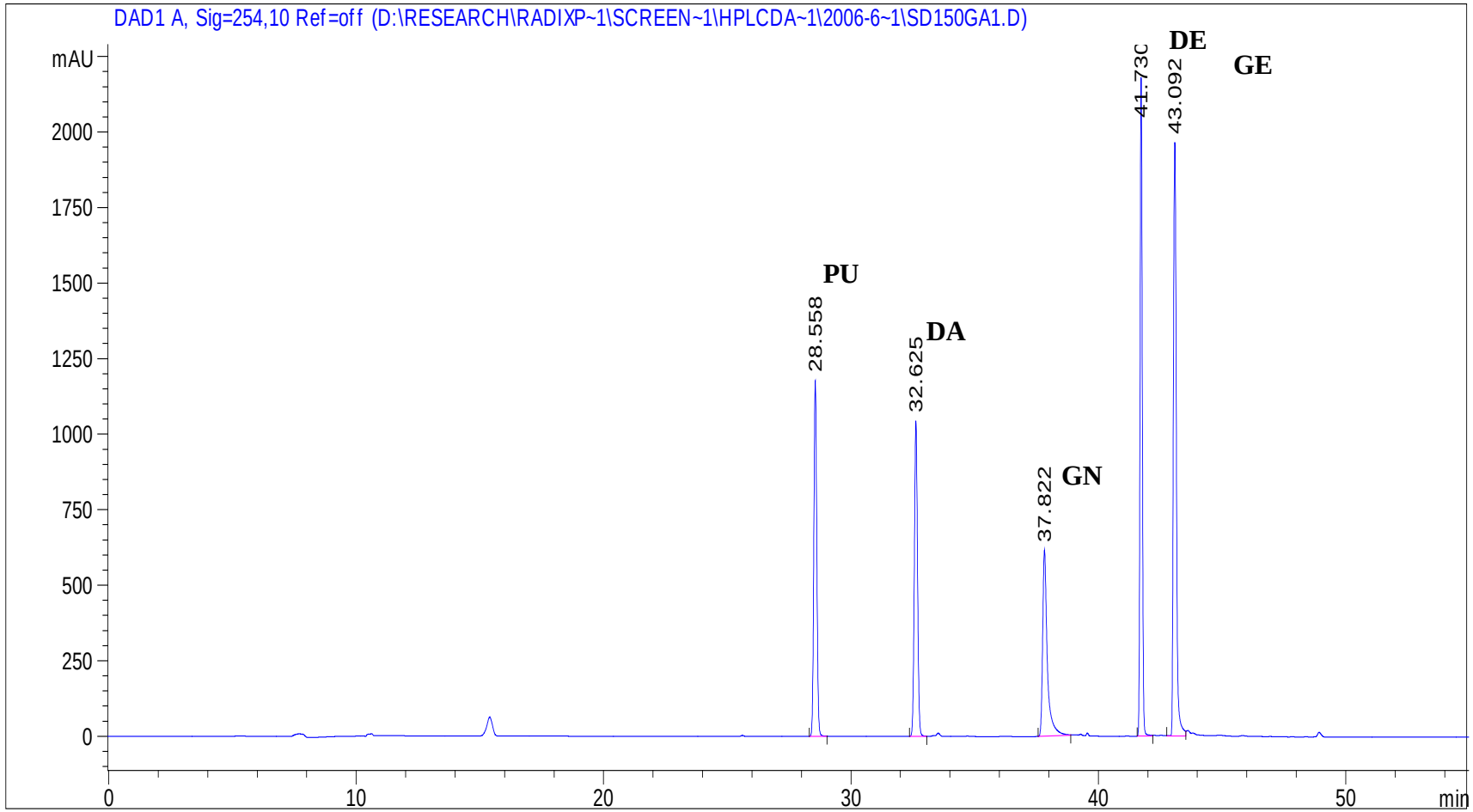
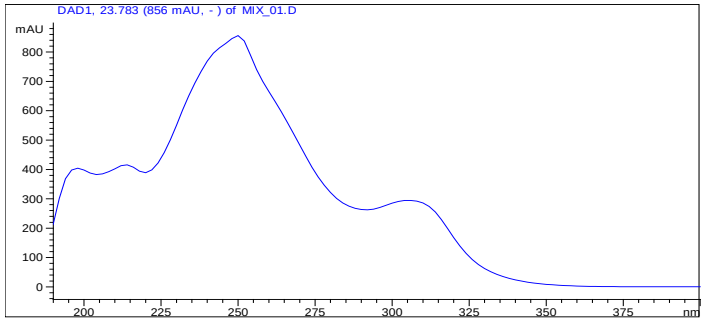
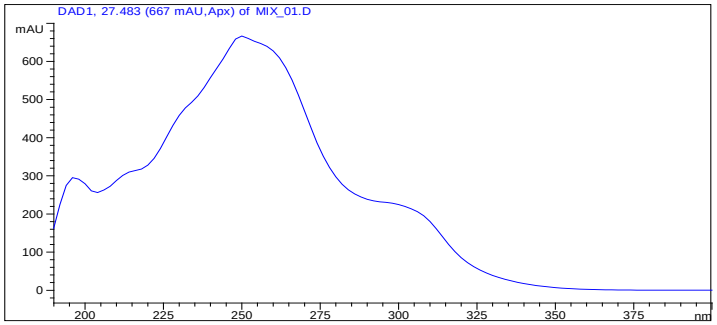


Figure 5.3 UV spectra and retention times of the five standards

| Name of Components | Puerarin (PU) | Daidzin (DA) |
|---|---|---|
| Retention time / mins | ~ 28.5 | ~ 32.6 |
| UV spectra |   |   |

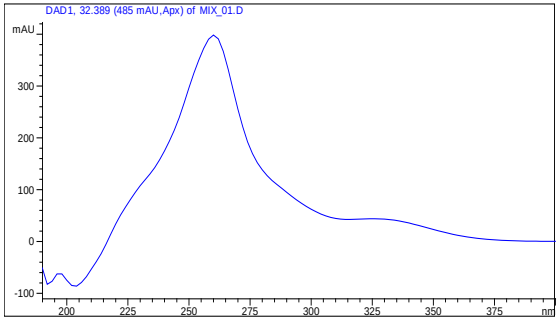
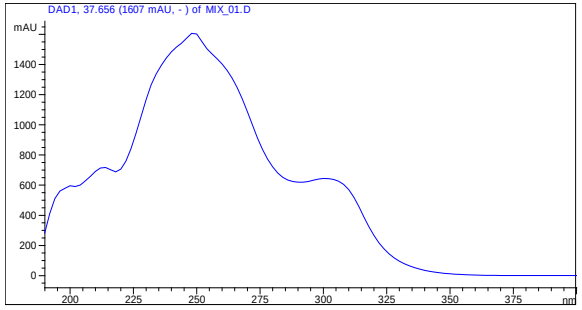
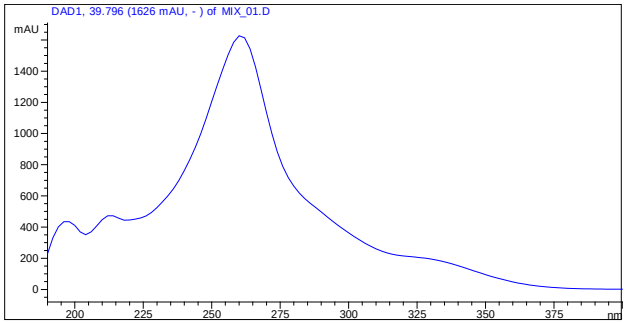
| Genistin (GN) | Daidzein (DE) | Genistein (GE) |
|---|---|---|
| ~ 37.8 | ~ 41.7 | ~ 43.1 |
|   |   |   |

Table 5.8 Linear range, regression equations and correlation coefficients of the standards PU, DA, GN, DE and GE

| **Compound** | **Linear range / ppm** | **Regression equations *** | **Correlation coefficients ($r^2$)** |
|---|---|---|---|
| PU | 5-150 | y = 65.569x + 17.118 | 0.9999 |
| DA | 5-150 | y = 62.997x + 16.198 | 0.9998 |
| GN | 5-150 | y = 55.475x + 75.585 | 0.9948 |
| DE | 5-150 | y = 90.631x + 108.45 | 0.9997 |
| GE | 5-150 | y = 109.88x + 75.134 | 0.9996 |

*** y : Peak area; x : Actual concentration (ppm)**

## 5.6.2 Screening of extraction methods

Reflux, ultrasonic and maceration were applied to extract P. Lobatae in this part because these three extraction methods are commonly employed in the chemical and biological studies of the isoflavonoids contents of P. Lobatae samples in the literature [9, 10, 12, 72, 87]. Three extracts are labeled as GG-R, GG-U and GG-M obtained by reflux, ultrasonic and maceration respectively. Different amounts of puerarin and total isoflavonoids in these three extracts were determined and hence their anti-oxidant activities could be measured. Figure 5.4 shows the overlay chromatograms of these three extract as obtained by three different extraction methods. Among them, component corresponding to peaks were identified by comparison their UV-spectra and retention times with those standards and the results are labeled in Figure 5.4. The antioxidant activities of these three extracts were also examined by DPPH assay. The

results of their antioxidant activities and the quantitative determination are tabulated in Table 5.9. The scavenging curves of these three extracts obtained by DPPH assay are shown in Appendix 5.2.

Figure 5.4 The overlay chromatographic fingerprints at 254nm of the three extracts P. Lobatae (1mg/ml) obtained by three different extraction methods

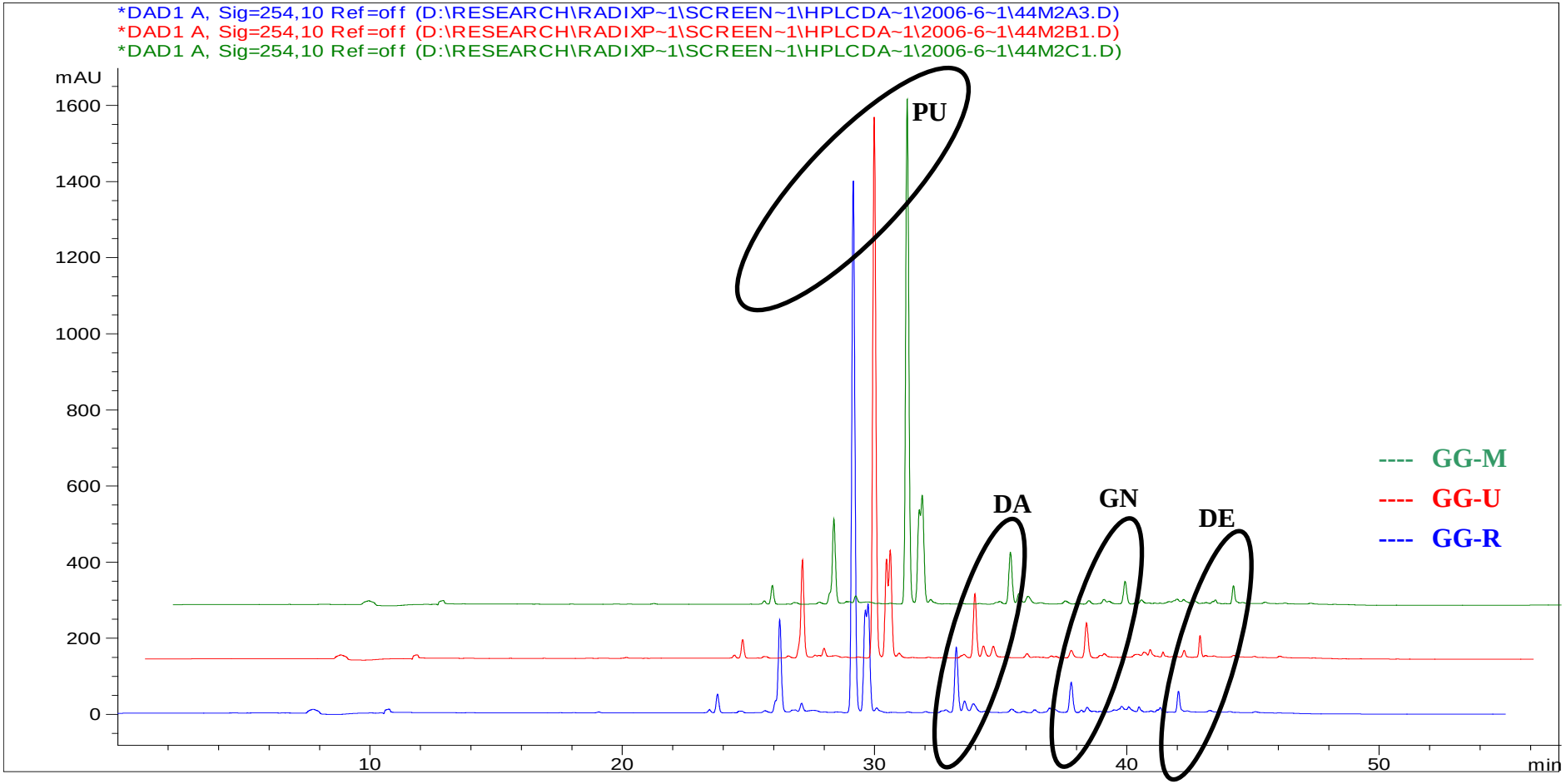


Table 5.9 The results of two chemical responses and biological response in screening the extraction methods of P. Lobatae

| Extract | Chemical response | | Biological response |
|---|---|---|---|
| | **Concentration of puerarin / ppm** | **Concentration of total isoflavonoids* / ppm** | **SRmax by DPPH**** |
| **GG-R** | 185±1 | 226±2 | 0.49±0.03 |
| **GG-U** | 157±1 | 187±2 | 0.57±0.02 |
| **GG-M** | 172±3 | 204±3 | 0.60±0.04 |

* Total isoflavonoids defined as the total concentration of the major five isoflavonoids of puerarin PU, daidzin DA, genistin GN, daidzein DE and genistein GE

** SRmax defined as the maximum scavenging capacity of P. Lobatae sample in the concentration of 5mg/ml at 10mins. The SRmax valued was expressed in form of mean± SD (n=3)

It can be seen that the amounts of puerarin and total isoflavonoids were obtained in GG-R as much as those in GG-M. In addition, GG-M gave higher biological response in DPPH assay than the other two. Moreover, the apparatus used and time spending on experimental set-up in maceration for GG-M is much less than that of the other two. Here we found out that maceration was very good for sample extraction of P. Lobatae which gave relatively high concentration of puerarin and total isoflavonoids but also produced the extract exhibiting the highest anti-oxidant activity. Therefore maceration was chosen as the sample extraction method of P. Lobatae in the following study.

## 5.6.3 Screening of extraction solvent

Methanol, ethanol and water are widely used in the chemical and biological

studies of isoflavonoids of P. Lobatae samples[9, 12, 72, 93, 94]. Three extracts were obtained in this work and, named as GG-MeOH, GG-EtOH and GG-water, by using the three respective solvents. The chromatograms of these three extracts were recorded and displayed in Figure 5.5. Four peaks as circled in the HPLC chromatograms were identified and labeled also. The concentration of puerarin and total isoflavonoids were determined and listed in Table 5.10. In addition, the antioxidant activities of these three extracts were examined by DPPH antioxidant assay and the results are listed also in Table 5.10. The scavenging curves of these extracts obtained via DPPH assay are shown in Appendix 5.3.

Table 5.10 The results of two chemical responses and two biological responses in screening of extraction solvent of P. Lobatae

| Extract | Chemical response | | Biological response |
|---|---|---|---|
| | Concentration of puerarin / ppm | Concentration of total isoflavonoids* / ppm | SRmax by DPPH** |
| **GG-MeOH** | 209±2 | 226±1 | 0.73±0.01 |
| **GG-EtOH** | 172±3 | 204±3 | 0.60±0.04 |
| **GG-water** | 88±1 | 110±3 | 0.78±0.06 |

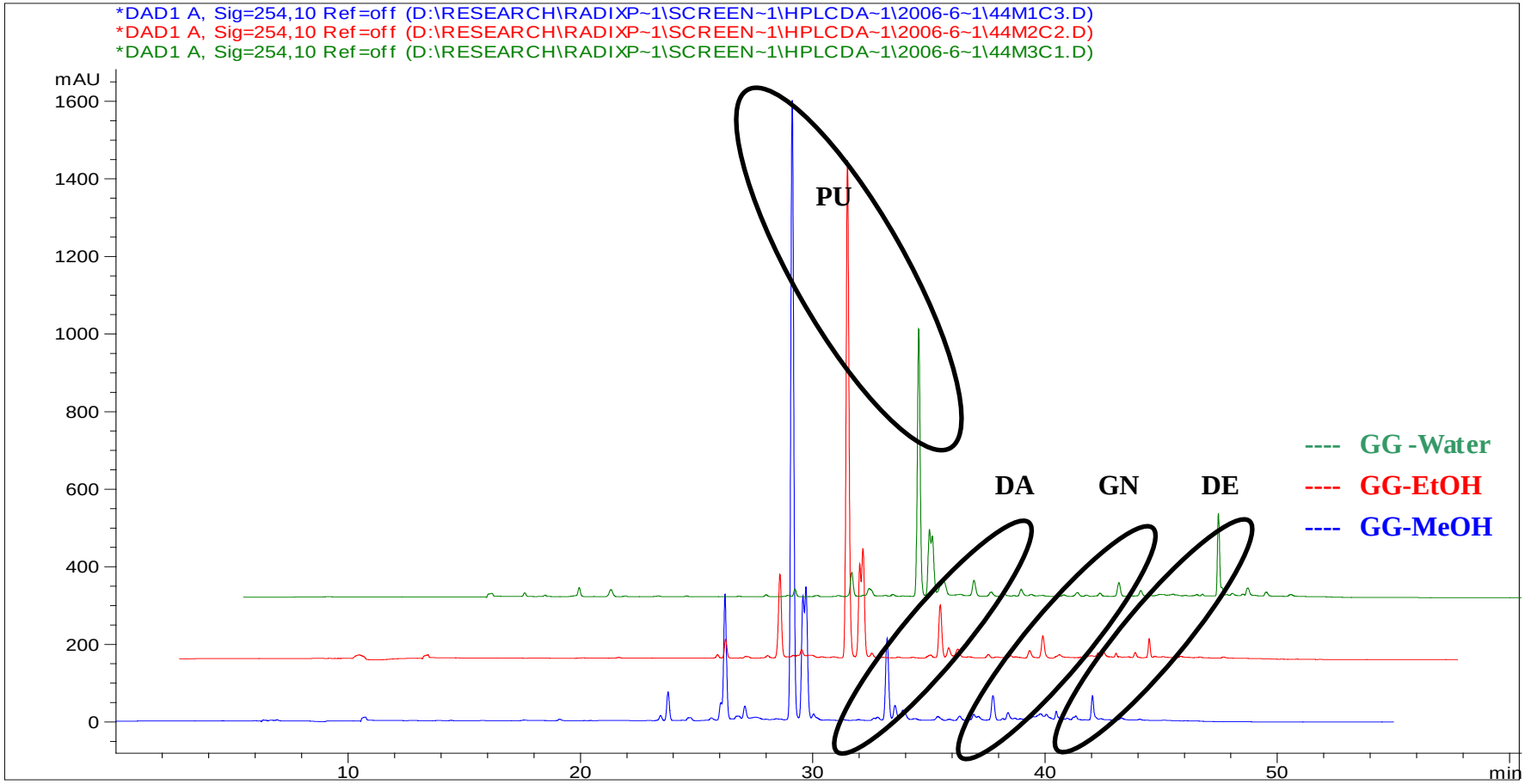
* Total isoflavonoids is defined as the total concentration of major five isoflavonoids that are (puerarin PU, daidzin DA, genistin GN, daidzein DE and genistein GE)

** SRmax defined as the maximum scavenging capacity of P. Lobatae sample in the concentration of 5mg/ml at 10mins. The SRmax valued was expressed in form of mean± SD (n=3)

As seen in Table 5.10, the concentration of puerarin and total isoflavonoids in GG-MeOH were significantly higher than those in the other two especially GG-water. In addition, GG-MeOH and GG-water gave relatively stronger

antioxidant activities than GG-EtOH. From the results of the chemical responses and biological response, the methanol and water were chosen as the solvents in the sample extraction. Different ratio of methanol and water were tried and described in the next section.

Figure 5.5 The overlay chromatographic fingerprints at 254nm of the three extracts of P. Lobatae (1mg/ml) obtained by three different extraction solvents



### 5.6.4 Optimization of Radix Puerariae Lobatae extraction by the use of pseudo-uniform experimental design

We used pseudo-level uniform technique to obtain the extracts of P. Lobatae from twelve groups having variations in five parameters of extraction including solvent, time, number of repeats of the extraction, solvent volume and temperature. The HPLC chromatograms of these twelve extracts of P. Lobatae are shown in Figure 5.6 with four peaks identified. The chemical corresponding to these identified peaks in are PU, DA, GN and DE. The amounts of these four isoflavonoids in these twelve extracts were determined and found to have significant variation under the twelve different extractions condition. The concentration variation of these isoflavonoids in these twelve extracts are shown in Figure 5.7 and tabulated in Appendix 5.4.

Figure 5.6 The overlay chromatographic fingerprints of P. Lobatae extracts (1mg/ml) in group 1 – 12 at 254nm

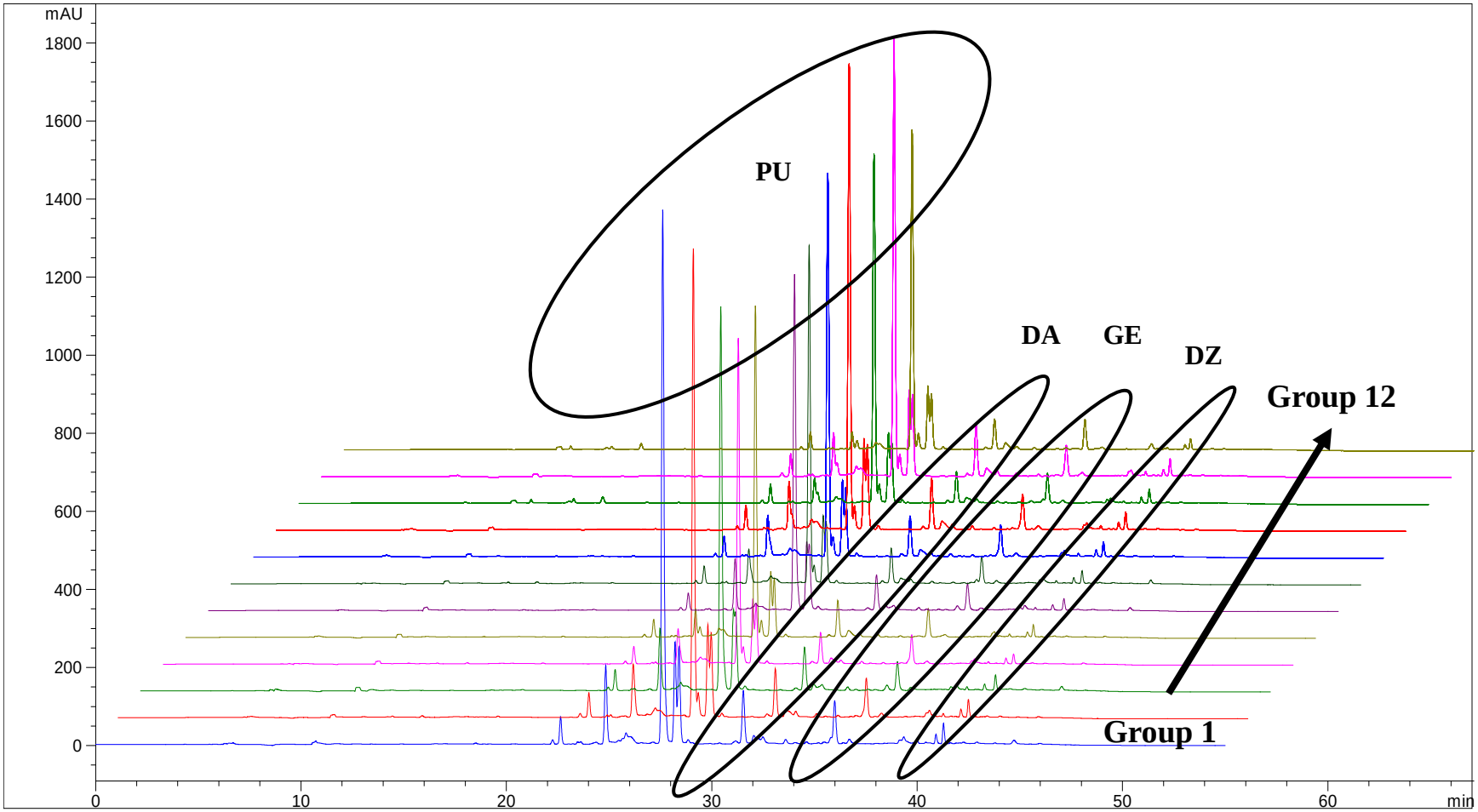
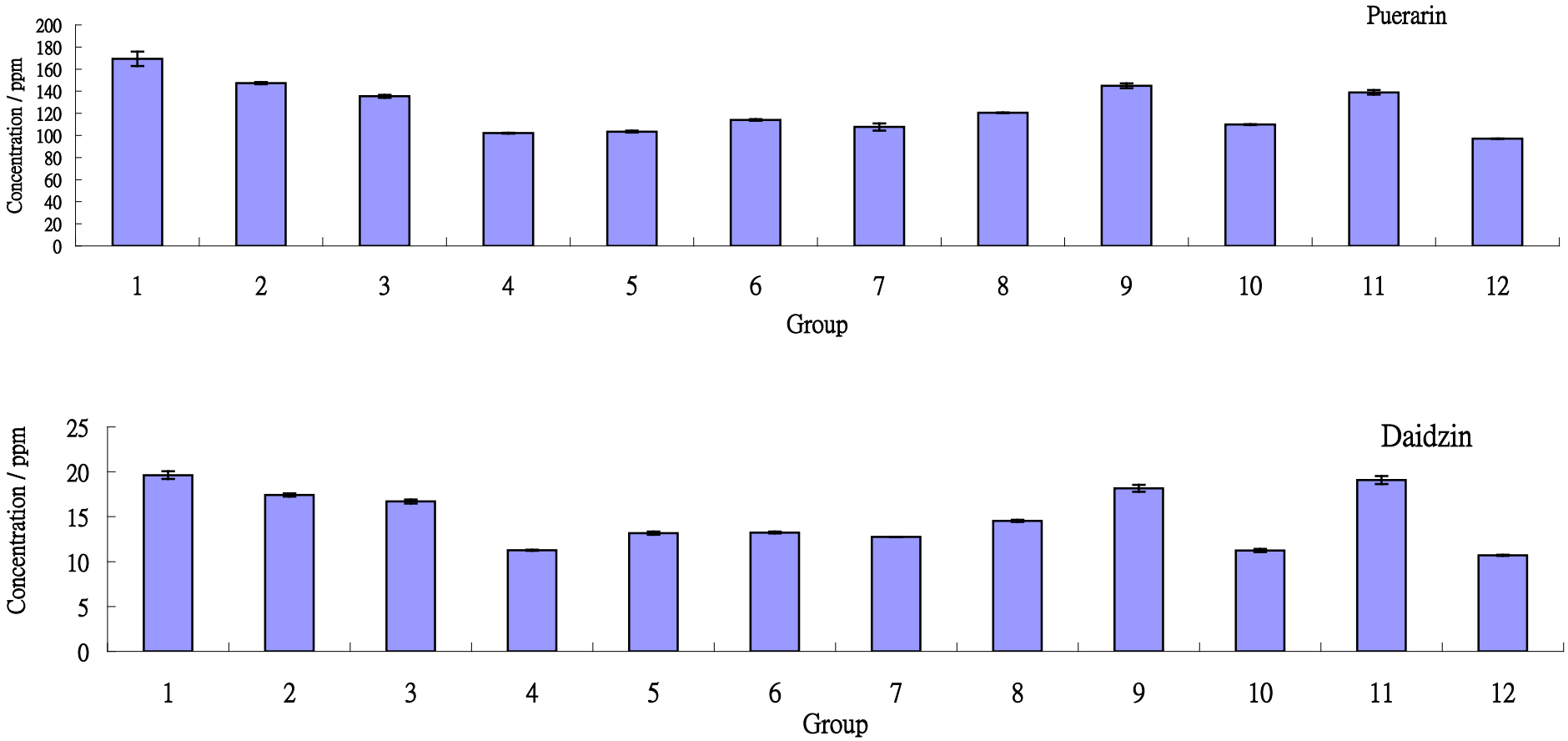
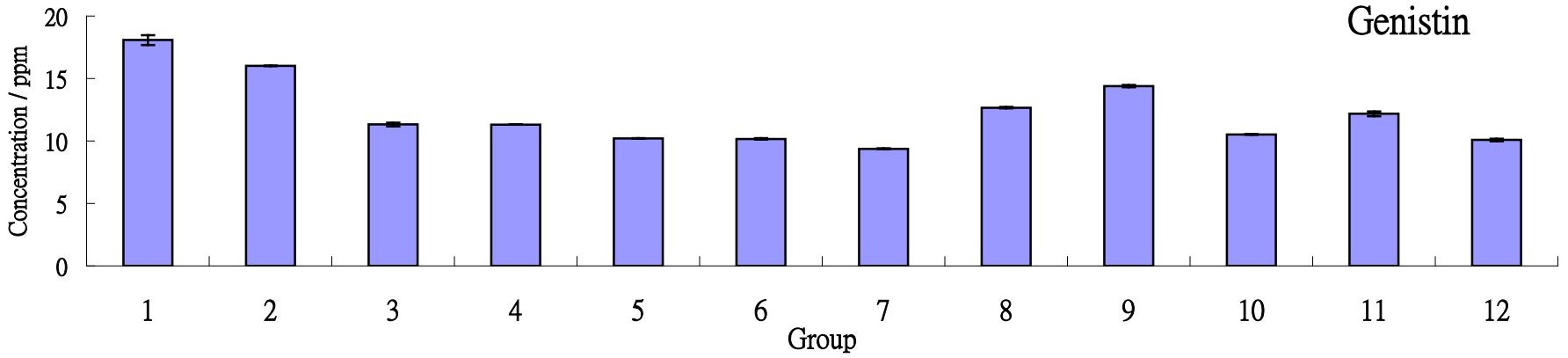
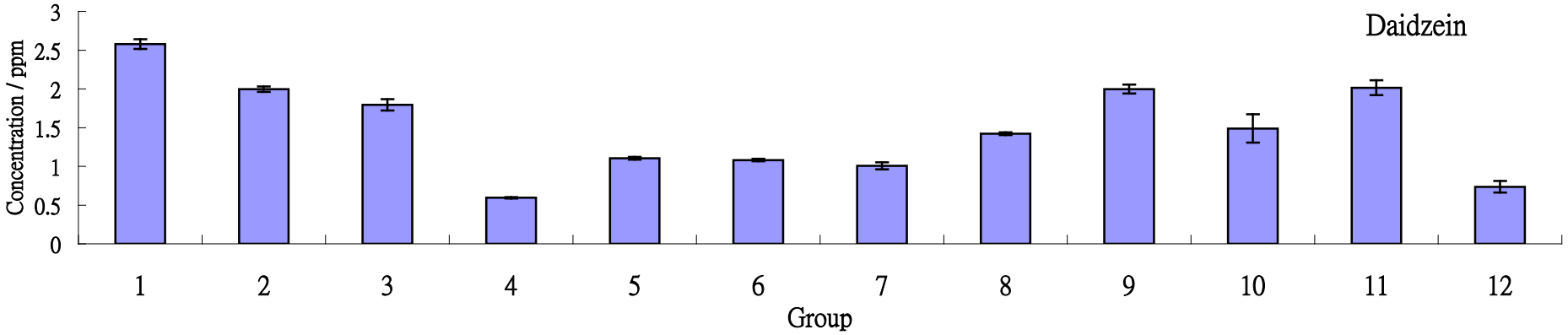


Figure 5.7 The concentration of puerarin, daidzin, genistin and daidzein in 1mg/ml of the twelve extracts of P. Lobatae produced under twelve different extraction conditions




Genistin
Concentration / ppm
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
Group


Daidzein
Concentration / ppm
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
10
11
12
Group

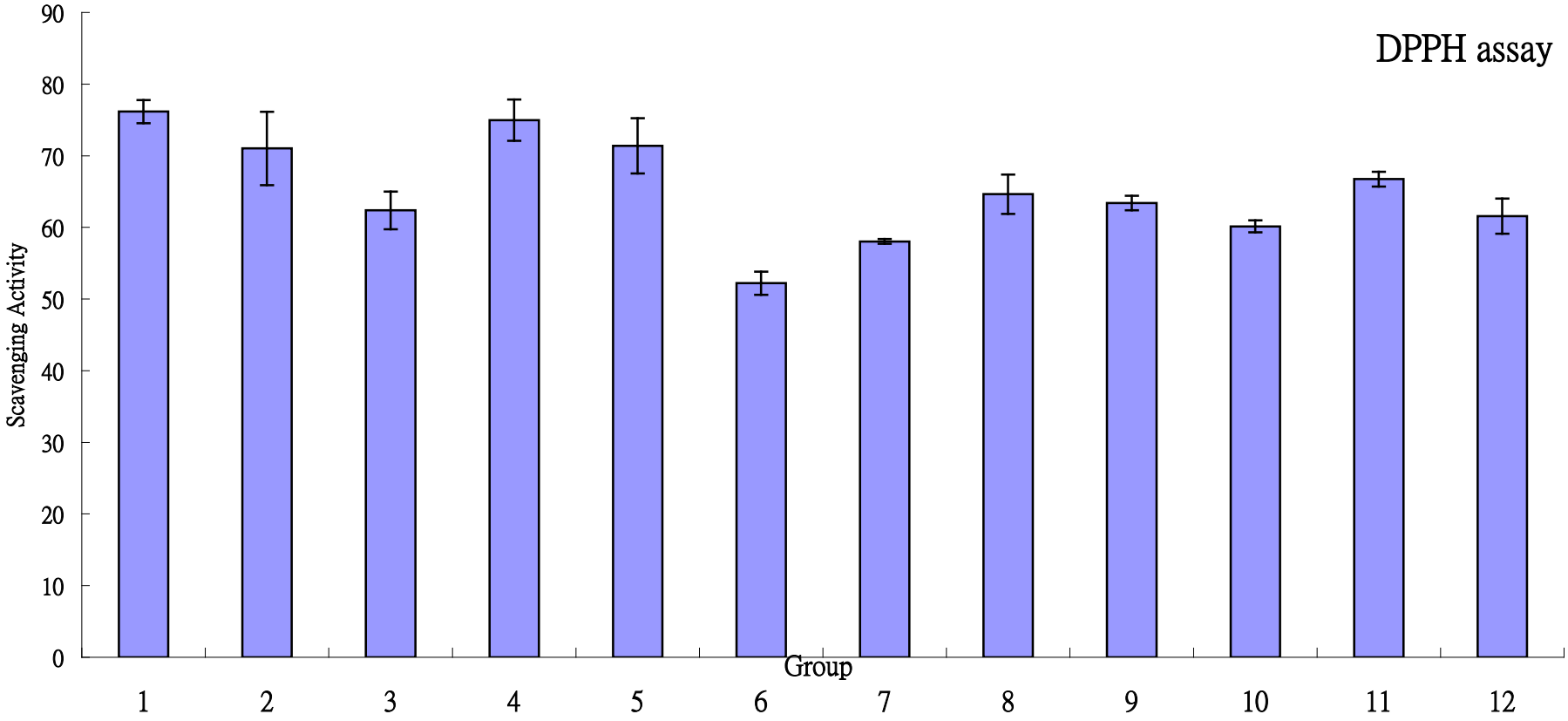
From Figure 5.7, it can be seen that the concentration of puerarin in group 1, 2 and 9 is higher than that in other groups, which its value is about double that of group 12 having the lowest value. As for daidzin, group 1, 9 and 11 contain the highest amounts, which are about 50% higher than that of the lowest one of group 12. Group 1, 2 and 9 contain about 2-fold higher amount of genistin than the lowest one group 7. Finally, the highest amount of daidzein is found in group 1,2 and 9 which is five times higher than the lowest group 4. According to the results of these chemical responses, four major isoflavonoids components with the higher contents can be obtained in two extracts of P. Lobatae under group 1 and 9 extraction conditions.

We tried further to determine which extraction methods not only extract more amounts of isoflavonoids but also provide a better biological response. The anti-oxidant activities of group 1 -12 extracts were studied by DPPH assay. The results are shown in Figure 5.8 and tabulated in Appendix 5.4. It can be seen from Figure 5.8, group 1, 2, 4 and 5 show a higher scavenging activity than others by about 12-32%. Among these four groups, group 1 exhibits the highest activity. Therefore, based on the result of DPPH assay, the extraction condition of group 1 is chosen.

Combined the results of chemical responses and biological response, extraction condition under group 1 is chosen for P. Lobatae sample. From these studies, we found that the result of biological response in only group 1 is consistent very well with the concentrations of the four identified major isoflavonoids by HPLC. However, the contradiction is found in other groups especially in group 4 and 9. As a result, the relationship between chemical response (the concentration of

four isoflavonoids) and biological response (scavenging activity) cannot be established with high confidence.

Figure 5.8 The antioxidant activities of group 1 to group 12 in DPPH assay



The concentration of these four components obtained in group 4 is almost two times lower than that in group 1, but surprisingly the scavenging activity of P. Lobatae extract in group 4 is as much as that in group 1. The opposite scene is observed in the results of group 9. This gives rise a question on that these four isoflavonoids are mainly responsible for giving scavenging activity of P. Lobatae extracts. The scavenging activities of these four components were then examined by DPPH assay in this work and the results are shown in Table 5.11.

Table 5.11 The scavenging activities of the four isoflavonoids at 1000μg/ml by DPPH assay

| **Components** | **SRmax*** |
|---|---|
| Puerarin (PU) | 0.34±0.04 |
| Daidzin (DA) | 0.11±0.01 |
| Genistin (GN) | 0.25±0.02 |
| Daidzein (DE) | 0.18±0.01 |

** SRmax defined as the maximum scavenging capacity of the component in the concentration of 1000μg/ml at 10mins. The SRmax valued was expressed in form of mean± SD (n=3)

It can be seen that single PU, DA, GE and DA at 1000μg/ml do not show significant scavenging effect in DPPH assay. The scavenging activities of these four isoflavonoids may be explained by their structures. The radical scavenging activities of isoflavonoids compounds are attributable to the hydroxyl groups on the backbone structure. As seem from their structures in Figure 5.1, the number of hydroxyl group in PU and GN are more than those in DA and DE and hence the scavenging activities of PU and GN are stronger than that of DA and DE. Although several hydroxyl groups exist in PU and GN, their scavenging abilities are still weak. This is probably due to their structures affecting the ease in

donation of hydroxyl group from their backbone structure. In addition, the scavenging activities of P. Lobatae extracts obtained are higher than that of these four isoflavonoids. This implies that the isoflavonoids compounds in P. Lobatae extracts would act synergistically each other or there are other components that had much stronger radical scavenging activities. Therefore the scavenging activity of P. Lobatae extract cannot be explained apparently by these four single components only in this moment.

It is a not an easy task and a challenging work to correlate the results of biological and chemical response from our studies. In doing so, the following things may be taken considerations. Firstly, interaction among these four identified isoflavonoids may exist in P. Lobatae extract and so the scavenging activity of the extract is probably due to synergetic effects of these four components. The work is undergoing in examining the scavenging activities of these four components at different ratio in concentration by DPPH assay. Secondly, other components as appeared as peaks in the HPLC chromatographic fingerprint are still not identified, but they may be responsible for the scavenging activity. With the help of Chemometric Resolution Methods (CRM), the relative concentrations of these unidentified peaks could be determined for comparison. Thirdly, the chromatographic technique used in this study was HPLC-DAD, only UV-active components were detected and therefore the UV-inactive components were hidden. In order to get a full picture of all the chemical components in P. Lobatae extract, LC-MS is suggested for further study so that more chemical information could be extracted for predication of the scavenging activity of P. Lobatae extract.

In this study, uniform design was employed to establish a model involving five parameters for finding optimal extraction condition for P. Lobatae. Indeed, the optimal extraction condition of P. Lobatae was found after just carrying out twelve experimental runs. This design used is very helpful especially in reducing the number of experimental runs. Therefore it is a more time- and resources-saving method that has significant advantages over the conventional methods such as the trial and error approach.

However, this model built was not good enough to find out which factors (solvent ratio, extraction time, number of extraction, solvent volume and temperature) in the extraction conditions of P. Lobatae play a significant role. One reason may comes from that the relationship between factors and the chemical or biological response in this model is quite complex, therefore a mathematical model developed describing this relationship is not evident. As a result, this model should be improved to perform much better. The improvement could be achieved by using sequential uniform design which is based on the procedure of UD. This design is carried out by that if some good points were found through the experimental results based on UD table, the optimum experimental point is possibly just around the good ones. Then another UD is selected in the sequential procedure to investigate a new selected domain. Such a procedure can be performed continuously until the satisfactory extraction result is obtained. The sequential UD does not require any mathematical model assumption and therefore it is a suitable tool for optimizing the extraction condition.

## 5.7 Conclusion

Radix Puerariae Lobatae (P. Lobatae), a rich source of isoflavonoid, is the fresh and dried roots of plant called *Pueraria Lobata* (Wild.) Ohwi and is famous on treating cardiovascular disease that are attributed to the antioxidant activity of isoflavonoids. The optimal extraction of P. Lobatae was found based on the concentration of puerarin which is the abundant isoflavonoids components in P. Lobatae. It is not enough to establish the optimal extraction just based on the content of chemical components, the biological activity of P. Lobatae is required to take into consideration.

In this study, with the help of pseudo-level uniform design (UD), the extraction condition of P. Lobatae with five parameters including solvent ratios of methanol and water, extraction time, number of repeat of extraction, solvent volume and temperature were optimized well according to the results of contents of major four identified compounds (puerarin, daidzin, genistin and daidzein) together with the results of antioxidant activity by DPPH assay. The P. Lobatae extract obtained in group 1 has the highest content of these four isoflavonoids and gave the strongest scavenging activity. The extraction condition of group 1 was done in 180ml of 100%methanol under 50 ℃ maceration for 75min in twice.

The purpose of the use of UD employed in this work was to establish a model for optimization of extraction condition of P. Lobatae extract. This purpose was achieved by using this design. We found that this design is better than the conventional methods in optimizing the extraction condition especially in saving time and resources.

Although the optimal extraction condition was found, the results of biological response were not consistent very well with the concentrations of the four identified major isoflavonoids by HPLC. This implies that these four single components may not be actively responsible for the antioxidant activity as found in DPPH assay. Further investigations like animal test are required to do this task. Three considerations mentioned above should be necessary to be involved when we carry out further investigation. In addition, the model established by UD could be improved by sequential UD to perform much better in order to find which factors in the experimental variables play more important role in the extraction condition of P. Lobatae and hence try to establish the relationship between the parameters and response.

# Chapter 6

# Conclusion

Traditional Chinese medicine (TCM) becomes more popular in these days over the world. Thus the quality of TCM products is getting more attention. TCM is a multi-component system with components mostly unknown and very limited knowledge is available on its chemical composition and pharmacokinetics. To tackle the problem, fingerprint analysis developed in recent years may play an important role. Fingerprint with the use of the entire chromatograms of the TCM is an effective way to represent more chemical information of the system with known and unknown components detectable by the techniques used. Combination of bioassays, biological activities and chemical information can give a better quality control of the TCM product. In this research work, we explored how well chromatography coupled with bioassays performs on fingerprint analyses of TCM. Two herbs Radix Paeoniae Alba (P. Alba) and Radix Puerariae Lobatae (P. Lobatae) were investigated in this study. A brief description of these studies is given in the following paragraphs.

In Chapter 2, two hyphenated chromatographic methods used to establish chromatographic fingerprint were mentioned. They were high-performance liquid chromatographic coupled with diode-array detector (HPLC-DAD) and preparative high-performance liquid chromatographic coupled with diode-array detector (PHPLC-DAD). Besides, this chapter also discussed the uses of some assays for investigation of the biological activities of TCM. One was (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt) MTS. It was used to examine the cell proliferation activity of P. Alba. Another one was 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) for determining the scavenging activity of P. Lobatae. These chromatographic techniques and bioassays were applied successfully to

investigate the chemical compositions and biological activities of two herbs.

In Chapter 3, we reported how P. Alba was extracted by two solvents with different ratio to obtain five P. Lobatae extracts of PR0, PR25, PR50, PR75 and PR100. The chromatographic fingerprint and cell proliferation activities of these five extracts on two human hepatoma cancer cells of HepG2 and Hep3B were investigated by HPLC-DAD and MTS bioassays respectively. Qualitative and quantitative analyses of these extracts were done. We found that the number of peaks and their peak heights representing different amounts of the corresponding components in their chromatographic fingerprints varied and this observation explain why they have different cell antiproliferation activities. Moreover, three known components C1, paeoniflorin (PF) and albiflorin (AF) were found in these five extracts with different concentration. Among them, PR100 was discovered to have the highest content of C1, PF, AF and another unknown component 2. Interestingly, the strongest growth inhibitory activities on HepG2 and Hep3B cells were also observed in PR100. Therefore C1, AF, PF and component 2 were inferred to be responsible for the anticancer activities. Further investigations are required to prove these components giving the antiproliferation effects and the works done were reported in Chapter 4.

The cell antiproliferation activities of C1, AF and PF on HepG2 were examined by MTS bioassay. The MTS results show that C1 displayed a marked cell growth inhibitory activity on HepG2 cells with IC50 about 16μg/ml but AF and PF gave no significant effect in the concentration range of 12.5μg/ml to 100μg/ml. Moreover, we know that the efficacy of a TCM is not attributed by just a single component, but a group of components work together. This scenario was

observed in P. Alba extract. We found that not only C1 but also other components in PR100 contributed to the inhibitory effect and therefore PR100 was fractionated into six fractions (F1-F6) by PHPLC for further investigations. Among the six fractions, F5 exhibited the strongest growth inhibitory effect with IC50=0.06mg/ml on HepG2 cells. Surprisingly, the cell antiproliferation effect of F5 was about six time higher than that of PR100 itself. It is may be due to higher concentration of the active ingredient(s) present and / or the removal of components in other fractions that may act antagonistic interaction on the components of F5 and therefore the growth inhibitory effect of F5 was enhanced.

As TCM has been increasingly used together with western drugs as complementary and alternative medicine, therefore the chemosensitivity effects of two anticancer drugs paclitaxel (PAC) and doxorubicin (DOX) to F5 on HepG2 cells were also investigated and the results were reported in Chapter 4. The data obtained from MTS bioassays revealed that the anticancer activity of F5 remained unchanged by the concomitant of PAC but decreased with the presence of DOX.

In Chapter 5, the use of uniform design was successful to build a model for optimization of sample extraction condition of P. Lobatae. The UD established a good model in which involved twelve groups with different levels of five experimental variables solvents ratio, extraction time, number of extractions, solvent volume and temperature. The extraction condition of group 1 contained the highest amount of the four identified isoflavonoids components and exhibited the strongest antioxidant activity in DPPH assay. The extraction condition in group 1 was preformed in 180ml of 100% methanol solvent for 75minutes in

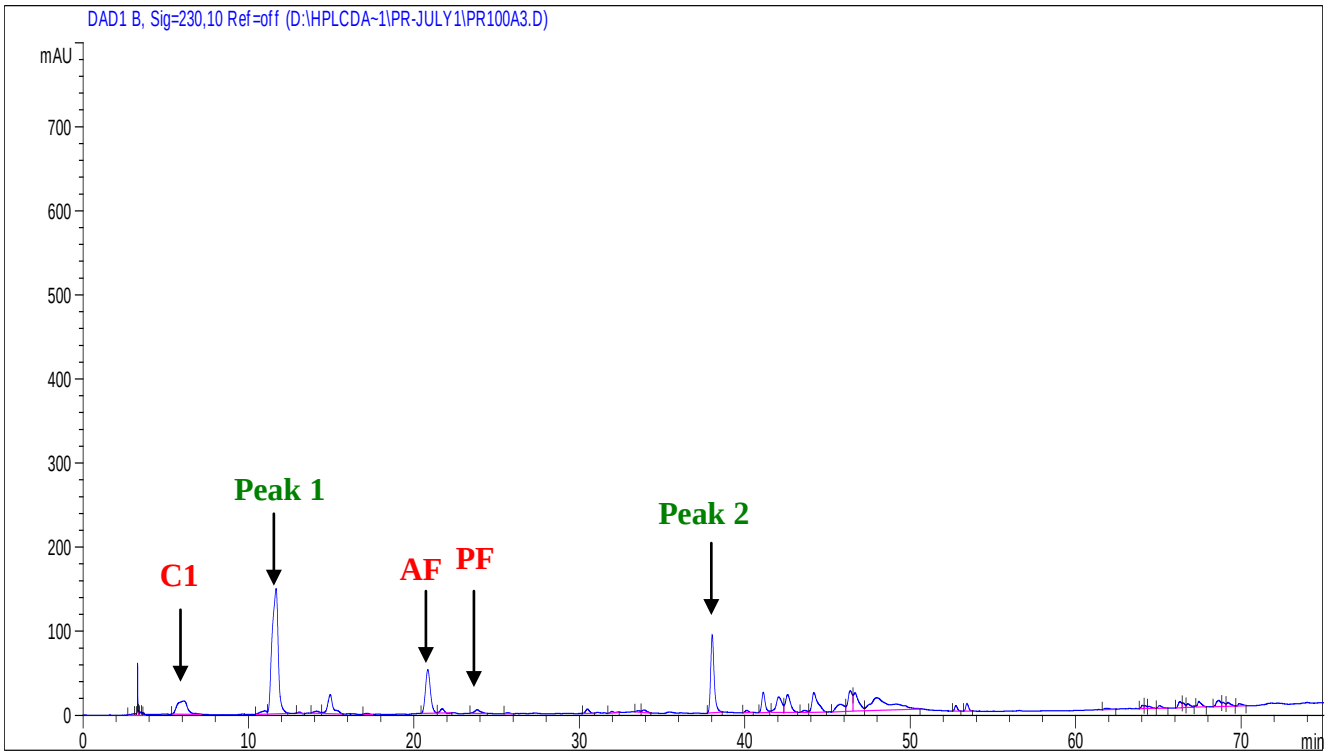
twice times under maceration at 50℃. In this chapter, we attempted to establish the relationship between the antioxidant activities and these four identified isoflavonoids components. However, the results of antioxidant activity measured by DPPH assay were not consistent with the concentration of these four components and hence this relationship has not been established successfully. Further investigations are required and some works are undergoing.

In conclusion, chemical and biological studies of the complicated multi-components system like TCMs were found to improve by using chromatographic fingerprint techniques combined with the inclusion bioassays. In this research work, Radix Paeoniae Alba and Radix Puerariae Lobatae were analyzed with good performance by using this combined approach. The results obtained are found to be useful and reliable for quality control. Yet, more detailed investigations are needed

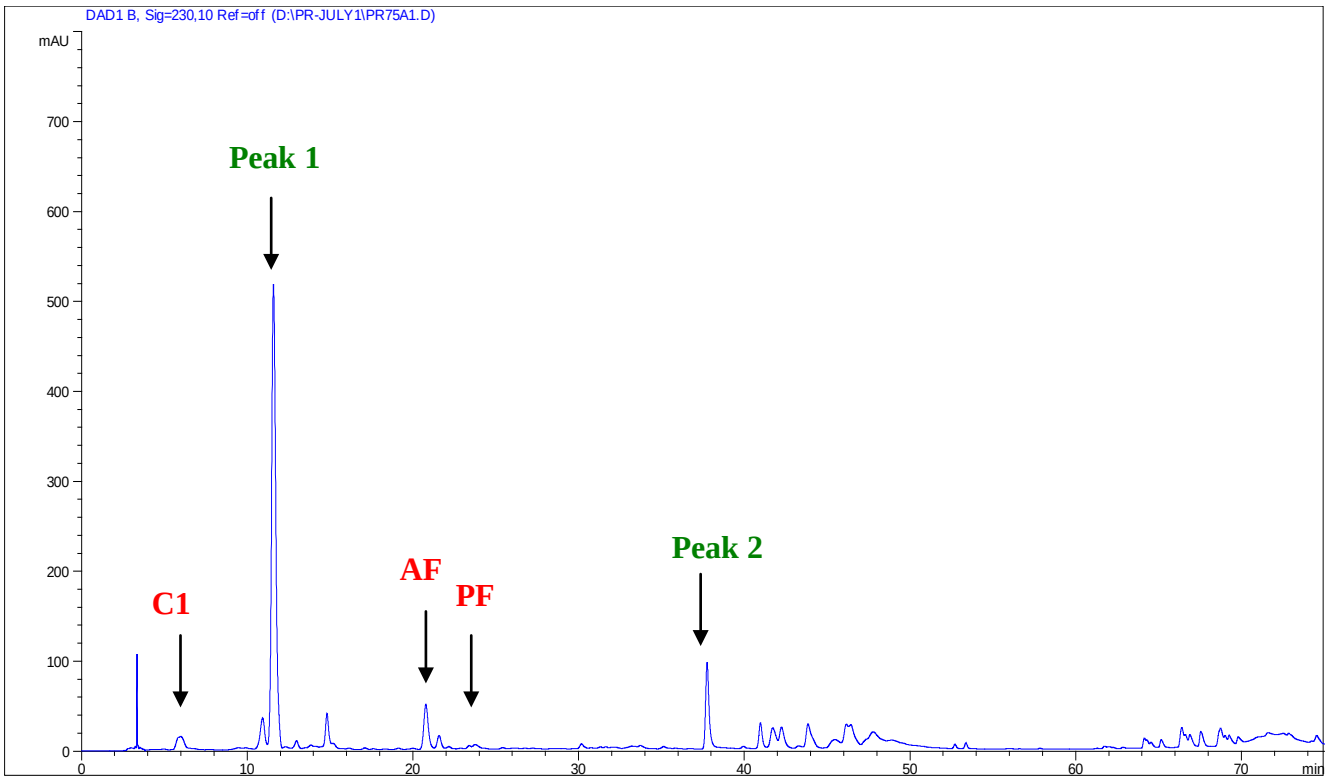
# Appendices

**Appendix 3.1 HPLC chromatographic fingerprints of the five extracts of P. Alba in the concentration of 5mg/ml at 230nm**

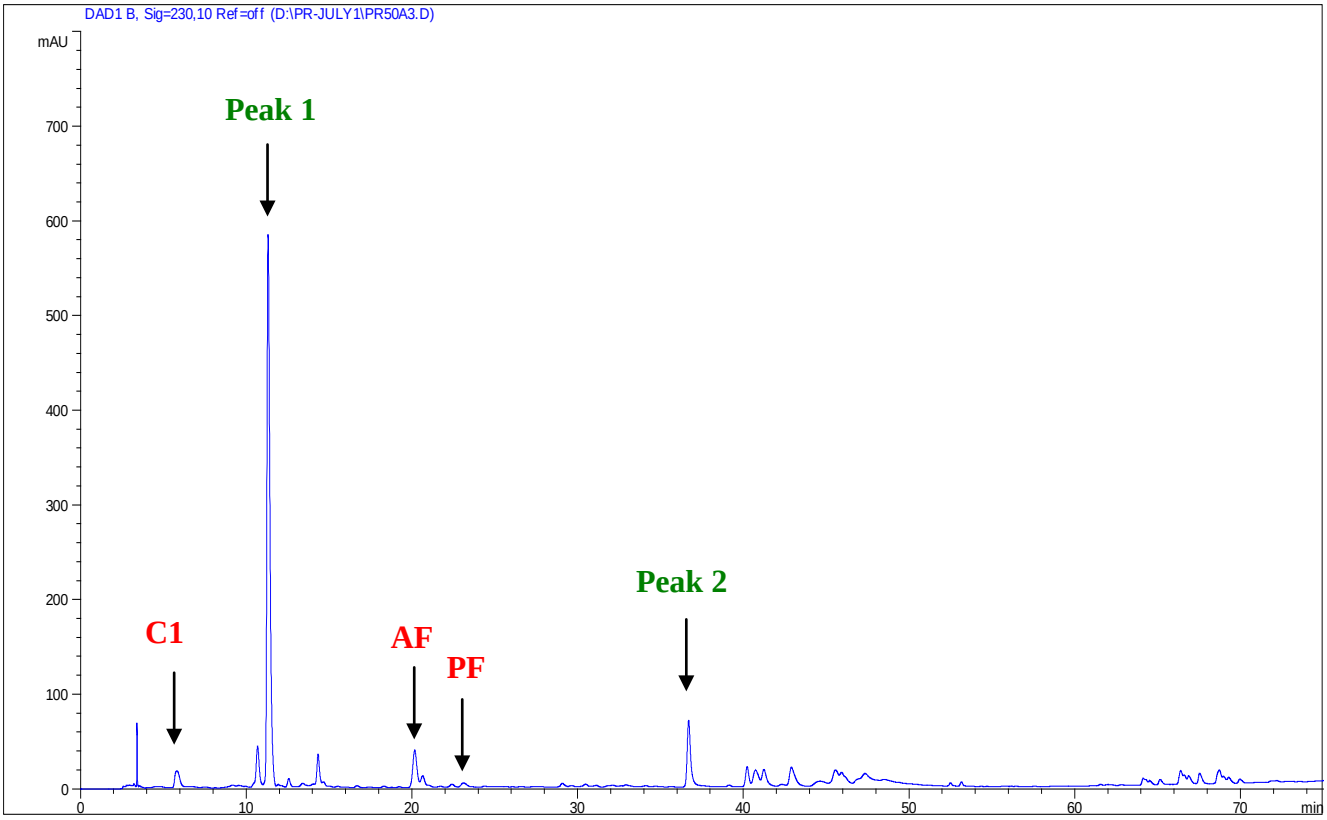
HPLC chromatographic fingerprint of PR100



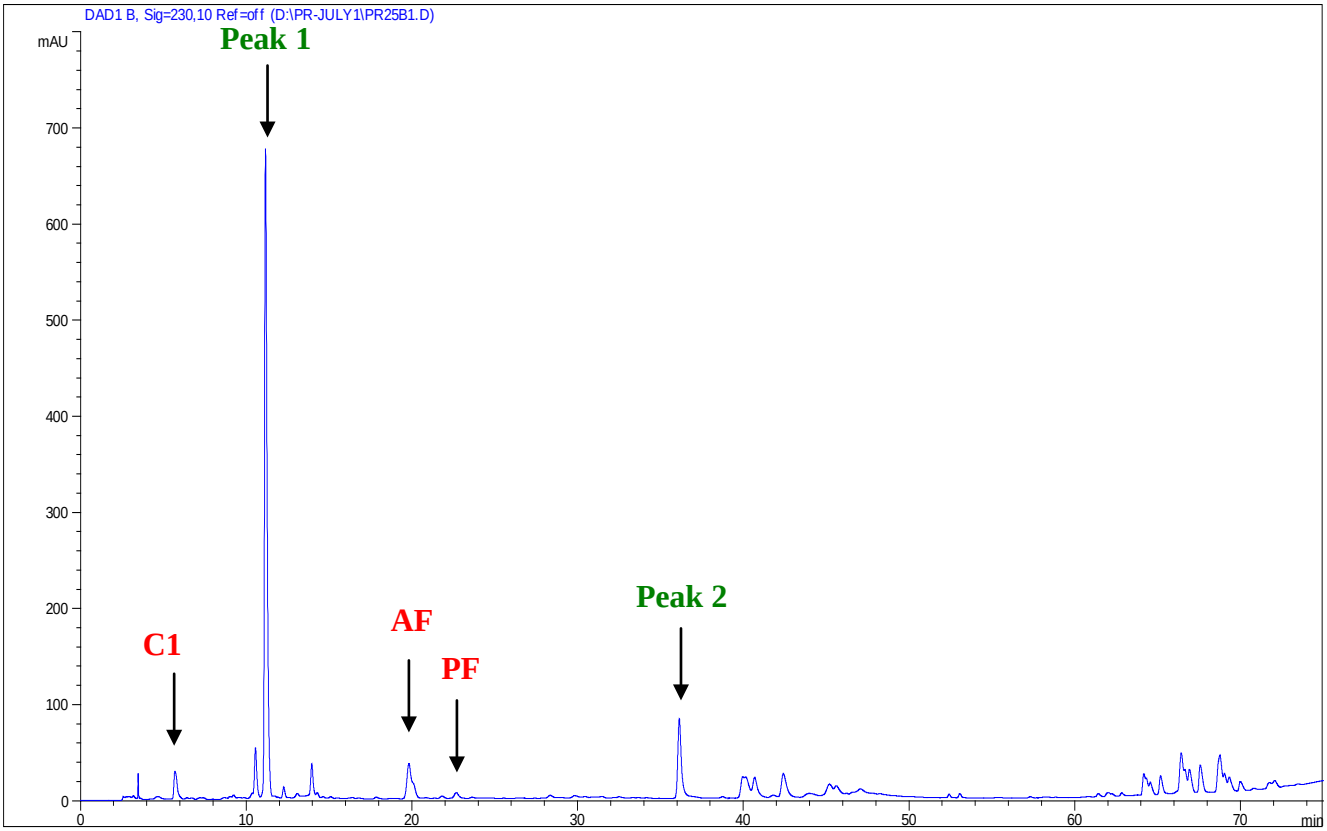

HPLC chromatographic fingerprint of PR75



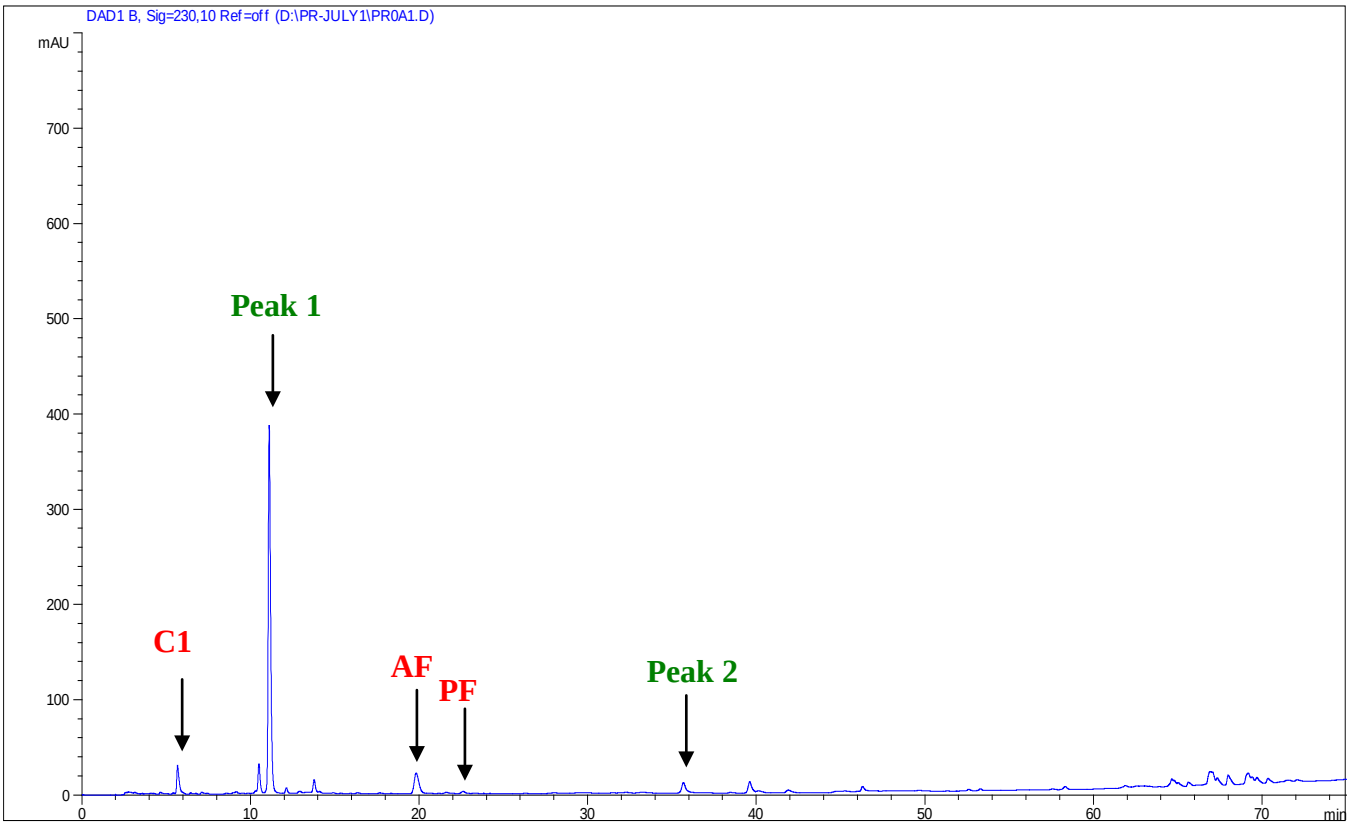
HPLC chromatographic fingerprint of PR50



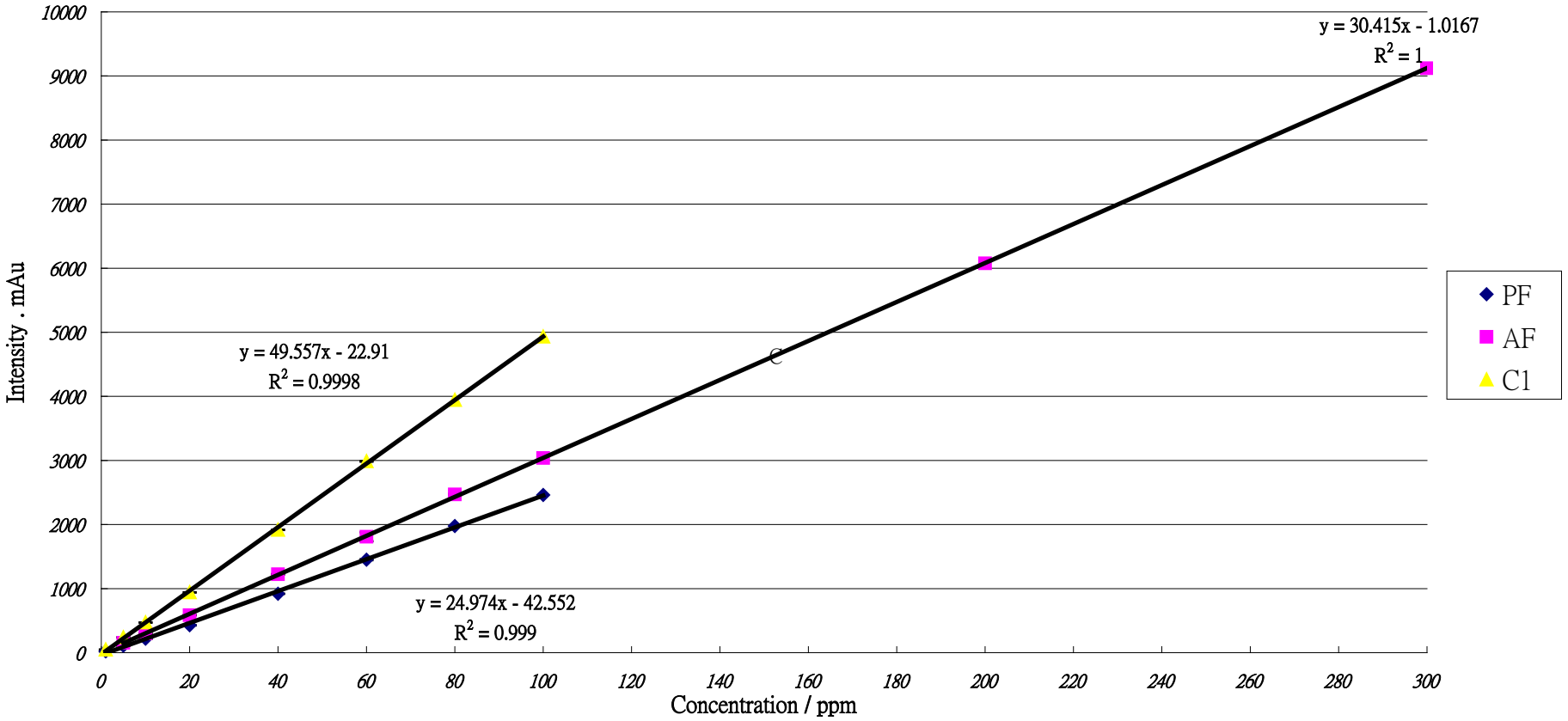

HPLC chromatographic fingerprint of PR25



HPLC chromatographic fingerprint of PR0

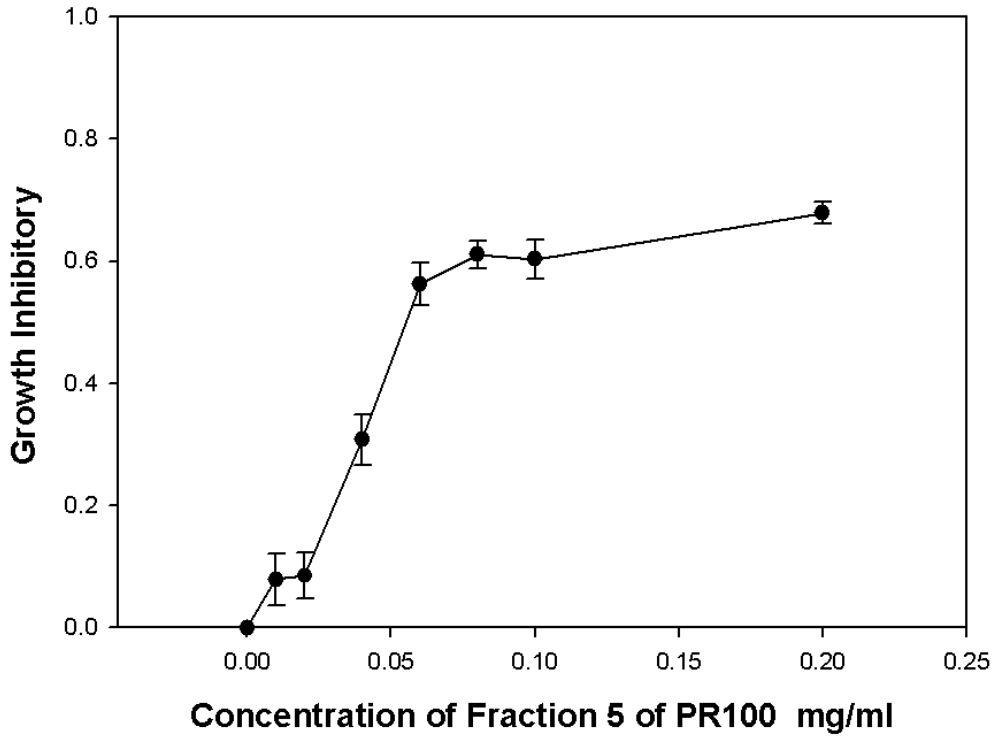


**Appendix 3.2 The calibration plot of the three standards (C1, albiflorin, paeoniflorin)**

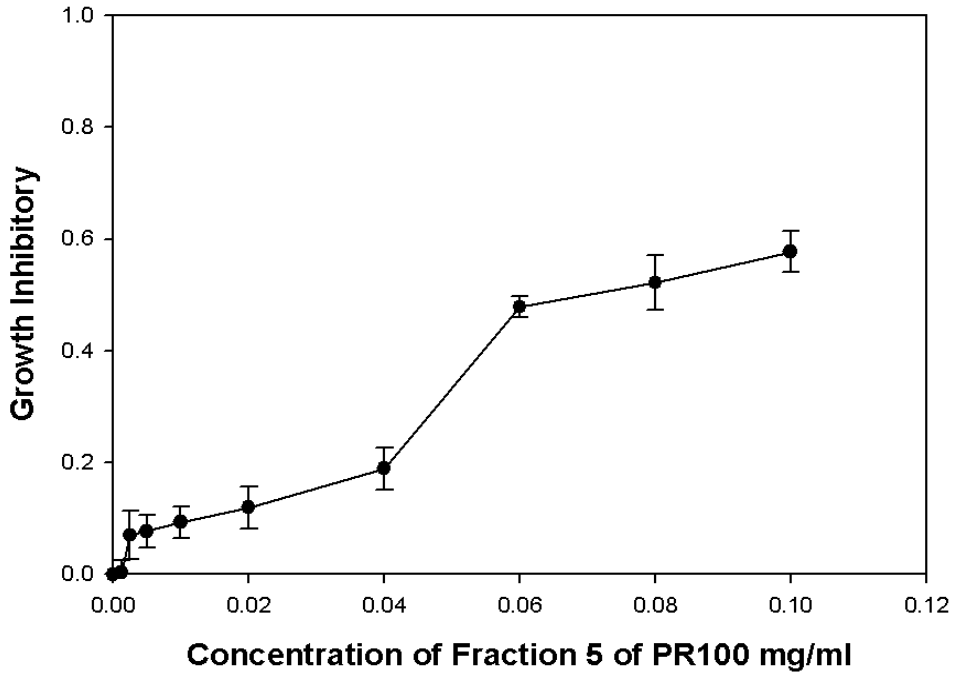


## Appendix 4.1 The antiproliferation effect of F5 on HepG2 and WRL-68 cells

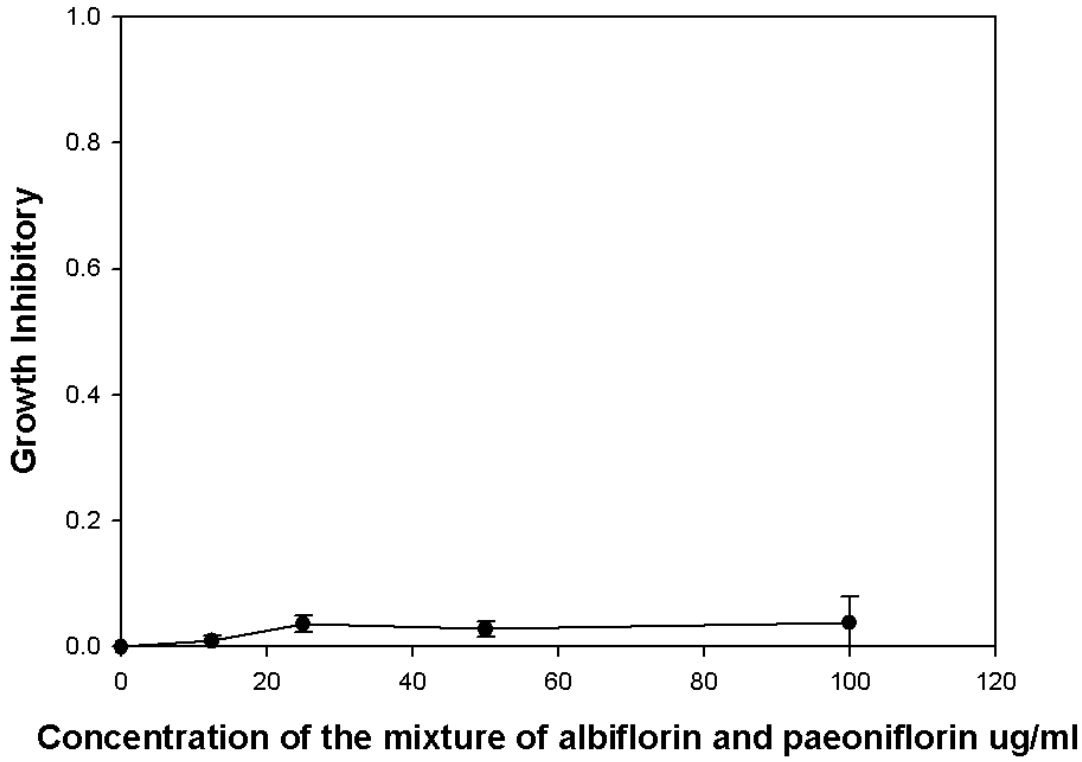
The antiproliferation effect of F5 (in the concentration range of 0.0125-0.2mg/ml) on HepG2 cells




The antiproliferation effect of F5 (in the concentration range of 0.0125-0.1mg/ml)on WRL-68 cells



## Appendix 4.2 The antiproliferation effect of the mixture of albiflorin and paeoniflorin on HepG2 cells



## Appendix 4.3 % of peak area and % mass of the six fractions

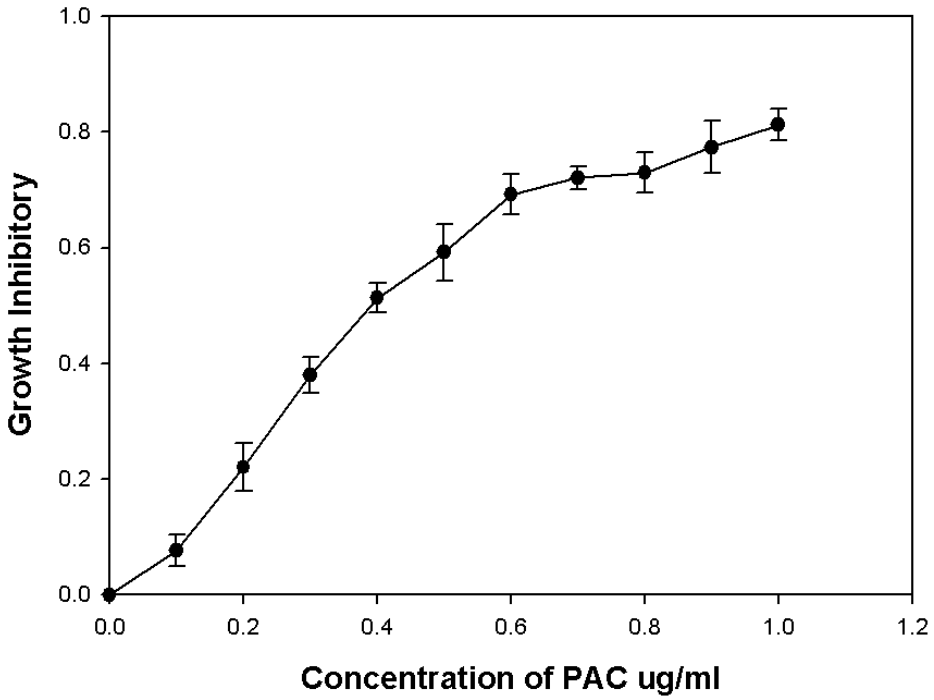
| | Percentage of peak area at 230nm* | Percentage mass** |
|---|---|---|
| **F1** | 36.56±1.61 | 78.05 |
| **F2** | 2.55±0.05 | 2.86 |
| **F3** | 3.75±0.04 | 0.95 |
| **F4** | 9.26±0.21 | 3.90 |
| **F5** | 40.19±1.18 | 7.58 |
| **F6** | 7.68±0.16 | 6.66 |

* % peak area = summation of peak area of a fraction / summation of peak area of the six fractions

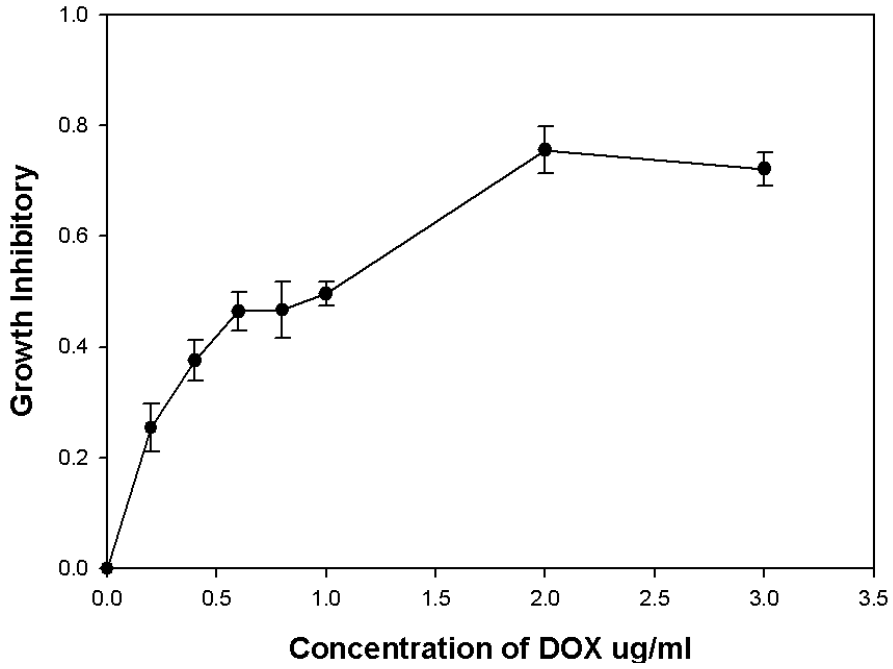
* * % of mass = dry mass of a fraction / total dry mass of the six fraction

## Appendix 4.4 The antiproliferation effect of paclitaxel (PAC) and doxorubicin (DOX) on HepG2 cells

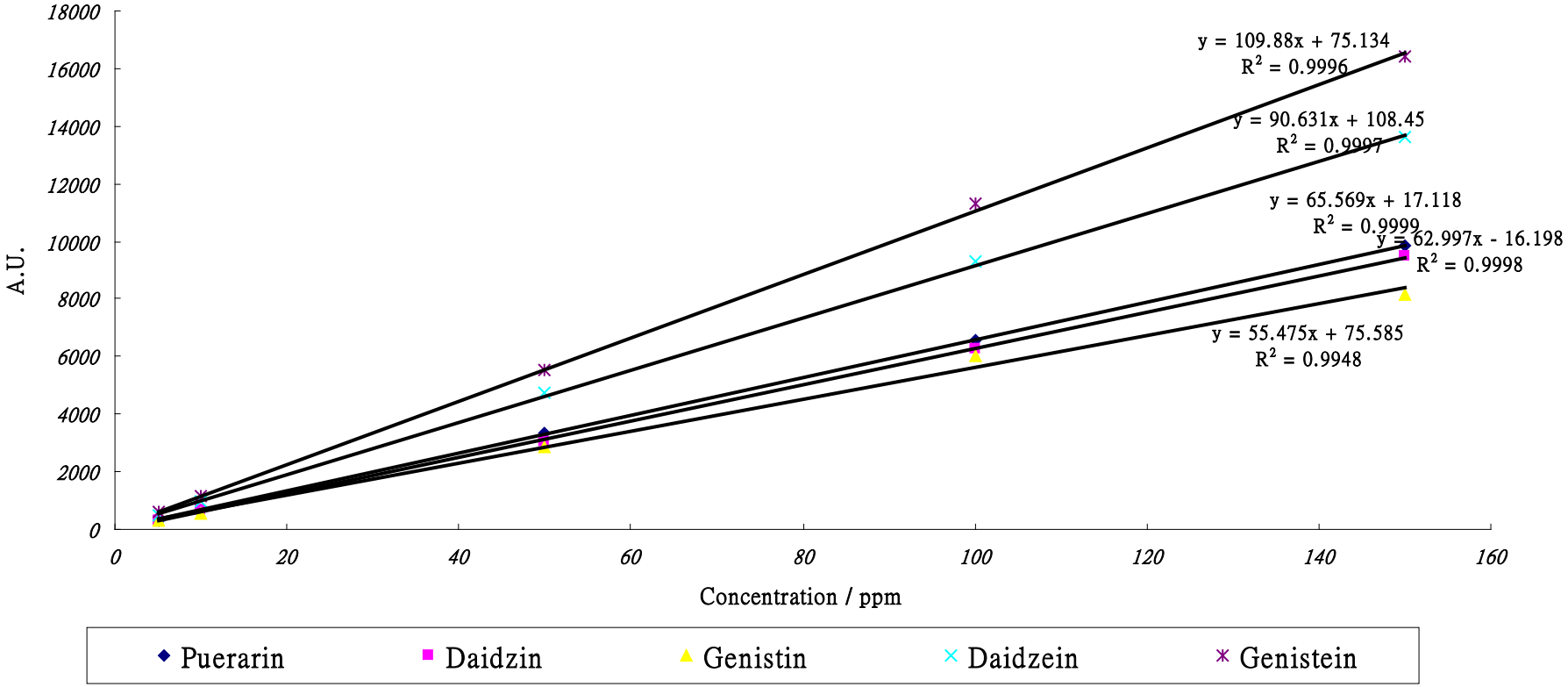
The antiproliferation effect of paclitaxel (in concentration range of 0.1-1.0ug/ml) on HepG2 cells



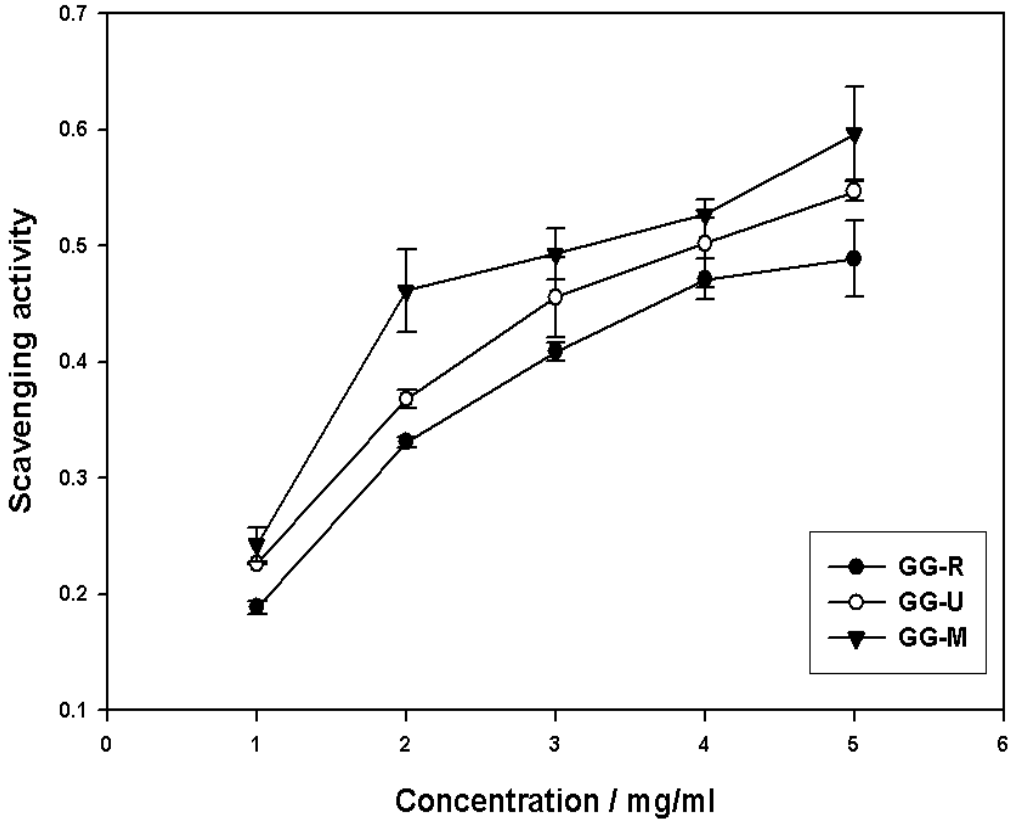

The antiproliferation effect of doxorubicin (in the concentration range of 0.2-3.0ug/ml) on HepG2 cells



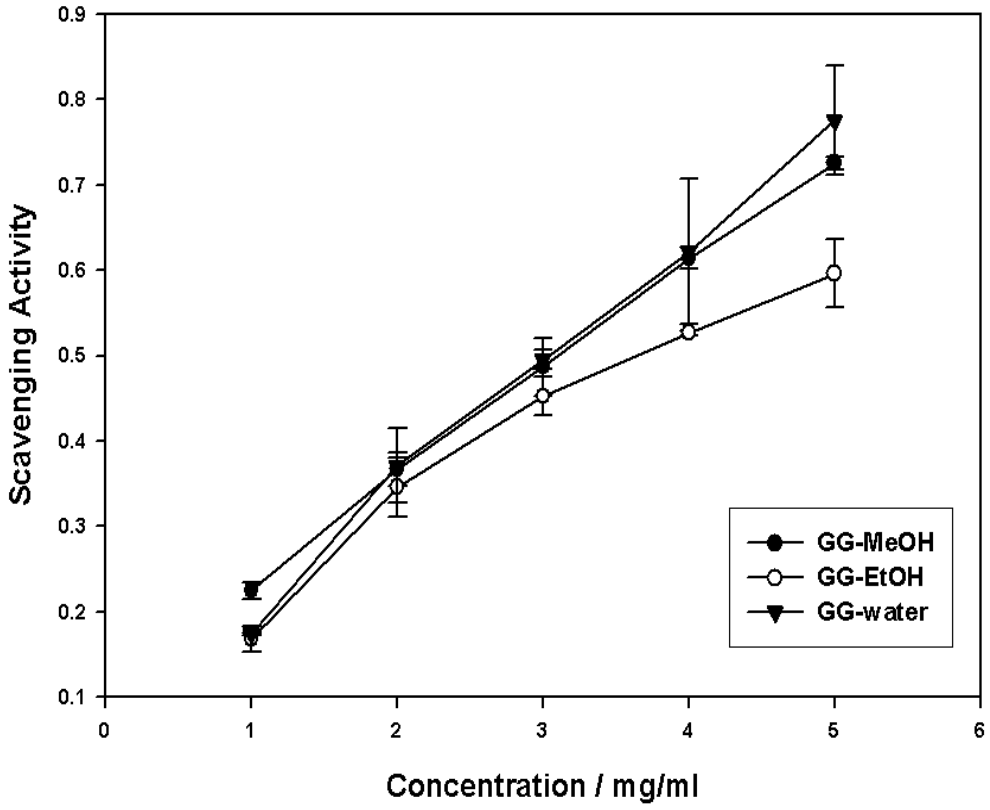
**Appendix 5.1 The calibration plot of the five standards (puerarin PU, daidzin DA, genistin GN, daidzein DE and genistein GE) in the concentration range 5-150ppm at 254nm**



**Appendix 5.2 The scavenging curves of three extracts obtained of P. Lobatae by reflux (GG-R), ultrasound (GG-U) and maceration (GG-M) in DPPH assay**



**Appendix 5.3 The scavenging curves of three extracts obtained of P. Lobatae by methanol (GG-MeOH), ethanol (GG-EtOH) and water (GG-water) in DPPH assay**



**Appendix 5.4 The concentration of puerarin, daidzin, genistin and daidzein and the antioxidant activities by DPPH assay in P. Lobatae extract of group 1 to group 12**

| Group | Concentration of isoflavonoids / ppm[a] | | | | Antioxidant activity |
|---|---|---|---|---|---|
| | Puerarin | Daidzin | Genistin | Daidzein | SRmax[b] |
| **1** | 169±7 | 20±0 | 18±0 | 2.6±0.1 | 0.76±0.02 |
| **2** | 147±1 | 17±0 | 16±0 | 2.0±0 | 0.71±0.05 |
| **3** | 135±1 | 17±1 | 11±1 | 1.8±0.1 | 0.62±0.03 |
| **4** | 102±0 | 11±0 | 11±0 | 0.6±0 | 0.75±0.03 |
| **5** | 103±1 | 13±1 | 10±0 | 1.1±0 | 0.71±0.05 |
| **6** | 114±1 | 13±0 | 10±1 | 1.1±0 | 0.52±0.02 |
| **7** | 108±3 | 13±1 | 9±0 | 1±0.1 | 0.58±0.01 |
| **8** | 120±1 | 15±1 | 13±1 | 1.4±0.1 | 0.65±0.03 |
| **9** | 145±2 | 18±0 | 14±1 | 2.0±0.1 | 0.63±0.01 |
| **10** | 110±1 | 11±1 | 11±0 | 1.5±0.2 | 0.60±0.01 |
| **11** | 139±2 | 19±1 | 12±1 | 2.0±0.1 | 0.67±0.01 |
| **12** | 97±0 | 11±0 | 10±1 | 0.7±0.1 | 0.62±0.02 |

[a] 1mg/ml of P. Lobatae extracts were injected into HPLC. The concentrations of each isoflavonoids were obtained from successive three injections, expressed in mean± SD (n=3)

[b] SRmax value of 5mg/ml P. Lobatae extract was expressed in term of SRmax ± SD (n=3)

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# PUBLICATIONS AND PRESENTATIONS

## Journal articles

1. Chui-yee Cheung and Foo-tim Chau, 'Finding bioactive components and fractions of Radix Paeoniae Alba on human hepatoma cancer cells', under preparation.

2. Chui-yee Cheung, Cheng-jian Xu, Foo-tim Chau, 'Optimization of extraction condition of Radix Pueraria Lobatae by Uniform Design', under preparation.

## Conference/ Symposium Presentations

1. Chui-yee Cheung and Foo-tim Chau, 'Finding bioactive components of Radix Paeoniae Alba for liver cancer', Poster presentation in the Twelfth Symposium on Chemistry Postgraduate Research in the City University of Hong Kong, Hong Kong, 23 April 2005.

2. Chui-yee Cheung and Foo-tim Chau, 'Finding bioactive components of Radix Paeoniae Alba for liver cancer', Poster presentation in 2005 Hong Kong – Macau Postgraduate Symposium on Chinese Medicine, Hong Kong, 12 August 2005.

3. Chui-yee Cheung and Foo-tim Chau, 'The study of Radix Paeoniae Alba extracts and their fractions on human hepatoma cell lines' Poster presentation in The Hong Kong University of Science and Technology, Hong Kong, 22 April 2006.

4. Chui-yee Cheung and Foo-tim Chau, 'Effects of concentrated extract of Radix Paeoniae Alba on proliferation of human hepatoma cell line and on the sensitivity to western drugs', Poster presentation in the 26th International Symposium on Chromatography , Copenhagen, Denmark, 21-25 August 2006

## Course Attendance

1. 'TCM Fingerprint Techniques and Chemometrics', offered by Prof. F.T. Chau, Prof. O. Kvalheim, Prof. Y.Z. Liang and Prof. P.S. Xie. A course offered in the Hong Kong Polytechnic Univerisity, 22 February 2006.

2. 'Liquid-phase Microextraction Fundamentals, recent developments and practical implementation' offered by Stig Pedersen-Bjergaard and Kunt Rasmussen. A course offered in 26th International Symposium on Chromatography, Copenhagen, Denmark, 21 August 2006.