MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN

Faculty of Chemical and Biopharmaceutical Technologies
Department of Industrial Pharmacy

Master's thesis

on the topic STUDY ON THE BIO-ACTIVE COMPOUNDS OF A CHINESE TRADITIONAL MEDICINE SPERANSKIA TUBERCULATA (BUNGE) BAILL

Completed: student of the group MPhch-20

of the speciality 226 pharmacy, industrial pharmacy

(code and name of the speciality)

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ASSIGNMENTS FOR THE MASTER'S THESIS

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6. Consultants of the master's thesis sections

Section	Surname, initials and positionof the consultant	Signature, date	
		the task wasissued	the task accepted
Section 1	Tetiana Derkach, Professor, ScD		Zeyuan Sun 24.09.21
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Summary

Sun, Zeyuan. "Study on the bio-active compounds of a Chinese Traditional Medicine Speranskia tuberculata (Bunge) Baill". – Manuscript.

Master's thesis on the speciality 226 – pharmacy, industrial pharmacy. – Kyiv National University of Technologies and Design, Kyiv, 2021.

The master's thesis is devoted to extracting the bioactive fractions of Speranskia tuberculata (Bunge) Baill and purifying the extracts by isolation. The extracts obtained are used in cellular experiments to explore their positive effects in preventing and treating atherosclerosis, liver cancer, cervical cancer, and melanoma. This research verifies that the active compounds in Speranskia tuberculata (Bunge) Baill have antiatherosclerotic and therapeutic effects on some cancers. The crude extracts of ethyl acetate had a significant ability to kill tumour cells. It was determined that the crude extracts of petroleum ether (PE) effectively inhibited the transition from macrophages to foam cells by the traditional method of labelling foam cells with neutral fat. The uptake and efflux of cholesterol by macrophages were characterized by fluorescently labelling cholesterol flow. The results showed that PE minimized cholesterol deposition in macrophages by inhibiting macrophage phagocytosis of cholesterol while promoting the dual effect of intracellular cholesterol efflux.

Keywords: Speranskia tuberculata (Bunge) Baill; Active compounds; Tumours; Atherosclerosis; MTT Cell Cytotoxicity Test; Oil red O staining

Аннотація

Сан, Ціуан. «Дослідження біологічно активних сполук китайської традиційної медицини Speranskia tuberculata (Bunge) Baill». – Рукопис.

Магістерська робота за спеціальністю 226 – фармація, промислова фармація. – Київський національний університет технологій та дизайну, Київ, 2021.

Магістерська робота присвячена екстрагуванню біоактивних фракцій Speranskia tuberculata (Bunge) Baill та очищенню екстрактів шляхом виділення. Отримані екстракти використовуються в клітинних експериментах для дослідження їх позитивного впливу на профілактику та лікування атеросклерозу, раку печінки, раку шийки матки та меланоми. Це дослідження підтверджує, що активні сполуки Speranskia tuberculata (Bunge) Baill мають антиатеросклеротичний та терапевтичний вплив на деякі види раку. Неочищені екстракти етилацетату мали значну здатність вбивати пухлинні клітини. Встановлено, що неочищені екстракти петролейного ефіру ефективно інгібують перехід від макрофагів до пінистих клітин традиційним методом мічення пінистих клітин нейтральним жиром. Поглинання та відтік холестерину макрофагами характеризувався флуоресцентним міченим потоком холестерину. Результати показали, що екстракт у петролейному ефірі мінімізував відкладення холестерину в макрофагах, пригнічуючи макрофагальний фагоцитоз холестерину, одночасно сприяючи подвійному ефекту внутрішньоклітинного витоку холестерину.

Ключові слова: Speranskia tuberculata (Bunge) Baill; біологічно-активні сполуки; пухлини; атеросклероз; МТТ тест на цитотоксичність клітин; забарвлення олійним червоним О

Аннотация

Сан, Циуан. Исследование биологически активных соединений китайской традиционной медицины Speranskia tuberculata (Bunge) Baill. – Рукопись.

Магистерская работа по специальности 226 – фармация, промышленная фармация. – Киевский национальный университет технологий и дизайна, Киев, 2021.

Магистерская работа посвящена экстрагированию биоактивных фракций Speranskia tuberculata (Bunge) Baill и очистке экстрактов путем выделения. Полученные экстракты используются в клеточных экспериментах для исследования их положительного воздействия на профилактику и лечение атеросклероза, рака печени, рака шейки матки и меланомы. Это исследование подтверждает, что активные соединения Speranskia tuberculata (Bunge) Baill оказывают антиатеросклеротическое и терапевтическое влияние на некоторые виды рака. Неочищенные экстракты этилацетата обладали значительной способностью убивать опухолевые клетки. Установлено, что неочищенные экстракты петролейного эфира эффективно ингибируют переход от макрофагов к пенистым клеткам традиционным методом мечения пенистых клеток нейтральным жиром. Поглощение и отток холестерина макрофагами характеризовался флуоресцентным меченым потоком холестерина. Результаты показали, что экстракт в петролейном эфире минимизировал отложения холестерина в макрофагах, подавляя макрофагальный фагоцитоз холестерина, одновременно способствуя двойному эффекту внутриклеточной утечки холестерина.

Ключевые слова: Speranskia tuberculata (Bunge) Baill; биологически активные соединения; опухоли; атеросклероз; МТТ тест на цитотоксичность клеток; окрас масляным красным О

List of abbreviations

ABCA1	-	ATP-binding Cassette Transporter A1
ABCG1	-	ATP-binding Cassette Transporter G1
ApoA-I	-	ApolipoproteinA-I
AS	-	Atherosclerosis
CA	-	Caffeic Acid
Caspase-1	-	Cysteine-requiring Aspartate Protease-1
CE	-	Cholesterol Ester
Dil-oxLDL	-	Dil labelled Oxidized Low-Density Lipoprotein
DMSO	-	Dimethyl Sulfoxide
DPPH	-	1,1-diphenyl-2-picrylhydrazyl
FC	-	Free Cholesterol
MTT	-	Methyl Thiazolyl Tetrazolium
ox-LDL	-	Oxidized Low-Density Lipoprotein
PBS	-	Phosphate buffer solution
PC	-	Positive control
PE	-	Crude Extracts of Petroleum Ether
QTOF-LC/MS	-	Quadrupole time-of-flight tandem liquid-chromatograph/mass-spectrometer
ROS	-	Reactive oxygen species
SDS	-	Sodium dodecyl sulfate
SR-BI	-	Scavenger receptor class B type I
TC	-	Total Cholesterol
TG	-	Total Triglyceride

Meetings attended and articles published:

1. Zeyuan Sun, Tao Wu, Shu Xing, Mingyang Zhou, T. M. Derkach. Antitumor: Effect of Bioactive Components of Speranskia Tuberculata (Bunge) Baill. In: Physical-Organic Chemistry, Pharmacology and Pharmaceutical Technology of Biologically Active Substances. 2021, Issue 4 (accepted for publication).

2. Zeyuan Sun, Tao Wu, Shu Xing, Mingyang Zhou, T. M. Derkach. Anti-hepatocellular Carcinoma: Effect of Bioactive Components of Speranskia Tuberculata (Bunge) Baill. II All-Ukrainian Conference “Innovation in Education, Science and Business: Challenges and Opportunities”. November 18, 2021, Kyiv, Ukraine.

CONTENT

INTRODUCTION

The relevance of the topic. Atherosclerosis is a cardiovascular disease with abnormal lipid metabolism and inflammatory response. It mainly occurs in large and medium-sized arteries and the cardiovascular system. It has a high mortality and disability rate. It has become a high-risk disease as dangerous as degenerative brain diseases and malignant tumours. In the past hundred years, medical researchers have never stopped exploring the treatment of cancer and atherosclerosis. At the same time, traditional Chinese herbal medicine can be directly used as an additive to prevent or treat various diseases because of its remarkable comprehensive therapeutic effect and fewer side effects. However, due to the diversity of components and complex mechanism of Chinese herbal medicine, its application is limited. Therefore, separating, extracting and purifying the active ingredients of Chinese herbal medicine to verify its efficacy has become an urgent problem to be solved.

Some studies have confirmed the efficacy of herbal extracts of Ginkgo, Salvia, Chuanqiong, and Apocynum venetum in preventing and treating atherosclerosis. Many active compounds have also been reported to have preventive and therapeutic effects against atherosclerosis. Sara [1] et al. found that virgin olive oil can have antiatherosclerotic effects by increasing high-density lipoprotein (HDL) levels and promoting cholesterol efflux. In addition, the active ingredients in herbs have excellent results in the prevention and treatment of some cancers. Huimin [3] et al. showed that sinomenine, extracted from Qingfengteng, had an inhibitory effect on the proliferation of IGROV1 and HeyA8 cells from human ovarian cancer. It also inhibited cell invasion and metastasis through oncogene McM2 and Wnt/β-catenin signalling pathway. The higher the dose, the stronger the inhibitory effect was.

Speranskia tuberculata (Bunge) Baill is a perennial herb. The chemical constituents in Speranskia tuberculata (Bunge) Baill are mainly alkaloids, organic acids, sterols, pyrimidines, progesterones and volatile oils. There are a large number of compounds present in it. Many of these compounds have excellent antioxidant and atherosclerotic effects. The following constituents have been reported in Speranskia

tuberculata (Bunge) Baill. Fan Yunbai [5] et al. found that nine compounds were isolated and identified from the leaves of Speranskia tuberculata (Bunge) Baill. The identification was confirmed as triacontanol palmitic acid, p-coumaric acid, ferulic acid, thymine and uracil. Among them, ferulic acid and vanillic acid have relatively good antioxidant potential. By extracting and crushing the whole herb of dried garden balsam stem, Gao Haixiang [6] et al. obtained fatty acid compounds with high content, accounting for 27.56% of the total volatile oil content and containing terpenoids. Hou Dongyan [7] et al. obtained volatile substances by distillation extraction of Speranskia tuberculata (Bunge) Baill. The volatile oil content was 1.06%, and the volatile oil composition identified terpenoids, alcohols, aldehydes, ketones, phenols, acids, oxygenates, and other compounds.

The purpose of the study. Tumours and cardiovascular diseases caused by atherosclerosis are the two top killers of human beings. Chinese herbal medicines have very excellent roles in the treatment of both diseases. However, herbal medicines have little international academic recognition, inhibiting their application because of their diverse components and complex mechanisms.

The active compounds soluble in petroleum ether and ethyl acetate of the Speranskia tuberculata (Bunge) Baill were tested in our laboratory concerning their antiatherosclerotic and anticancer effects respectively.

In this study, firstly, we extracted the bio-active fraction of Speranskia tuberculata (Bunge) Baill, then purified the extracts by isolation and used them in cellular experiments to explore their positive effects in the prevention and treatment of atherosclerosis, liver cancer, cervical cancer and melanoma diseases.

The research objectives of the study. This research verifies that the active compounds in garden balsam stems have antiatherosclerotic and therapeutic effects on some cancers. Following the objectives, the following tasks were developed:

1. Extraction of a bio-active fraction of Speranskia tuberculata (Bunge) Baill.
2. Isolation and purification of active compounds.
3. Screening and efficacy evaluation of anticancer compounds by MTT Cell

Cytotoxicity Test and antiatherosclerotic compounds by oil red O staining.

Research methods.

1. Extraction and isolation of active compounds from Speranskia tuberculata (Bunge) Baill. The powdered Speranskia tuberculata (Bunge) Baill was extracted by reflux using 95% ethanol solution so that most of the compounds were dissolved in the ethanol solution. The extracts were separated by polar extraction. The crude extract solution obtained by rotary evaporation was evaporated from the ethanol solution and then separated by extraction using four different polar solvents (petroleum ether, ethyl acetate, n-butanol and water) in turn. After that, the extracts of the four polar parts were concentrated by spin evaporation.

2. Chromatographic column separation and purification. The four extracts of different polarities were firstly separated using silica gel column and afterwards further separated by using gel column according to the molecular weight of the compounds. Finally, the extracts with higher purity were obtained.

3. Anticancer activity screening (compounds in ethyl acetate extracts). The method was MTT Cell Cytotoxicity Test. The cells used were: endothelial cells and macrophages for atherosclerosis, HEPG2 cells for liver cancer; Hela cells for cervical cancer; A375 cells for melanoma.

4. Anti-atherosclerosis (compounds in petroleum ether extract). In vitro LDL oxidation inhibition and macrophage lipid phagocytosis tests were used.

Practical value. Chinese herbal medicine is an integral part of Chinese pharmaceutical treasure, and it plays a vital role in developing the Chinese population, health and pharmaceutical industry. With the progress of science and the enhancement of human self-care, consciousness is rising day by day—countries worldwide focus on researching natural drugs. Therefore, drugs with Chinese herbal extracts as raw materials are increasingly favoured by governments worldwide. The extraction, separation and efficacy test of active components in Chinese herbal medicines have become a critical significance of this study.

CHAPTER 1. LITERATURE REVIEW

Speranskia tuberculata (Bunge) Baill is a perennial herb. It is one of the standard clinical Chinese medicines [8], with a long history of medicinal use. It is mainly found in Gansu, Heilongjiang, Jilin, etc. The whole herb can be used as medicine. The main effects include antioxidant, anti-inflammatory, anti-tumour, heat-clearing and blood-activating [9-11]. The active compounds in Speranskia tuberculata (Bunge) Baill dissolved in petroleum ether; ethyl acetate was previously revealed in our laboratory to have positive effects against atherosclerosis and cancer, respectively. As a traditional herbal medicine, it can be directly incorporated as an additive in preventing or treating various diseases due to its combined therapeutic and low side effects. However, the application of Speranskia tuberculata (Bunge) Baill is primarily limited by its diverse composition and complex mechanism. Therefore, it has become an urgent problem to isolate, extract and purify the active ingredients in Speranskia tuberculata (Bunge) Baill to verify their efficacy. This chapter first introduces Speranskia tuberculata (Bunge) Baill and its classification and then lists the extraction, isolation, and purification of the active ingredients of herbal medicines. Then, the chemical composition of Speranskia tuberculata (Bunge) Baill, confirmed by research, is summarised. Finally, the pathological mechanisms of the two diseases studied in this topic, tumours and atherosclerosis, and their therapeutic drugs are presented.

1.1 Classification of Speranskia tuberculata (Bunge) Baill and its efficacy profile

Speranskia tuberculata (Bunge) Baill, as a clinically used herbal medicine, is widely available and less expensive. Five species of Speranskia tuberculata (Bunge) Baill most occur in China: Herba Speranskiae Tuberculatae, used in the northwest and north China and Henan and Shandong. Impatiens balsamina L., used in East China and Guangxi, Xinjiang, etc. Several species of wild peas in the genus Leguminosae (Vicia amoena Fisch.) are often used in north-eastern China. Incarrillea

sinensis Lam is used primarily in Liaoning, Jilin, etc. Several species of plants of the genus Gaultheria L. (Small tougucao) in the Rhododendron family are used in southwest China, including Yunnan, Guizhou, and Sichuan.

1.1.1 Herba Speranskiae tuberculatae

Herba Speranskiae Tuberculatae is derived from the whole dried herb of Euphorbiaceae, and its main chemical components are alkaloids and organic acids. Herba Speranskiae Tuberculatae leaves are mostly curled and crinkled grey-green, grey-white pilose, with rounded florets and fruits on the inflorescence. The whole dried herb is aromatic and has low toxicity. Its roots and stalks help prevent wind-cold dampness, dispelling wind and dampness, detoxifying and relieving pain, and also for rheumatic joint pain [13].

1.1.2 Incarrillea sinensis Lam

Incarrillea sinensis Lam is derived from the dried whole grass of Artemisia angustifolia, family Zweiwei, with a flat round crutch-shaped stem, with branches. The plant is 10-95 cm in length, 2-7 mm in diameter, up to 10 mm in diameter in the lower part. The surface is light green to yellowish-green, with fine longitudinal lines, glabrous. Light and brittle, easily fractured, yellowish-white in section. Hairs are spiny, pith white. The upper end of the stem is sometimes with a racemose arrangement of fruits, ramshaped, 4-10 cm long, 4-6 mm in diameter. Most seeds are flat, without smell, taste bland and slightly bitter. It is mainly produced in Shandong, Heilongjiang, Jilin, and Liaoning in China. It grows wild on mountain slopes, hills and grasses and is cold-resistant. It can disperse wind, dispel dampness, and detoxify and relieve pain [14].

1.1.3 Impatieans balsamina L.

Impatiens balsamina L. is obtained by cutting the plant's above-ground parts in summer and autumn when growing luxuriantly, by removing the leaves and flowers and fruits, washing and drying them. It can dispel wind and dampness, invigorate blood and detoxify the blood, and is mainly used to treat rheumatism and paralysis,

bruises and swelling pain, and snake and insect bites [13].

1.1.4 Small tougucao

Small tougucao comes from the Rhododendron family. The corolla is often white, light green or pink, bell-shaped or altar-shaped, the fruit is subglobose, berry-like, usually black to blue; there are about 130 species worldwide. When crushed, part of the whole plant will emit a strong odour of holy oil. It has the efficacy of clearing heat and detoxifying, activating blood circulation, dispelling wind and dampness, and relieving gas and asthma. It is mainly used to treat rheumatoid arthritis and the treatment of wind chill, cough, asthma, etc. It can also be used externally to treat skin eczema [14].

1.1.5 Vicia amoena Fisch.

Vicia amoena Fisch. is derived from the legume family with a quadrangular stem. The leaves are doubly pinnate, mostly convoluted and crinkled. Flowers are blue or purple, occasionally with brown or dark brown pods containing black seeds. The smell is slight; the taste is light. Mainly produced in Northeast China, Gansu and Shaanxi [15].

In addition to the effects mentioned above, most species of Speranskia tuberculata (Bunge) Baill also have potent antioxidant effects. Guan et al. [9] first established a free radical and reductive reaction system in 2011 to study the antioxidant activity of different polar components of Speranskia tuberculata (Bunge) Baill. The antioxidant capacity of the total extract, ethyl acetate layer, n-butanol layer and aqueous layer extracts of Speranskia tuberculata (Bunge) Baill at different concentrations was determined using ABTS, DPPH radicals using spectrophotometric method. The various polar components of Speranskia tuberculata (Bunge) Baill showed strong scavenging and reducing abilities against DPPH and ABTS+ radicals. The n-butanol layer extract had the strongest antioxidant capacity and could be developed as a new natural edible antioxidant. Li et al. [10] in 2017 used cellulase-assisted extraction of total flavonoids from Speranskia tuberculata (Bunge)

Baill and investigated the antioxidant activity of flavonoids. The total flavonoids in Speranskia tuberculata (Bunge) Baill were extracted by cellulase enzymatic digestion followed by the ethanol reflux method. The results showed that the scavenging activity of total flavonoids from Speranskia tuberculata (Bunge) Baill was significantly better than that of dibutylhydroxytoluene and rutin at the same concentration.

1.2 Extraction method of chemical components of Chinese herbal medicine

1.2.1 Solvent extraction method

Solvent extraction is a method of dissolving the active ingredients from the medicinal tissues by selecting solvents with high solubility for active ingredients and low solubility for impurity components according to the different solubility of various components in Chinese herbal medicines in different solvents. According to the nature of the components to be extracted, select the appropriate solvent and add it to the appropriately crushed Chinese herbal medicine materials. The solvent will gradually enter the cell through the cell wall due to diffusion and osmosis, dissolving soluble substances, resulting in poor concentration inside and outside the cell. As a result, the concentrated solution inside the cell continues to diffuse outward, and the solvent outside the cell continues to enter the medicinal tissue cells. This operation will repeat many times until the solution concentration inside and outside the cell reaches a dynamic equilibrium. The saturated solution is filtered out, concentrated, and then filtered. A new solvent is added to the later dregs, and the above process will repeat. The required ingredients can be almost wholly dissolved or basically dissolved, and then all the concentrated liquids are combined, which will be a mixed solution containing the required active ingredients. The solvent extraction method is as follows:

1) Decoction.

The decoction method is the earliest traditional extraction method used in China. This method is simple and easy to implement, and the most effective ingredients can

be decocted. Still, the decoction liquid contains many impurities and is prone to mildew, and some are not resistant to volatile components and are easily damaged. Available medicinal materials should be fried twice. It is best to stir frequently when heating to avoid the local medicinal materials from being heated too high and easy to scorch.

2) Dipping method.

The dipping method is suitable for the extraction of Chinese herbal medicines whose active ingredients are volatile and easily destroyed when exposed to heat. According to the temperature of the solvent, it is divided into a hot dip, warm dip and cold dip. This method is relatively simple and easy to implement. Still, the extraction rate is low, and if the extraction solvent is water, the extract is prone to mould and deterioration, so care must be taken to add appropriate preservatives. In addition, it is best to use 2 or 3 times immersion to reduce the loss caused by the adsorption of medicine residue and increase the extraction rate.

3) Reflow method.

When the method uses organic solvents for heating and extraction, a reflux device must be used to avoid solvent volatilisation loss and reduce the toxicity of toxic solvents to experiment operators. The amount of medicinal materials should be about 1/3-1/2 of the volume of the round-bottomed flask. The solvent immerses the surface of the medicinal materials by about 1-2 cm. The laboratory is mainly heated in a water bath, which is relatively safe.

4) Continuous extraction method.

The continuous extraction method is commonly used in organic solvent extraction when the laboratory analyses the effective components of traditional Chinese medicine. It is usually done with a fat extractor or a Soxhlet extractor. This kind of extraction method requires less solvent and extracts the components more thoroughly, but it usually takes several hours (usually 6-8h) to complete. Therefore, it is not suitable to use this method for traditional Chinese medicine components that are unstable and changeable when exposed to heat. Nevertheless, when volatile

organic solvents are used to extract the effective ingredients of Chinese herbal medicines, continuous extraction is better regardless of small-scale experiments or large-scale production.

1.2.2 Steam storage method

The steam storage method is only suitable for extracting Chinese herbal medicines that are insoluble or insoluble in water, do not react with water, and can be stored with steam without being destroyed. The boiling point of this type of ingredient is mostly above 100℃. When the temperature is close to 100℃, there is certain vapour pressure. When heated with water, when the sum of its vapour pressure and the vapour pressure of water is one atmosphere, the liquid begins to boil. The water vapour brings out the volatile substances together. For example, volatile oils in Chinese herbal medicines are extracted mainly by this method. This method also commonly extracts monomer components such as Pulsatilla, paeonol, rhododendron, eugenol, and cinnamaldehyde. In the specific laboratory operation, the total volume of the medicine powder and water in the steaming storage bottle should be 1/2 of the volume of the steaming filling bottle and should not exceed 2/3. The condensing efficiency of the condenser tube must be high. When the outflowing liquid changes from turbidity to clear, the steam storage has been basically completed.

1.2.3 Sublimation method

Some solid materials will vaporise directly after heated and solidify into the original solid compound when cold. This phenomenon is called sublimation. Some ingredients in Chinese herbal medicine have sublimation properties to be directly extracted by the sublimation method. For example, caffeine in tea leaves can be sublimated without decomposing when the temperature reaches 178°C or higher. Therefore, the sublimation method is often used when extracting caffeine. In addition, some alkaloids, coumarins, and organic acids also have sublimation properties, such as swainsonine and benzoic acid. Although the sublimation method is simple and

easy to implement, it is rarely used in actual extraction. Chinese herbal medicines can easily be carbonised because of the high temperature required for sublimation. The volatile tar produced after carbonisation is easy to adhere to the sublimation and challenging to refine and remove. Secondly, the sublimation is not complete, the yield is low, and sometimes it is accompanied by decomposition.

1.2.4 Squeezing method

This method can be used when the content of active ingredients in certain Chinese medicines is relatively high and exists in the sap of plants. For example, the essential oil content of the rutaceous plant is high, and it is mainly found in the peel. Most of the essential oils are extracted by this method, such as orange peel oil, lemon oil, etc.

1.2.5 Ultrasonic extraction technology

Ultrasound, like sound waves, is an elastic mechanical wave in a material medium, but the frequency is different. Physics stipulated that the frequency higher than 20 kHz is ultrasonic, and the upper limit can be as high as overlap with the microwave region (> 10 GHz) of electromagnetic waves. As a form of physical energy, ultrasound is widely used in metal flaw detection, underwater positioning, medical diagnosis and treatment, pharmacy, industry, chemical and chemical processes, environmental protection, food industry, biological engineering, etc. The basic principles of ultrasonic extraction technology are as follows [28]:

1) Cavitation mechanism.

Due to the cavitation effect, the intense pressure destroys the biological cell wall, and the entire crushing process is completed instantly. At the same time, the vibration generated by the ultrasonic increases the permeability of the solvent into the Chinese herbal medicine cell. It strengthens the violent movement of the bubbles due to cavitation during the mass transfer process. The strong shearing force formed on the cells can rupture the Chinese herbal medicine cells, make the cells easy to release the contents and accelerate the conversion speed of the components in each stage of the

traditional extraction method. Therefore, ultrasonic extraction shortens the extraction time and can completely extract the ingredients contained in Chinese herbal medicines.

2) Mechanical mechanism.

Ultrasonic or even low-intensity ultrasonic action can make the particles of the medium compress and stretch alternately, produce linear or non-linear alternating vibrations, cause the interaction of Bernoulli force, viscous force, etc., thereby enhancing the movement of the particles of the medium and accelerating the mass. It can produce mechanical effects, such as stirring, dispersion, crushing, degassing, fogging, cohesion, and orientation.

Ultrasound avoids the destruction of active ingredients caused by high-temperature heating but requires relatively high requirements on the thickness of the container wall and the placement position during operation. At present, the experimental research is in the small-scale stage. If used for large-scale production, it is necessary to solve the magnification problem of related engineering equipment. At the same time, the effective ingredients proposed by conventional methods are used as a control. They indicate that ultrasonic extraction will not change the structure of the effective ingredients, shorten the extraction time, increase the extraction rate, thereby providing a fast and high-yield new method for extracting Chinese herbal medicine components.

1.2.6 Microwave extraction technology

Microwave-assisted extraction, also known as microwave extraction or microwave extraction, is a new type of extraction technology that combines microwave and traditional solvent extraction. Microwave-assisted extraction has many advantages, including simple equipment, wide application range, high extraction efficiency, strong selectivity, good repeatability, time-saving, solvent saving, energy-saving, and low pollution. The application range has been rapidly expanded from the initial preparation of environmental analysis samples to the food, chemical and agricultural fields. In recent years, domestic and foreign scientific

researchers have applied microwave technology to the extraction process of active ingredients of natural products, which has effectively improved the yield. At present, the use of microwave technology to extract biologically active ingredients has involved several major categories of natural compounds, such as volatile oils, sesames, polysaccharides, sugars, alkaloids, flavonoids, tannins, and organic acids.

1.2.7 Semi-bionic extraction method

The semi-bionic extraction method is a new method proposed in recent years. From the perspective of biopharmaceutics, it combines the holistic drug research method with the molecular drug research method to simulate the environment of oral drug transport and absorption through the gastrointestinal tract. The drug material is first extracted with acidic water with a specific pH, followed by a certain pH. Alkaline water extraction, the extracts are filtered and concentrated on preparing preparations for oral administration. The semi-biomimetic extraction method embodies the characteristics of the comprehensive effects of the clinical use of traditional Chinese medicine. It conforms to the principle of oral drug transport and absorption through the gastrointestinal tract. At the same time, without ethanol treatment, more effective ingredients can be extracted and retained, shortening the production cycle and reducing costs.

1.2.8 Enzymatic extraction

Enzymes are a particular type of biocatalyst in the form of proteins produced by living cells of organisms. The application of enzymes is extensive, and the active ingredients of Chinese herbal medicines are extracted by the enzymatic method. The yield is obviously improved, and it has great application potential. However, enzymatic extraction requires higher experimental conditions. It is necessary to determine the optimal temperature, pH value, and optimal time through experiments to make the enzyme play a more significant role. The effects of concentration, temperature, pH, inhibitors and agonists on the extract are discussed in [29].

1.3 Analysis method of the chemical composition of Chinese herbal medicine

1.3.1 Chromatography

Chromatography is a new type of analysis technology developed in modern times, and it is a separation and analysis method of physical and physical chemistry. It has the characteristics of high separation efficiency, strong selectivity, fast separation and analysis speed, and increased sensitivity, so it is widely used in separation analysis in various fields. Commonly used chromatographic methods in the study of chemical components of natural products include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), high-speed countercurrent chromatography (HSCCC), high-performance capillary electrophoresis (HPCE), gas chromatography (GC), supercritical fluid chromatography (SFC), etc.

1.3.2 Spectroscopy

The chemical components of Chinese herbal medicines are complex. For the structural identification of compounds, chemical reactions were the primary means before the 1950s. The amount of information in chemical experiments is minimal, and it is often not always possible to get a clear conclusion smoothly. The emergence and rapid development of new instrumental analysis methods have considerably changed the situation in recent years. The commonly used instrumental analysis methods are "four spectrums": infrared spectroscopy, ultraviolet spectroscopy, mass spectrometry, and nuclear magnetic resonance.

1.3.3 Combined techniques

Although different analytical methods have their advantages, a single analytical method cannot reflect the complexity of the chemical components in Chinese herbal medicines. In recent years, the combined use of a variety of modern analytical instruments has provided a lot of information for the research on the chemical components of Chinese herbal medicines. Commonly used combined methods are:

GC/MS, HPLC/MS, HPLC/NMR, HPLC/IR, CE/MS, HSCCC/MS, etc. High-performance liquid chromatography-nuclear magnetic resonance online combined technology is one of the best methods for simultaneous separation and structural identification of unknown mixtures. It can be achieved directly using multiple chromatographic combined technologies without obtaining pure products and can confirm the structure of each component in the mixture [30].

1.4 Separation and purification methods of active ingredients

Standard separation and purification technology methods include sedimentation separation, filtration separation, and centrifugal separation. Standard refining methods include water extraction and alcohol precipitation, alcohol extraction and water precipitation, acid-base method, salting out, adsorption chromatography, ion exchange and crystallisation. Some new separation and purification techniques have appeared in recent years, such as flocculation sedimentation method, supercritical CO_2 fluid extraction technology, macroporous resin adsorption method, high-speed countercurrent chromatography, and high-speed centrifugation, membrane separation technology, etc.

1.4.1 Supercritical CO_2 fluid extraction technology

Compared with the traditional steam storage and solvent extraction methods, the supercritical fluid extraction method has fast mass transfer speed, strong permeability, high dissolution extraction efficiency, and low extraction temperature. It can be operated at room temperature, which is more conducive to extracting heat-sensitive components. The advantages of no solvent residue, no pollution, convenient operation, fast speed, and low cost have attracted more and more attention. Substances that can be used as supercritical fluids include carbon dioxide, water, ethane, nitrogen dioxide, etc., of which carbon dioxide is the most commonly used. Supercritical carbon dioxide has very low polarity and is suitable for extracting volatile oils, small molecules, and some alkaloids. The extraction of materials with high polarity is subject to certain restrictions by adding entrainers, such as methanol,

ethanol, acetone, ethyl acetate, water, etc. Increasing the pressure can improve the dissolution properties of the fluid. It has aroused widespread interest in chemical, pharmaceutical, food, biochemical and other fields. When Chinese medicine is gradually modernising, supercritical fluid extraction technology is called high-efficiency extraction and separation technology for traditional Chinese medicine.

1.4.2 Macroporous resin adsorption method

Macroporous resin is a kind of organic polymer adsorbent with good adsorption performance developed in the late 1970s. Macroporous resin adsorption technology was first used in wastewater treatment, pharmaceutical industry, chemical industry, analytical chemistry, clinical verification and treatment and other fields. The macroporous resin adsorption chromatography method is effective, simple in process, low in production cost, unaffected by inorganic substances, and convenient in regeneration. In recent years, it has been widely used in the extraction, separation and purification of active ingredients of Chinese herbal medicines in China.

Compared with the traditional Chinese medicine preparation process, the extract obtained using the macroporous resin adsorption technology is small in size, non-hygroscopic, and easy to be made into various dosage forms with beautiful appearance, especially suitable for granules, capsules and tablets [31].

1.4.3 High-speed countercurrent chromatography

High-speed countercurrent chromatography technology is a continuous liquid-liquid distribution chromatography technology without solid carrier or support. It combines the directionality of the spiral tube with the high-speed planetary motion. Such a technique can produce a particular dynamic phenomenon and make the two-phase solvent efficient mixing, distribution and transfer in the spiral tube can achieve high-efficiency separation and preparation quickly. The high-speed countercurrent chromatography technology has the characteristics of simple operation, wide application range, no solid carrier, high product purity, and is suitable for

preparative separation. Based on its characteristics, it is used to extract, separate and purify the effective ingredients of Chinese herbal medicine has significant advantages.

1.4.4 High-speed centrifugal technology

Centrifugal technology is a centrifuge as the main equipment. Through the high-speed operation of the centrifuge, the centrifugal acceleration exceeds the acceleration of gravity hundreds of thousands of times, thereby increasing the sedimentation speed to accelerate the precipitation and removal of impurities in the liquid medicine. The principle is to use the density difference of the mixed liquid to separate the material liquid. The sedimentation centrifuge has the characteristics of time-saving, labour-saving, complete liquid recovery, high active ingredient content, and high clarity. It is more suitable for separation and filtration with difficult sedimentation, such as suspensions of fine particles or flocs.

1.4.5 Membrane separation technology

Membrane separation technology is a new high-tech and interdisciplinary science and technology. In recent years, membrane separation technology has been widely used in industrial. The mechanism is to achieve separation between two or more substances by overcoming the osmotic pressure of the membrane [32]. Membrane separation technology has the advantages of operating at room temperature without phase change and low energy consumption. It is particularly suitable for treating heat-sensitive substances and biologically active substances. Standard membrane separation technologies include microfiltration, ultrafiltration, and reverse osmosis. Compared with traditional separation methods, membrane separation technology separates peptides and amino acids, significantly saving energy, simplifying the process, reducing costs, and improving separation efficiency and quality.

1.5 Chemical composition study of Speranskia tuberculata (Bunge) Baill

The chemical composition of Speranskia tuberculata (Bunge) Baill varies

significantly due to its relatively complex species and origin. In recent years, scholars from home and abroad have conducted systematic and in-depth studies on the chemical constituents of various Speranskia tuberculata (Bunge) Baill. Finally, various constituent compounds with biological activity have been isolated and identified [17].

According to the literature, lignans are most abundant in Speranskia tuberculata (Bunge) Baill. The phrymarolin-II and ursolic acid were obtained from the ethyl acetate layer of the above-ground parts of Speranskia tuberculata (Bunge) Baill. Extraction of phrymarolin-I, phrymarolin-II and leptostachyol acetate was obtained from the roots of Speranskia tuberculata (Bunge) Baill. The compounds obtained in Speranskia tuberculata (Bunge) Baill are lignin-like compounds, except for ursolic acid. Their chemical structures are shown in Figure 1 [18].

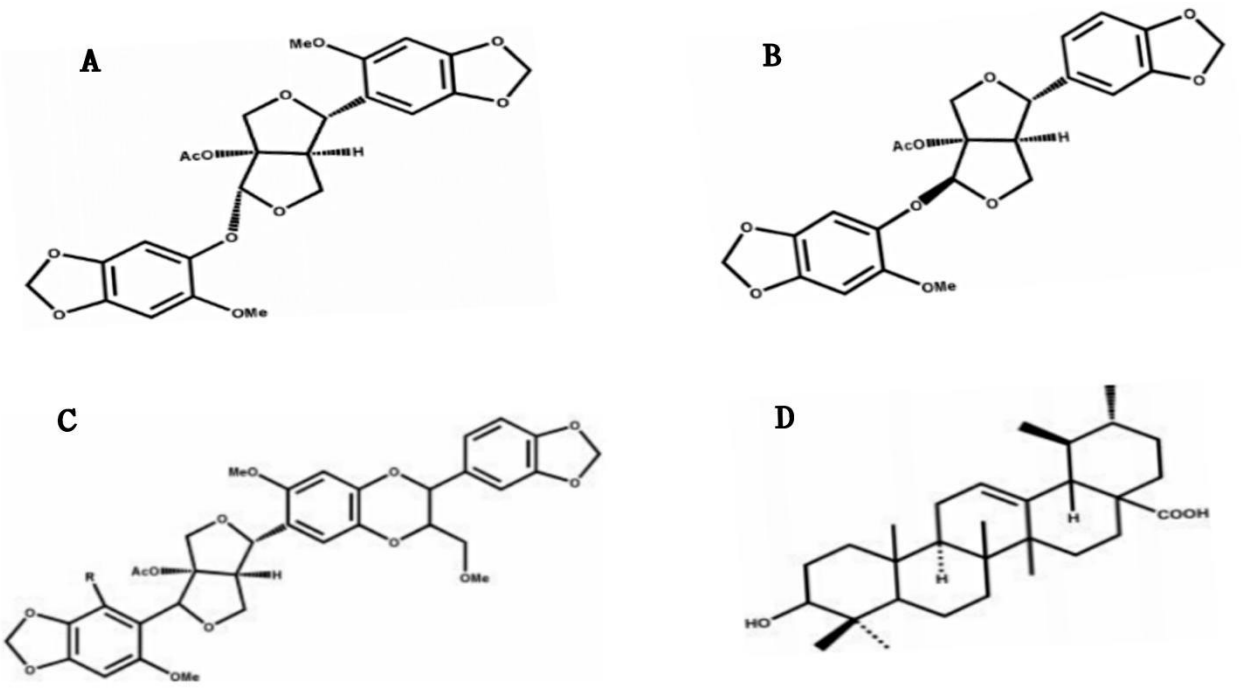


Figure 1.1 – The lignin-like compounds in Speranskia tuberculata (Bunge) Baill. A is phrymarolin-I; B is phrymarolin-II; C is Leptostachyol acetate; D is Ursolic acid.

Researchers have isolated many medicinal components from the stem and leaves of Herba Speranskiae Tuberculatae. Dong et al. [19] isolated nine compounds from

the above-ground parts of the leaves of Herba Speranskiae Tuberculatae. Based on their physicochemical properties and wave spectra characteristics, the authors identified their structures as chondroitin, β-sitosterol, triacontanol, coumaric acid, ferulic acid, monoterpenoids, p-coumaric acid, thymine and uracil. Five flavonoids were isolated from the leaves of Dictyostelium, which were identified according to their properties and spectral characteristics as geranylin (3, 5, 7-trihydroxy-4-methoxyflavone), lignan (3, 4, 5, 7-tetrahydroxyflavone), 4, 5, 7-trihydroxydihydroflavone, spikenard bisflavone, and lignan-7-O-rutinoside.

Chen et al. [20] also isolated many medicinal components from the flowers, seeds, pericarp and roots of Impatiens balsamina L.: for example, flavonoids, quercetin, coumaric acid, protocatechuic acid, rutin and coumarin. Among them, flavonoids, coumarins and naphthoquinones are the main components. Kaempferol and its derivatives, including kaempferol, kaempferol-3-glucoside, kaempferol-3-rutinoside and kaempferol-3-p-hydroxycinnamoyl glucoside, were isolated from the pink petals of Bromeliads by capillary electrophoresis. The 5A-reductase inhibitor di- (2-hydroxy-1, 4-naphthoquinone-3-)-ethane was isolated from the stem and leaves of Fenugreek, a class of drugs commonly used in the treatment of symptoms such as benign prostatic hyperplasia, male pattern baldness, and female hirsutism.

Li et al. [21] isolated seven compounds from the ethanolic extract of Impatiens balsamina L. They are as follows: 2-methoxy-1, 4-naphthoquinone, vanillic acid, heptaerythrolactone, protocatechuic acid, rutin, soybean brain glycoside, and 1, 2, 4 -trihydroxynaphthalene-1,4-bis-B-D-pyroglucoside. Guan et al. [22] isolated and identified four compounds from the ethanolic extract of Xinjiang impatiens balsamina, namely, dousterol, kaempferol, kaempferol glucoside, and kaempferol glucosyl rhamnoside. Among them, dousterol and kaempferol have biological activities such as antibacterial, anti-inflammatory and anti-cancer. Among them, quercetin has a variety of biological activities, such as dilating coronary arteries, reducing capillary permeability, anti-platelet aggregation, anti-oxidation, anti-allergy,

analgesia and other pharmacological effects. Recent studies have found that quercetin has inhibitory effects on various carcinogenic and pro-carcinogenic substances, inhibits the growth of various tumour cells, and induces apoptosis [23].

Wang et al. [24] obtained several monoterpene alkaloids and macrocyclic spermine alkaloids from Incarrillea sinensis Lam. A total of 18 compounds were identified and isolated. Yang et al. [25] used GCMS to isolate and confirm 72 chemical components from the volatile oil of Incarrillea Sinensis Lam, accounting for 97.19% of the total amount detected. The percentages of their main chemical components in the volatile oil were: terpenoids (27.48%), alcohols (14.91%), aldehydes (5.42%), ketones (10.58%), phenols (5.34%), acids (7.86%), oxygenates (16.10%), and other compounds (9.50%). The main terpenoids in the volatile oil composition are A-pinene, linalool, camphor, linalyl acetate, B-elemene, B-malmene, B-stilbene, A-turmerene and A-rutinene. Most of them have various biological activities such as sweating, anthelmintic, expectorant, antibacterial and antiviral. Tan [25] concluded that Incarrillea Sinensis Lam was extracted and purified by ultrasonication and then analysed by the GC-MS method. A total of 52 compounds were isolated and identified in the volatile oil of Speranskia tuberculata (Bunge) Baill, with the highest content of esters, accounting for 24.53% of the total volatile oil. Acid compounds with 19.82% followed this. There were also alkanes, olefins, aldehydes and ketones. Ester compounds are the main components found in roots, stems, leaves, flowers and fruits, and have certain physiological activities, including analgesic, cough suppressant, expectorant, sweating and wind repellent. Among them, hexadecanoic acid, also known as palmitic acid, has a relative content of 17.74% in the volatile oil of Speranskia tuberculata (Bunge) Baill, which is colourless, odourless, and a saturated higher fatty acid.

Liu et al. [26] isolated various compounds from Small tougucao such as Dianbaijurin, ranunculin, palmitic acid, carotene, ferulic acid, chlorogenic acid, catechin, rutin, quercetin, salicylic acid and vanillic acid. Zhao et al. [27] isolated quercetin and kaempferol mainly from Vicia amoena Fisch. Six compounds were

isolated from the ethanolic extract of the whole grass of wild pea. They were identified by spectroscopy and chemical analysis as kaempferol, quercetin, kaempferol, quercetin-3-O-A-L-rhamnoside, rhamnoside, quercetin-3-O-B-D-glucoside, kaempferol-3,7-O-A-L-dirhamnoside.

1.6 Pathological mechanism of atherosclerosis and therapeutic drugs

Atherosclerosis is a type of cardiovascular disease mainly caused by abnormal lipid metabolism and accompanied by inflammation. It primarily occurs in the large and middle arteries and the cardiovascular system. It has a high mortality and disability rate. It has become a high-risk disease with the same risk as degenerative brain diseases and malignant tumours. The pathogenic mechanism of atherosclerosis is more complicated. It starts with the lipid deposition in the cell leading to the conversion of macrophages to foam cells, and the endpoint is the rupture of plaque in the arteries caused by inflammation. The process of atherosclerosis mainly includes lipid metabolism in macrophages, adhesion and migration of monocytes, abnormal endothelial cell proliferation and differentiation, foaming of macrophages, early foam cell apoptosis, early plaque formation. The inflammatory response is activated, and the plaque ruptures.

1.6.1 Cholesterol metabolism imbalance

The imbalance between the intake and efflux of intracellular cholesterol is an essential factor leading to the high content of total cholesterol in the cell and the formation of lipid-rich foam cells [33]. Abnormal cholesterol metabolism will cause lipid accumulation and induce oxygen free radicals to accelerate the reaction in the body, oxidatively modify low-density lipoprotein (LDL), and cause oxidative damage to endothelial cells [34]. Endothelial cell injury causes the increase of endothelial cell membrane permeability. It makes LDL easily migrate through the endothelial cell membrane barrier to the artery's inner wall, deposit a large amount, and undergo oxidative modification with oxygen free radicals to form oxidised low-density lipoprotein (ox-LDL). While ox-LDL continues to damage endothelial cells,

macrophages recognise it as abnormal lipids and are phagocytosed in large quantities. It is decomposed into free cholesterol under the action of intracellular lysosomes. A large amount of intracellular cholesterol accumulates and deposits to form free lipids. Lipid-containing macrophages are called "foam cells" [35]. Foam cells promote inflammation in the process of AS disease and are also prone to apoptosis. The apoptotic foam cells accumulate and aggregate to form the early AS plaques' core and continue to condense lipids to cause the plaques to expand and become more prominent.

1.6.2 Oxidative stress causes endothelial dysfunction

Oxidative stress refers to the process of losing the balance between the production of reactive oxygen species (ROS) and anti-oxidation in cells or the body, leading to excessive accumulation of oxygen free radicals and their oxidative metabolites, resulting in oxidative damage [36]. Under normal circumstances, ROS in the body's environment can kill harmful microorganisms, regulate the body's immunity, and play an essential role in the body's defence system. However, excessive oxidative stress has damaging effects on the body, mainly manifested in two aspects. First, oxidative stress can interfere with the proliferation and apoptosis of endothelial cells, damage blood vessels, and affect vasoconstriction. Oxidative stress can release a large amount of O^{2-}, the reaction of O^{2-} and NO^{-} will lead to the infiltration of highly reactive peroxynitrite, induce endothelial cell apoptosis, and cause endothelial dysfunction [37]. At the same time, the accumulation of free oxygen-free radicals will cause the endothelium to reduce the availability of the vasodilation regulator NO. Free radicals cause the vasodilation rate to slow down. The blood flow rate slows down. It is easy to cause tissue ischemia and anaemia symptoms [38]. Oxygen-free radicals produced by oxidative stress oxidise and modify LDL to create ox-LDL. The latter has physiological toxicity to cells and biological macromolecules, causing cell necrosis and apoptosis and leading to body damage. It promotes macrophages to foam cells, leading to the formation of plaques [39]. Also, ox-LDL can enter the blood vessel wall from the site of endothelial cell

injury; increase endothelial cell function damage, induce macrophages to enter the injury site to expand the inflammatory response; infiltrate the lipid plaque; accelerate the rupture of the lipid plaque; form thrombus to promote the process of AS [40].

1.6.3 Inflammation

The accumulation of lipids and immune cells in the arterial wall is an important cause of AS. Whether in the process of lipid metabolism or oxidative stress damage to endothelial function, inflammatory cells and inflammatory mediator factors directly or indirectly promote the pathology of AS Process [41]. In the initial stage of AS, the endothelium is functionally damaged. Many lymphocytes adhere to the inner membrane and enter the sub-intimal space under the induction of cell adhesion factors and chemokines secreted by monocytes [42]. Subsequently, neutrophils and monocytes infiltrate the vascular disease site and affect the normal metabolism of lipids by secreting cytokines. Monocytes are transformed into macrophages under the induction of monocyte chemotactic factor (MCP-1). It further activates the inflammatory response and releases pro-inflammatory factors such as TNF-α, IL-1β, IL-6, etc., aggravating local inflammation's progress [43]. In addition, under the induction of inflammatory factors, foam cells are prone to apoptosis and deposit to form lipid plaques, promoting the further deterioration of AS disease [44]. The inflammatory response promotes the formation of early AS plaques and, more importantly, encourages the transition of plaques from a stable state to an unstable state and the construction of thrombus, which pushes the AS disease process to a high-risk period [45]. The formation of unstable plaques is mainly due to inflammation in the plaques. Many macrophages, lymphocytes, and other immune cells migrate and accumulate to the diseased cells, producing many proteolytic enzymes to hydrolyse the lipid-encapsulated fibrous sheath and make the plaque's surface fibres thinner, easier to rupture [46].

1.6.4 Drugs for treatment of atherosclerosis

At present, the clinical treatment of AS is mainly to reduce the body's blood

lipid level by adjusting the body's response. There are fewer types of drugs, mainly divided into the following types:

1) Statins. They act mainly by inhibiting the effect of hydroxymethylglutarate-coenzyme. A reductase (HMG-CoA), reducing its cholesterol production, such as lovastatin, simvastatin, etc. [47].

2) Niacin drugs. It has the function of improving the enzyme activity of lipoprotein esterase, thereby accelerating the decomposition of cholesterol. For example, asimimus has a better effect [48].

3) Fibrate drugs. Similar to niacin drugs, it can accelerate the decomposition of cholesterol but at the same time has the function of increasing HDL-c in serum. Such drugs mainly include bezafibrate, phenafibrate, etc. [49].

4) Bile acid sequestrant drugs. It can inhibit bile acid circulation in the liver and reduce cholesterol intake by cells. Representative drugs mainly include cholestyronic acid, colestipol, etc. [50].

There are relatively few drug treatments at present. They can only participate in the treatment of AS by directly or indirectly inhibiting the synthesis of one's cholesterol or promoting cholesterol decomposition. Although these drugs have a particular therapeutic effect on AS, they also have many side effects, such as loss of liver function, causing gastrointestinal dyspepsia, allergic reactions, etc., which cause certain damage to the human body.

Traditional Chinese herbal medicine has significant comprehensive treatment effects and low side effects. It can be directly used as a medicine or food additive to participate in the prevention or treatment of AS. Wang et al. [51] found that E3317 can promote cholesterol efflux by increasing the expression of ABCA1, thereby playing a role in treating AS. Yu et al. [52] found that ApoE mice fed with a high-fat diet can effectively prevent AS after consuming quercetin and further studied that quercetin has the function of inhibiting oxidative stress and protecting endothelial damage. At the same time, Zhang et al. [53] found that quercetin has the function of regulating the secretion of ROS and cytokines IL-8, NF-κB, etc., inhibiting the

progress of inflammatory response, controlling cell apoptosis, and playing an anti-AS effect. Zhou et al. [54] found that retinoic acid can effectively reduce arterial plaque in ApoE mice by regulating ABCA1 and ABCG1 to promote cholesterol efflux. He et al. [55] found that Metformin inhibited the formation of foam cells and delayed AS disease progression by inhibiting the uptake of ox-LDL by cells and increasing the expression of ABCG1.

1.7 Carcinogenesis mechanism of tumours and classification of anti-tumour Chinese herbal medicines

The tumour is a common clinical disease, and due to the influence of many factors, it also poses more and more severe threats to human health. In particular, tumour diseases have a rapid onset, rapid onset, and poor prognosis, so it is essential to take timely, effective, safe and reliable treatment for patients after the beginning. In the past, when treating cancer patients, the primary methods were western medicine and surgical treatment. For example, they were radical tumour resection, chemotherapy and radiation therapy, etc. [56]. Although these treatment methods can achieve a particular effect, their safety is not high. For example, various adverse reactions and complications often occur after chemotherapy, radiotherapy, and surgery, significantly impacting patients' bodies.

1.7.1 Canceration mechanism of tumour

A tumour is a genetic disease whose biological basis is the abnormality of genes. Tumours occur when cells in local tissues of the body lose the normal regulation of their growth at the genetic level under the action of various factors, leading to the formation of new tissues due to abnormal cell proliferation. The pathogenic factor is the deletion of normal genes caused by somatic gene mutations and disorder of gene expression, which affects cells' biological and genetic activities and forms the morphology of normal cells. Tumour cells are different in metabolism and function. The current theories on the mechanism of cancer mainly include:

1) Gene mutation theory. The genetic information of genes determines the

morphology and function of cells. Carcinogenesis is the effect of carcinogens, or the integration of foreign genes (such as tumour viruses) into the cell genome, resulting in changes in the structure of the cell's genetic material DNA. As a result, normal cells acquire new genetic characteristics (irrepressible growth and immature differentiation) and transform into cancer cells.

2) The theory of gene expression disorders. Under normal circumstances, the DNA fragments of normal somatic cells of the human body only activate certain parts to synthesise particular proteins and enzymes. This gene expression is strictly controlled and regulated. When a specific carcinogen disrupts the gene regulation process, genes that should not be activated under normal circumstances are activated. Such processes can be cell division and differentiation losing regulation, which causes the cells to continue to divide and lose the ability to differentiate and mature, leading to cell cancer.

3) Oncogene theory. This theory believes that the DNA of human cells contains homologous sequences of viral oncogenes, which are called cellular oncogenes. Under normal circumstances, it is only expressed at a low level and may also have physiological functions. However, under the action of physicochemical or viral carcinogenic factors, it may cause the rearrangement, amplification or mutation of cell oncogenes to activate, thereby enhancing or improper expression, producing excessive or abnormal proteins, and causing normal cells. Thus, a malignant change occurred.

1.7.2 Classification of anti-tumour Chinese herbal medicines

The treatment of cancer by western medicine has attracted more attention in treating tumours. It is precise because western medicine has the disadvantages of high treatment cost, low safety, and many adverse reactions after surgery. Through Chinese herbal medicine treatment, safe and effective treatment effects can be achieved. Compared with western medicine, Chinese herbal medicine is very safe. Especially if the corresponding Chinese herbal medicine treatment is selected strictly according to the patient's condition, it will not lead to severe complications and

adverse reactions. At the same time, the safety of using Chinese herbal medicine is also higher. Therefore, Chinese herbal medicine has been used more and more widely in treating tumours. There are currently four types of drugs involved in the implementation of Chinese herbal medicine treatment for cancer patients: cytotoxic drugs, metabolism regulation, immunity promotion, and blood circulation and blood stasis removal.

1.7.3 Cytotoxic drugs

Cytotoxic drugs mainly include cell cycle-specific drugs (Cephalotaxus, Nongjili, etc.), cell cycle non-specific drugs (cantharidin, Indigo Naturalis, comfrey root, etc.), acting on cell membranes (West bean root), inhibiting cell respiration (Centipede). These drugs can have a deadly effect on cells [57]. Especially for cancer cells, it can achieve a better killing effect. Through cytotoxic drugs, it can act on the proliferation cycle of cancer cells and avoid the proliferation of cancer cells. At the same time, it can also destroy the cell membrane of cancer cells and inhibit the respiration of cancer cells, eventually leading to the death of cancer cells.

1.7.4 Metabolism-regulating drugs

Metabolism regulating drugs mainly include regulating cancer cell metabolism (Polyporus umbellatus), regulating overall metabolism (ginseng), and phytoagglutinin (canavalin). The use of metabolism-regulating medications can directly or indirectly cause changes in the concentration of substances that regulate metabolism in cancer cells, thus causing cancer cells to be in an abnormal state and ultimately leading to their death. For example, using metabolic regulation drugs, Wu et al. [58] regulate the cAMP and cGMP in cancer cells. These two types of substances are closely related to the metabolism of cancer cells. Therefore, it is concluded that once the two kinds of substances are adjusted, the synthesis of cancer cells will be blocked. It can reduce the spread of cancer cells and help cancer cells transform into normal cells.

1.7.5 Immunisation-promoting drugs

Immunity-promoting drugs mainly include specific immunity-promoting

(Longbai Yunzhi) and non-specific immunity-promoting (oldenlandia diffusa). If it only kills cancer cells, it may not be effective. Under such a premise, immune-promoting drugs have begun to receive more attention. Cao et al. [59] found that the use of immune-stimulating drug therapy can improve the function of the patient's lymphatic system and other immune organs and significantly enhance immune cells' phagocytic ability. Due to the emergence of diseases, cancer patients' immunity will be reduced, and they will eventually enter a vicious circle. Promoting immune drug therapy can better promote the recovery and improvement of the patient's immune function so that the patient's immune system can kill cancer cells.

1.7.6 Activating blood and removing blood stasis drugs

Chinese herbal medicines for promoting blood circulation and removing blood stasis mainly include red peony root and Salvia miltiorrhiza. Once they stay in the blood, cancer cells will cause their aggregation and development, and eventually, cancer cells will spread. The blood circulation and blood stasis treatment can promote blood circulation in the patient's body, make it difficult for cancer cells to stay in the blood tumour, and avoid the spread and metastasis of cancer cells. In addition, by promoting blood circulation and removing blood stasis drugs, anti-tumour other medicines can be spread to various parts of the patient's body in a short period to achieve significantly better therapeutic effects.

Conclusions to Chapter 1

This chapter firstly introduced Speranskia tuberculata (Bunge) Baill and its classification and then listed the extraction, isolation, and purification of the active ingredients of herbal medicines.

Secondly, the chemical composition of Speranskia tuberculata (Bunge) Baill, confirmed by research, is summarised.

Finally, the pathological mechanisms of the two diseases studied in this topic, tumours and atherosclerosis, and their therapeutic drugs are presented.

CHAPTER 2. STUDY OF ANTI-TUMOUR EFFECT OF BIO-ACTIVE COMPONENTS OF SPERANSKIA TUBERCULATA (BUNGE) BAILL

Tumour is a common clinical disease, and due to the influence of many factors, it poses more and more severe threats to human health. In particular, tumour diseases have a rapid onset, rapid onset, and poor prognosis, so it is essential to take timely, effective, safe and reliable treatment for patients from the very beginning [61].

When treating tumour patients, the primary methods were western medicine and surgical treatment, such as radical mastectomy, chemotherapy and radiotherapy. Although this treatment method can achieve a particular effect, its safety is not high. For example, various adverse reactions and complications often occur after chemotherapy, radiotherapy, and surgery, significantly impacting patients' bodies.

Because of these shortcomings of western medicine, Chinese medicine has begun to receive more attention in treating many tumour diseases. Through Chinese herbal medicine treatment, safe and effective treatment effects can be achieved. Chinese herbal medicine is very safe [62] and will not cause severe complications and adverse reactions. Therefore, Chinese herbal medicine has been used more and more widely to treat tumour diseases.

Currently, there are several studies have proven that some herbal medicines have been shown to slow down the clinical signs and symptoms in patients with hepatocellular carcinoma. For example, ursolic acid UA, an extract of Hedyotis diffusa, significantly inhibited the growth of R-HepG2 cells in a time- and dose-dependent manner by a mechanism mediated by cell cycle blockade and induction of apoptosis [63].

Chrysanthemum extract effectively attenuated the mitosis effects of isoprenaline (ISO) on HepG2 and MH-CC97H cells by inhibiting the sub β2 receptor agonism of ISO on tumour cells, thereby blocking the activation of MAPL/ERK1/2 signalling pathway [64].

Sinomenine can reduce the expression level of all proteins including Cy-clinDl, cDK4 and cDK6 in human ovarian cancer Caov3 and SKOV3 cells, thereby reducing

the activity of Caov3 and SKOV3 cells staying in the G0/G1 phase and inhibiting cell proliferation [65].

Carvacrol can induce apoptosis in human non-small cell lung cancer NCI-H1299 cells by activating Caspase-9 and inhibiting cell invasion by down-regulating MMP-9 [66]. These studies have shown that many kinds of Chinese herbal medicine could inhibit the metastasis of tumour cells.

Speranskia tuberculata (Bunge) Baill is a kind of Euphorbiaceae Phyllanthus plant containing various nutrients, including polysaccharides, vitamins and amino acids, and a variety of drug structural components, including polyphenols, flavonoids, sterols, alkaloids and trace elements.

Speranskia tuberculata (Bunge) Baill has the effects of resolving phlegm, relieving cough, strengthening the stomach, clearing heat and detoxification, and is widely used in antioxidant, lipid-lowering and anti-inflammatory aspects. In recent years, the bioactive components of Speranskia tuberculata (Bunge) Baill have been widely studied, but there are few studies on antitumor, anti-atherosclerosis and cardiovascular diseases.

This chapter examines the antitumour effect of the extract of Speranskia tuberculata (Bunge) Baill. It is mainly divided into two parts:

1. the extraction and polar separation of active biological parts of Speranskia tuberculata (Bunge) Baill;

2. the screening and efficacy evaluation of antitumour polar parts in MTT Cytotoxicity Test.

2.1 Preparation and polar separation of crude extracts of Speranskia tuberculata (Bunge) Baill

2.1.1 Experimental apparatus

Table 2.1 – Experimental Apparatus

Apparatus	**Model**	**Manufacturer**
Rotating evaporator governor	RE-52AA/RE-20 1D	Shanghai Yarong Biochemical Instrument Factory
High-speed freezing centrifuge	Neofuge 1600R	Shanghai Lishen Scientific Instrument Co., Ltd
Collector constant temperature heating magnetic stirrer	DF-101S	Gongyi Yingyu High-tech Instrument Factory
KQ-300DE ultrasonic instrument	KQ-300E	Kunshan Ultrasonic Instrument Co., Ltd

2.1.2 Experiment reagent

Table 2.2 – Reagents

Experimental Drugs and Reagents	**Specification**	**Manufacturer**
Petroleum benzine	analytically pure	Tianjin Fuyu Fine Chemical Co., Ltd.
Ethyl acetate	analytically pure	Tianjin Fuyu Fine Chemical Co., Ltd.
n-butanol	analytically pure	Tianjin Fuyu Fine Chemical Co., Ltd.
Dehydrated alcohol	analytically pure	Tianjin Fuyu Fine Chemical Co., Ltd.

2.1.3 Experimental Methods

The Chinese herbal medicine Tougucao was crushed to 20 mesh by a crusher, and the experiment was carried out according to the liquid-solid ratio of 1: 10. The

Soxhlet extractor was used to extract four different polar solvents (petroleum ether, ethyl acetate, n-butanol and water) in order of polarity from small to large.

1) The powder was weighed 50 g, and the filter paper was wrapped in the bottom of the soxhlet extractor. 500 mL petroleum ether was added into the flask of the soxhlet extractor, 85℃, and heated for 4 h.

2) After cooling to room temperature, the petroleum ether extract was centrifuged at 6000 RPM for 5 min, and the supernatant was collected.

3) Subsequently, 500 mL ethyl acetate was added into the soxhlet extractor flask and extracted at 72 ℃ for 4 h. After cooling to room temperature, the ethyl acetate extract was centrifuged at 6000 RPM for 5 min, and the supernatant was collected.

4) n-butanol and water were extracted according to the above process. The above different polar solvent extracts were vacuum distillation, rotary distillation extract to extract, dried and weighed.

2.2 Cell Experiment

2.2.1 Experimental apparatus

Table 2.3 – Experimental Apparatus

Apparatus	**Model**	**Manufacturer**
Multifunctional microplate reader	Spectra Max M5	Molecular Device
High-speed freezing centrifuge	Neofuge 1600R	Shanghai Lishen Scientific Instrument Co., Ltd
Cell ultra-clean workbench	ZHJH-C115B	Shanghai Zhicheng Instrument Analysis and Manufacturing Co., Ltd.

Apparatus	Model	Manufacturer
Ultralow temperature freezer	DW-86L728J	Haier Biomedical Co., Ltd
Pure water producer	STU4100	Shanghai Suitian Environmental Protection Technology Co., Ltd.
Vortex mixer	Vortex-2	Shanghai Huxi Industry Co., Ltd.
Decolorising shaker	TS-1	Kylin-Bell Instrument Co., Ltd.
Cell culture box	311	Semerfeld Technology Co., Ltd
Phase-contrast microscope	DM IL LED	Leica BIOSYSTEMS
HH Series Digital Display Constant Temperature Water Bath Pot	XMTD203	Jiangsu Science and Technology Instruments Co., Ltd

2.2.2 Experimental drugs

Table 2.4 – Experimental Drugs

Experimental drugs and reagents	Specification	Manufacturer
Thiazolyl blue (MTT)	98%	Sigma-Aldrich
Lipopolysaccharide	biotechnology level	Ita biology
Disodium hydrogen phosphate	analytically pure	Guoyao Group Chemical Reagent Co., Ltd.
Potassium dihydrogen phosphate	analytically pure	Guoyao Group Chemical Reagent Co., Ltd.
Phosphate buffer solution (PBS)	biotechnology level	Sangon Biotech
RPMI Medium Modified		HyClone
FETAL BOVINE SERUM		GEMINI
TRYPSIN 0.25%（1X）		HyClone

2.2.3 Cell culture

1) Cell types: B16-F0, HEPG2, A375, RAW 264.7, A549, Hela. The cell morphology is shown in Figure 2.1.

2) Medium types: A549, A375, Hela, HEPG2cells were cultured in DMEM + 10 % FBS medium; RAW 264.7 and B16-F0 cells were cultured in RPMII1640 + 10 % FBS medium.

3) Culture conditions: 10 cm diameter cell culture dish, cell culture box is set as follows: constant temperature 37°C, 5% CO_2 concentration; according to the cell growth condition, exchange fluid or passage every two or every other day.

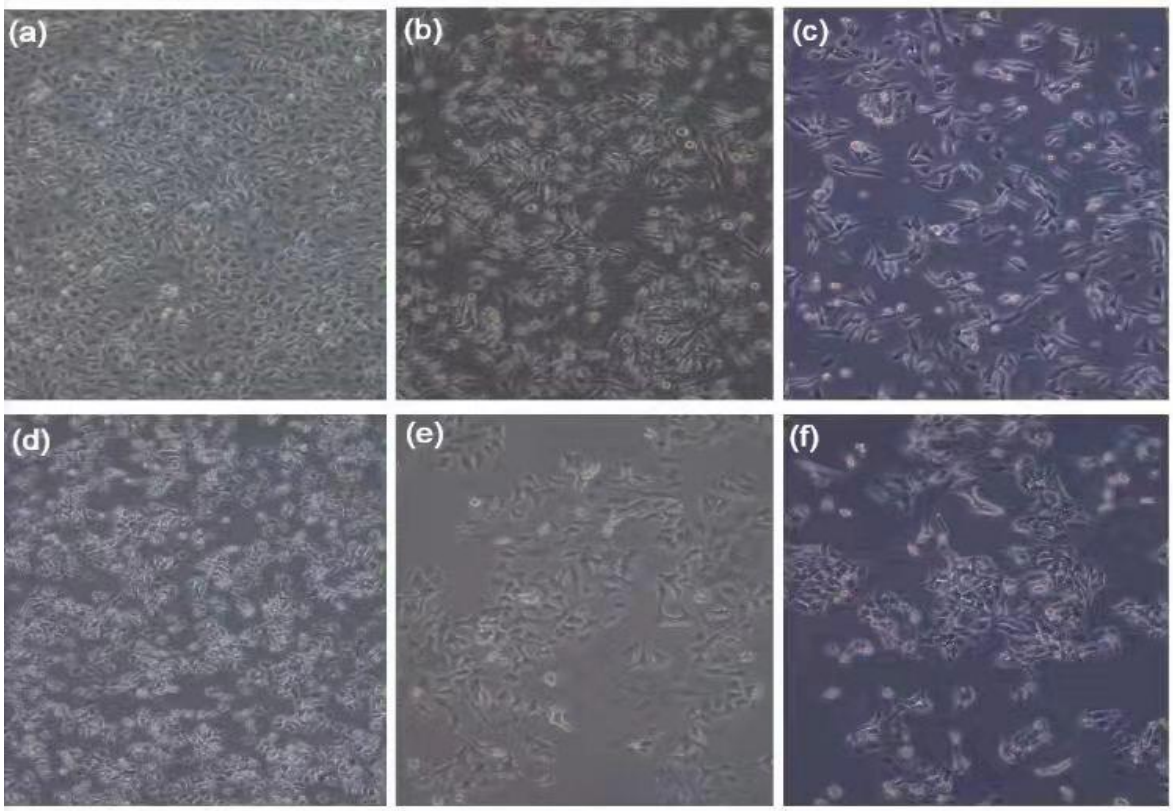


Figure 2.1 – Different types of cell morphology: (a) is B16-F0; (b) is HEPG2; (c) is A375; (d) is RAW 264.7; (e) is A549; (f) is Hela.

2.2.4 Cell passages

Cultured cells were passaged in different ways according to different cells. Adherent cells were passaged by digestion. Part of the adherent growth but not firm cells can also be passaged by direct blowing. Suspended cells can be separated and

passaged by direct blowing or centrifugal precipitation, or directly by natural sedimentation to remove the supernatant, and then blowing and passaged.

Experimental steps:

1) The growth state of cells was observed. When the intercellular space was small, and the cells gradually became round, the cells were subcultured.

2) Absorb the original culture medium from the culture dish, and then absorb 8 mL PBS slowly into the cell culture dish, gently shake, clean the residual culture medium and cell metabolites.

3) 1 mL trypsin digestion solution was added to the cell culture dish and gently shaken. The cells were digested in the incubator for 0.5-1 min. Observation under the microscope showed that the cells were round and shiny, indicating that the cells had been completely digested. The trypsin digestion solution was sucked out and added to the medium containing an appropriate serum.

4) Take a small amount of medium gently blow the bottom of the culture dish cells, along the clockwise direction, each turn 90°, blow the same area, each blow 3-4 times, so that cells into the culture medium, under the microscope to observe cells without the adherent state.

5) Appropriate amount of cell suspension was taken and transferred into a dish. An appropriate amount of medium was added and gently mixed. Cell density was observed under a microscope.

2.2.5 Cell cryopreservation

Slowly freeze cells when frozen. Because when cells are directly frozen without any protective agent, the water inside and outside the cells will quickly form ice crystals, and the formation of ice crystals will cause a series of adverse reactions. First, cell dehydration increases the local electrolyte concentration and changes the pH value. It causes a few adverse effects, namely: the disorder of the internal spatial structure of the cell, the formation of intracellular ice crystals and the denaturation of proteins and enzymes on the cell membrane system, causing the damage of the lysosomal membrane, the release of lysosomal enzymes, the destruction of

intracellular structural components, the swelling of mitochondria, the loss of function and the disorder of cell capacity metabolism. Some proteins are denatured due to the above factors.

The lipoprotein-like complex on the cell membrane is prone to damage during freezing, causing changes in cell membrane permeability and loss of cell contents. DNA in the nucleus is also the vulnerable part of cells during freezing. Suppose more ice crystals are formed in cells with the decrease of freezing temperature. In that case, the volume expansion of ice crystals will cause irreversible damage to the spatial configuration of DNA, resulting in cell death. During cell cryopreservation, it is necessary to reduce intracellular water as evenly as possible, and reducing the formation of intracellular ice crystals is the key to reducing cell damage. At present, glycerol or dimethyl sulfoxide are often used as protective agents. These two substances have no apparent toxicity to cells after deep cryogenic freezing. They have a small molecular weight, high solubility, and easy to penetrate cells, reducing the freezing point and improving membrane permeability to water. In addition, the slow freezing method can make the intracellular water exudate from the cells and form ice crystals outside the cells, reducing the formation of ice crystals inside the cells, thereby reducing the cell damage caused by ice crystals.

Experimental steps:

1) Open the water bath to 37°C, put the medium, PBS, trypsin for preheating, open the ultraviolet lamp in the ultra-clean worktable, irradiated for 30 minutes;

2) Preparation of cell cryopreserved solution: 10% FBS was added to 90% DMEM medium and 90% RPMII1640 medium, respectively. Each cryopreserved tube was calculated according to 1 mL of liquid, and a specific volume of cryopreserved solution was prepared according to the proportion. When the cells were covered at the bottom of the whole culture dish, generally, a culture dish with a diameter of 10 cm could be cryopreserved for 3-4 branches.

3) Remove the frozen cells, discard the supernatant, add 2-3 mL PBS, rinse the cells twice, then add 1-2 mL trypsin digestion for 1-2 minutes;

4) When the cells turn round, use a 2mL medium, neutralise the effect of trypsin, blow repeatedly make the cells fall off, then turn into 15 mL centrifuge tube, 1000 rpm centrifuge 3 minutes; the supernatant was discarded, and 3 mL frozen solution was entirely suspended. After thoroughly mixing, the supernatant was transferred into three cell frozen tubes according to the volume of 1 mL per tube, and the label (name, date of cryopreservation, name) was written.

5) Put the freezer tube into the freezer box and put it in the refrigerator at -80°C overnight. After 24 hours, transfer it into the liquid nitrogen tank.

2.2.6 Cell recoveries

Resuscitation requires rapid melting, which ensures that extracellular crystals melt in a short time. To avoid the damage caused by the slow water softening into the cells to form intracellular recrystallisation.

Experimental steps:

1) Open the water bath to 37°C, put it into the medium for preheating, open the ultraviolet lamp in the ultra-clean workbench, irradiate for 30 minutes.

2) Defrosting. According to the slow freezing and rapid dissolution principle, the target cell line was quickly taken out from the liquid nitrogen tank, prepared for resuscitation, and promptly thawed in a 37 °C water bath.

3) Centrifugation. After melting, it was transferred into a 15 mL centrifuge tube; 2-3 mL medium was thoroughly mixed, centrifuged at 1000 rpm for 3 minutes.

4) Cell culture: Abandon the original supernatant, gently blow with 2 mL medium to make cells thoroughly mixed into the petri dish, adding 8-9 mL medium cross mixing into the cell culture box for culture.

2.2.7 Cytometry

The cell counting method is a basic technology in cell culture research. It is an essential means to understand the growth state of cultured cells and determine the biological effects of medium, serum and drugs. Commonly used cell techniques are the blood counting plate and electronic cell counter counting methods.

Experimental steps:

1) Preparation of single-cell suspension: the cells were thoroughly mixed and diluted according to the cell volume, which was generally diluted by 50 times. If the cell density was large, the dilution multiple could be appropriately increased.

2) Cell suspension dilution: Take a clean 1.5mL Ep tube, calculate the amount of cell suspension and PBS according to the dilution ratio. The total volume is not more than 1 mL, thoroughly mixed.

3) Counting plate: Absorbing 10 μL diluted single-cell suspension, dropping at the cell entrance of the counting plate, standing for 1-2 minutes, counting under the microscope, the area is four squares on the four corners, and then the total number of cells is recorded as N, the counting principle is as follows: count innumerable, count left or right (just one side).

4) Cell density: Cell density (cell number / mL) = $N/4 \times 10^4 \times$ dilution cells / mL.

2.2.8 Cytotoxicity of four polar Extracts on five kinds of tumour cells by MTT cytotoxicity test

The experimental principle is as follows. MTT can react with succinate dehydrogenase in living cells mitochondria to form blue-purple crystalline formazan (Figure 2.2), insoluble in water deposited in living cells.

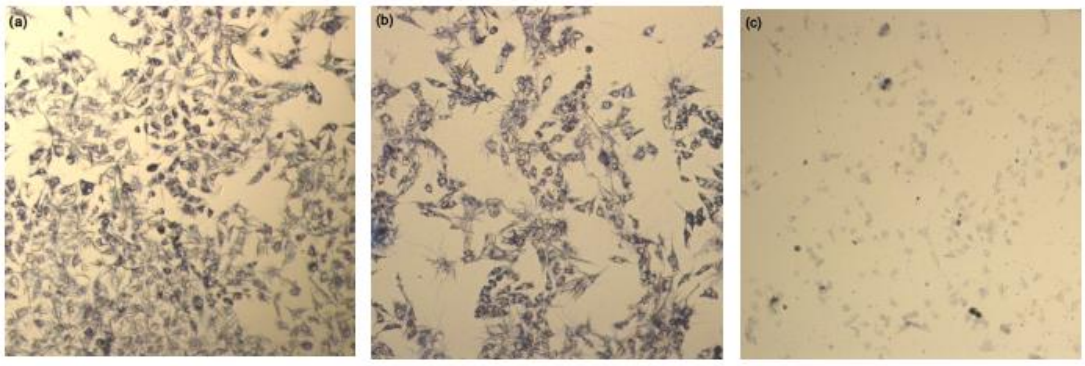


Figure 2.2 – Different amounts of formazan observed under a microscope

The blue-violet formazan has a particular absorption peak at 492 nm after dissolved in DMSO. When the number of collected cells was within a specific range,

the number of living cells was proportional to the absorbance at 570 nm after the dissolution of formazan.

Experimental steps:

1) A549 cells were spread in a 96-cell plate, diluted to 0.5-1×10^4 / well with DMEM + 10 % FBS, and cultured overnight (37°C, 5 % CO_2).

2) The experimental design is shown in Fig. 2-2-8-2, and the cells were cultured for 24 h. When the cells are entirely adherent, the sample is added to stimulate the cells. 12.5, 25, 37.5, 50 and 62.5 (μg/ml) of four polar extracts were added, and the culture system was 100 μL per well. Three replicate wells were set in each group, with the control wells (only cells and DMEM + 10 % FBS medium). 100μl PBS solution was added to the outermost hole.

3) Remove the medium containing the sample, add the appropriate amount of PBS to clean two times, then add 100 μL medium containing 0.5 % MTT (5 mg / mL) per well, continue to culture for 3-4 h.

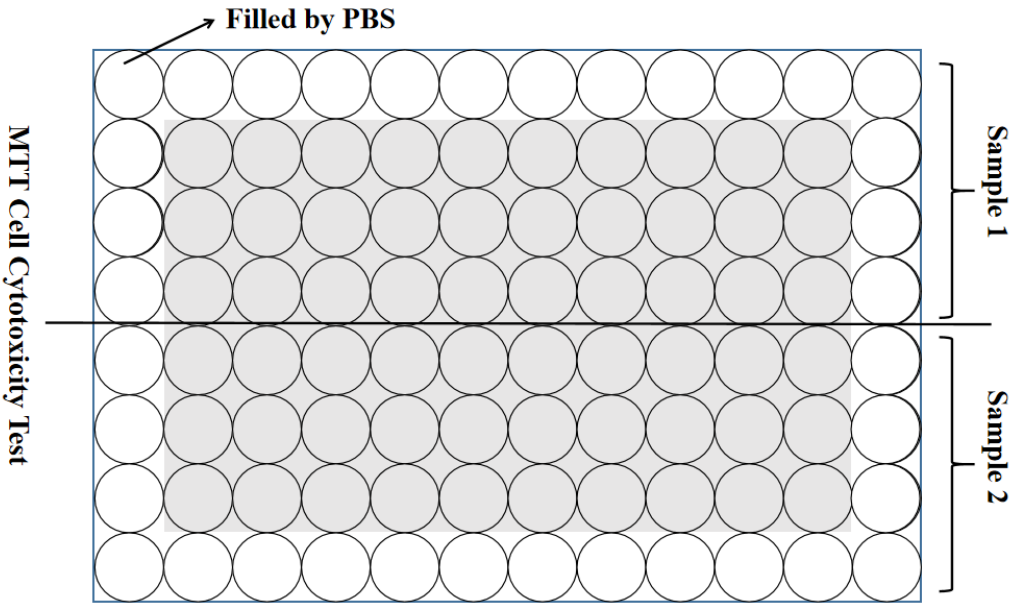


Figure 2.3 – A 96-cell plate schematic diagram of MTT cytotoxicity test

4) Remove the medium containing MTT and buckle the 96-well plate on clean paper three times. 100 μL DMSO was added to each well for 10 min, and the absorbance of the cell lysate was measured at 492 nm. The types of tumour cells are detected following this step.

5) Cell viability was calculated according to the following formula:

Cell viability % = A_X / A_0 × 100 %; A_X is the absorbance of the experimental group, and A_0 is the absorbance of the blank control.

2.2.9 Cell scratch test

The scratch method studied the effects of different ethyl acetate extracts on the cell migration of five tumour cells. The experimental results were used to calculate and analyse the cell healing rate after 24 h of administration and determine whether the tested compounds promoted cell migration or repair. Cell scratch tests can reflect cell repair or migration ability to some extent. After administration, until the end of the experiment, the growth and migration of the surrounding cells before and after the scratch can be observed and compared. The healing rate can be calculated to reflect the migration or repairability of the selected test sample to five tumour cells.

Experimental steps:

1）Before the experiment, the marker pen was used to mark three horizontal lines behind the 6 - well plate. Five tumour cells in the logarithmic phase were taken for cell cultivation. The cells were further cultured to monolayer adherent cells to start the experiment — experimental reaction system 2 mL cell culture medium. The three test concentrations were 37.5, 50 and 62.5 μg/mL, respectively. The three wells were selected as the control group, and only cells and medium were added.

2）Before administration, a 200 μL sterile gun head was used to draw a line perpendicular to the marker line in the centre of the cell plate, washed three times with PBS, added to the medium, and photographed.

3）After treatment, the cells were cultured in the cell culture box for 24 h and photographed until the end of the experiment. Scratch width before and after administration. Photo processing, 0 h scratch width L_0, 24 h scratch width L_{24}.

4）The calculation formula of scratch healing rate is as follows: Scratch healing rate % = (L_0 – L_{24}) / L_0 * 100 %

2.3 Experimental results and analysis

2.3.1 Different polarity crude extracts treatment

Through the extraction and concentration of 100g Speranskia tuberculata (Bunge) Baill, each component is shown in Table 2.5. The concentrated extract was dissolved in dimethyl sulfoxide to prepare 50 mg / mL for the cell model experiment.

Table 2.5 – Quality of crude extracts from Speranskia tuberculata (Bunge) Baill by 4 different polar solvents

Solvent	Petroleum Ether	Ethyl Acetate	n-Butanol	Water
Weight (g/100 g powder)	3.63	1.53	1.32	0.98

2.3.2 Screening and efficacy evaluation of anti-tumour polar parts

MTT cytotoxicity test was used to detect whether the crude extracts of different polarities have killing effects on five kinds of tumour cells. Five tumour cells were stimulated with different types and concentrations of polar crude extracts. All experiments were repeated three times, and the obtained data were analysed by GraphPad Prism software.

The t-test was used for comparison among groups, * $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$. The experimental results are shown in Figures 2.4 - 2.8.

As shown in charts (a) and (d) from Figure 2.4 to Figure 2.8, crude extracts from the polar fraction of petroleum ether and water had no significant effect on the viability of the five tumour cells at different concentrations.

Charts (c) from Figure 2.4 to Figure 2.8 allowed one to conclude that when the sample concentration was greater than 50 μg/mL, the crude extract of the n-butanol polar fraction slightly inhibited the five tumour cells.

Charts (b) from Figure 2.4 to Figure 2.8 showed that the crude extracts of the ethyl acetate polar fraction showed significant killing on all five tumour cells.

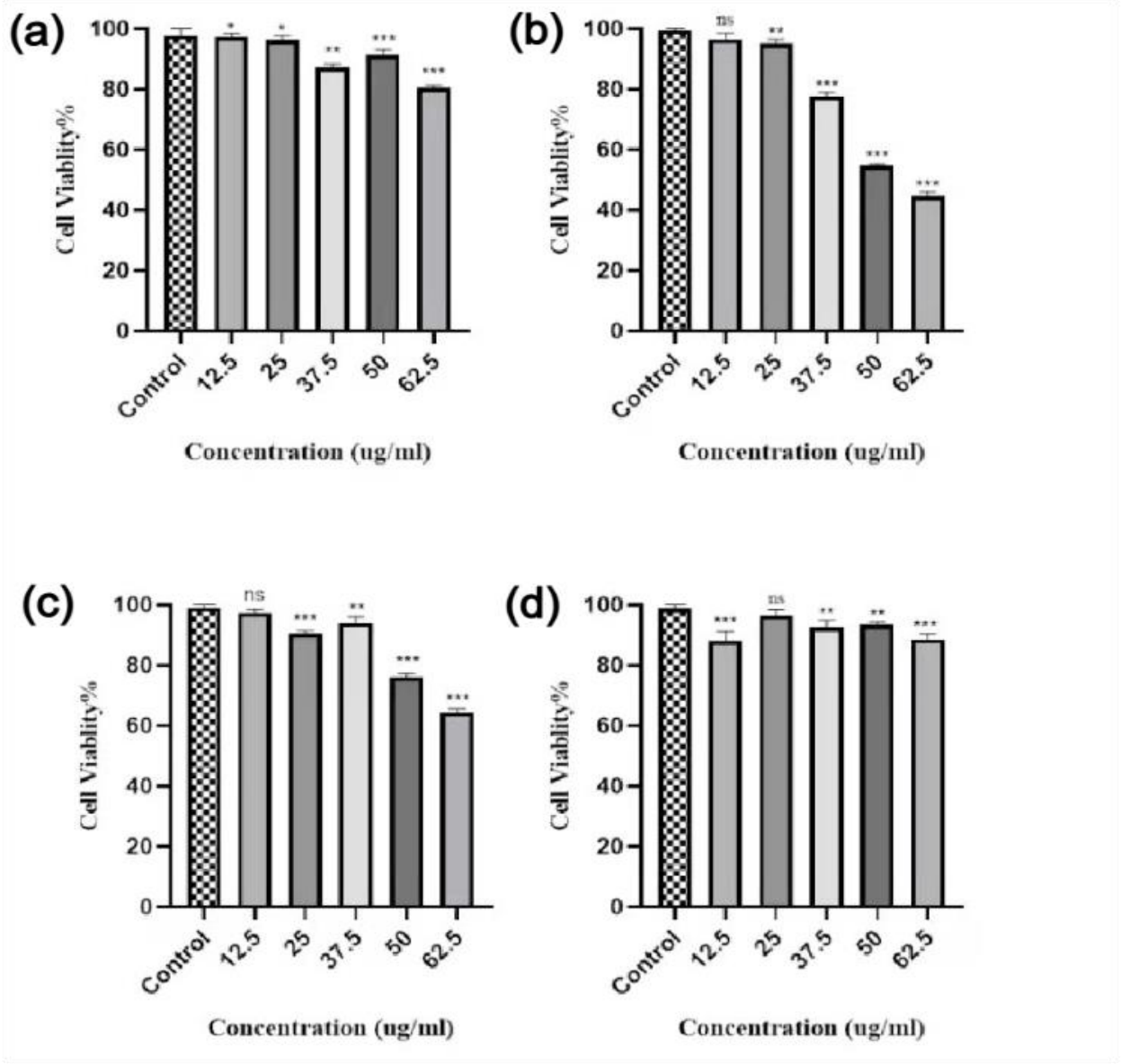


Figure 2.4 – The effects of 4 polar crude extracts on A549 cells. (a) is a crude extract of petroleum ether polarity; (b) is a crude extract of ethyl acetate polarity; (c) is a crude extract of n-butanol polarity; (d) is a crude extract of water polarity

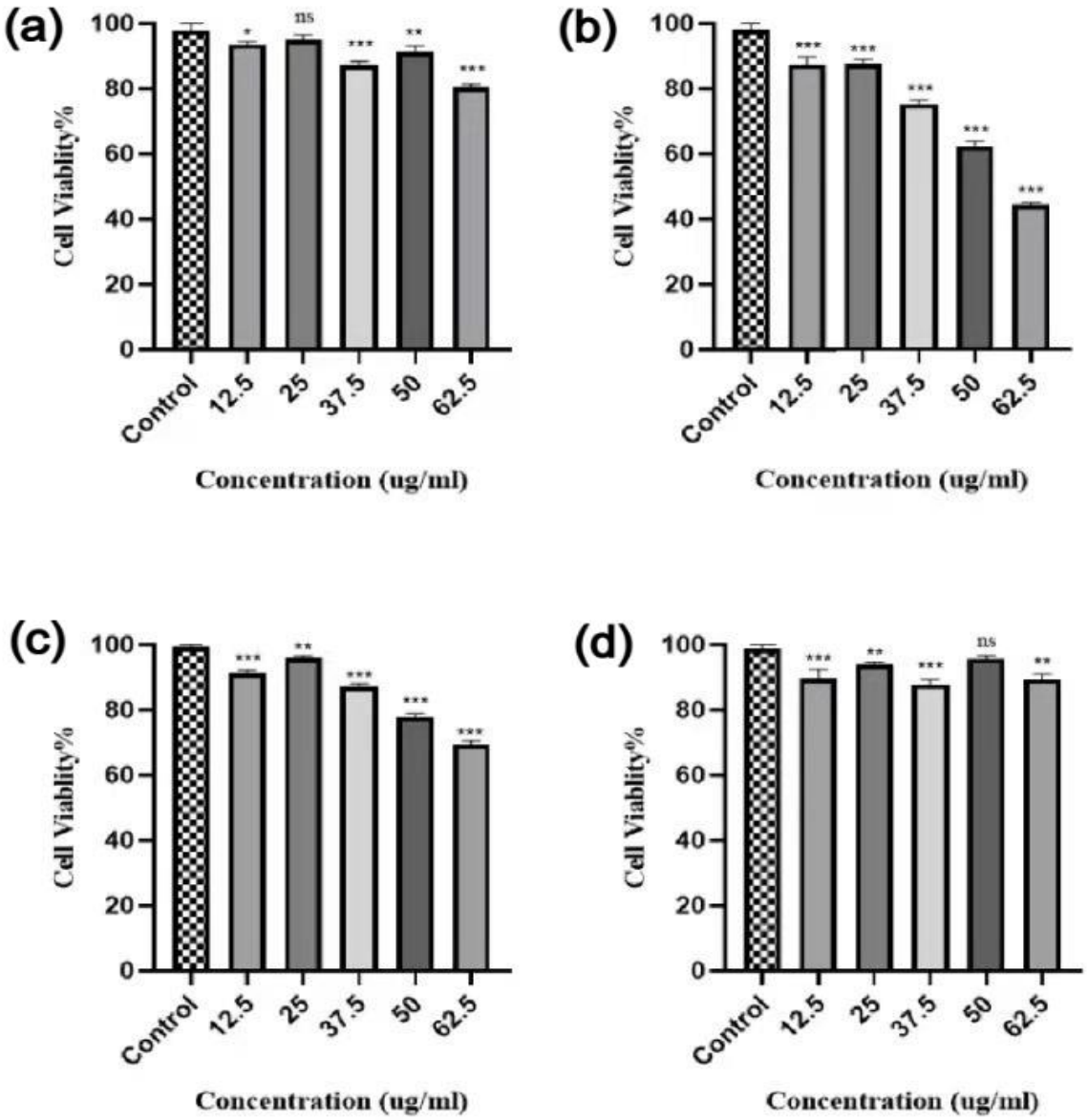


Figure 2.5 – The effects of 4 polar crude extracts on Hela cells. (a) is a crude extract of petroleum ether polarity; (b) is a crude extract of ethyl acetate polarity; (c) is a crude extract of n-butanol polarity; (d) is a crude extract of water polarity

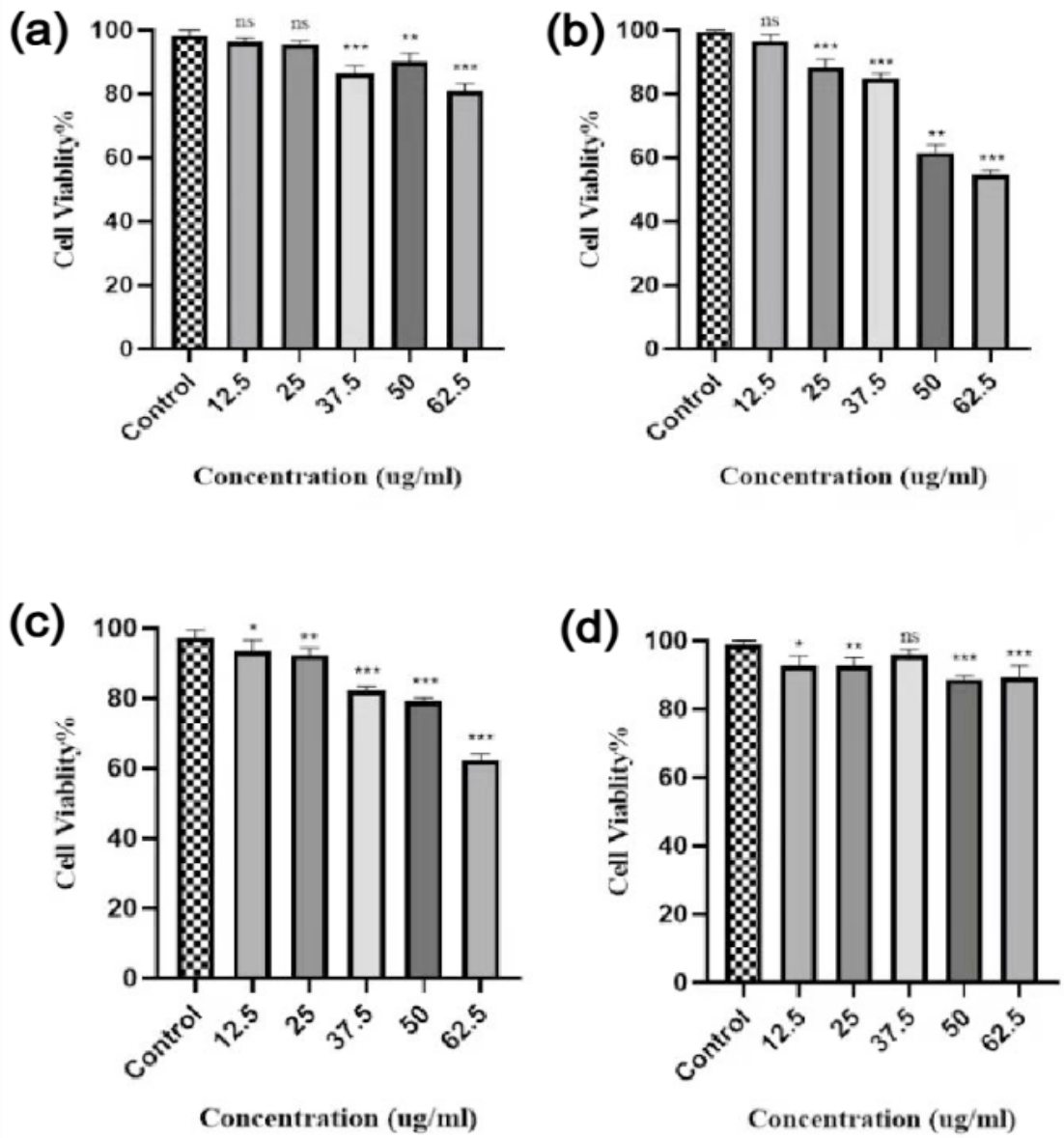


Figure 2.6 – The effects of four polar crude extracts on B16-F0 cells. (a) is a crude extract of petroleum ether polarity: (b) is a crude extract of ethyl acetate polarity; (c) is a crude extract of n-butanol polarity; (d) is a crude extract of water polarity

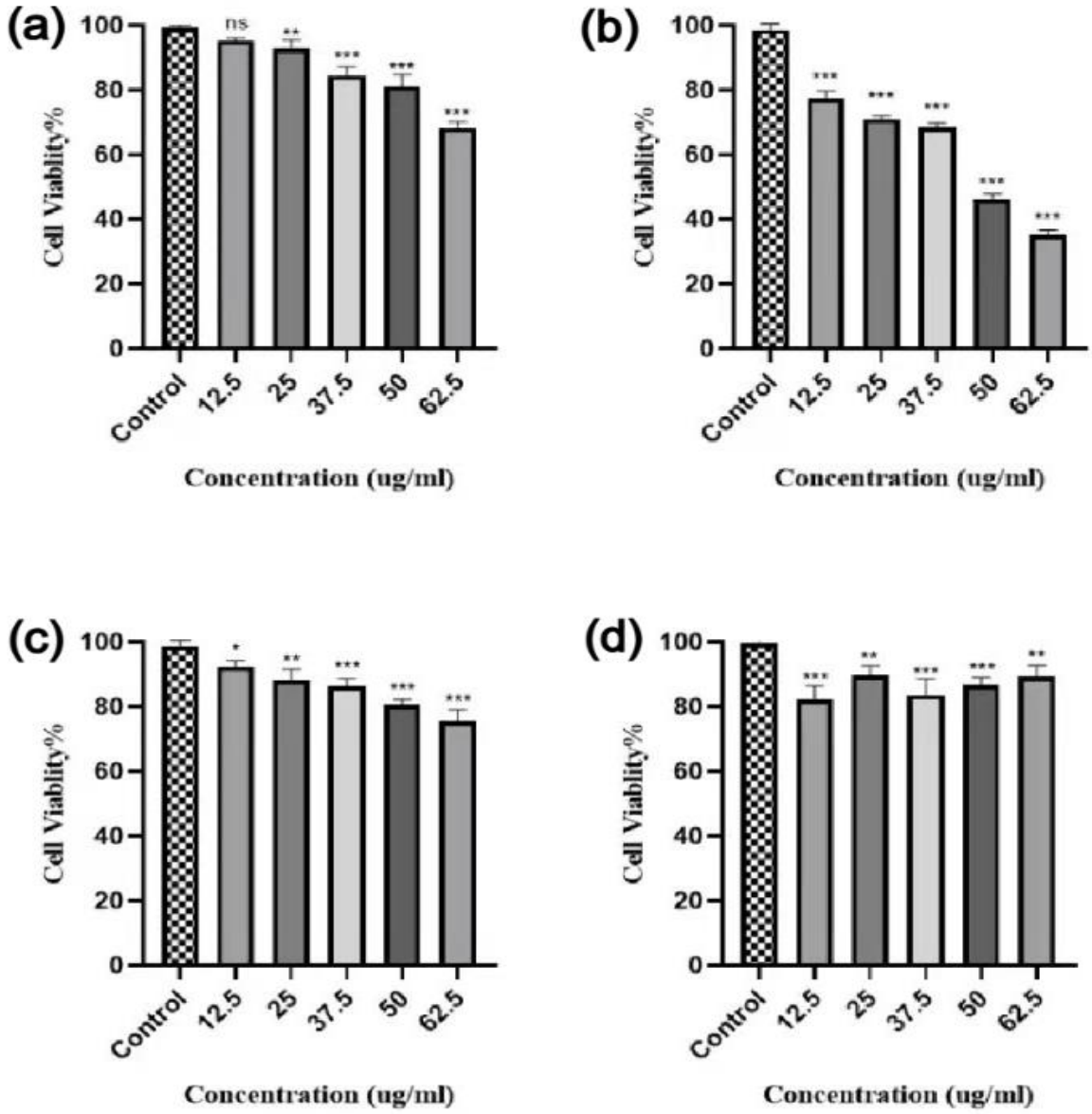


Figure 2.7 – The effects of 4 polar crude extracts on HEPG2 cells. (a) is a crude extract of petroleum ether polarity; (b) is a crude extract of ethyl acetate polarity; (c) is a crude extract of n-butanol polarity; (d) is a crude extract of water polarity

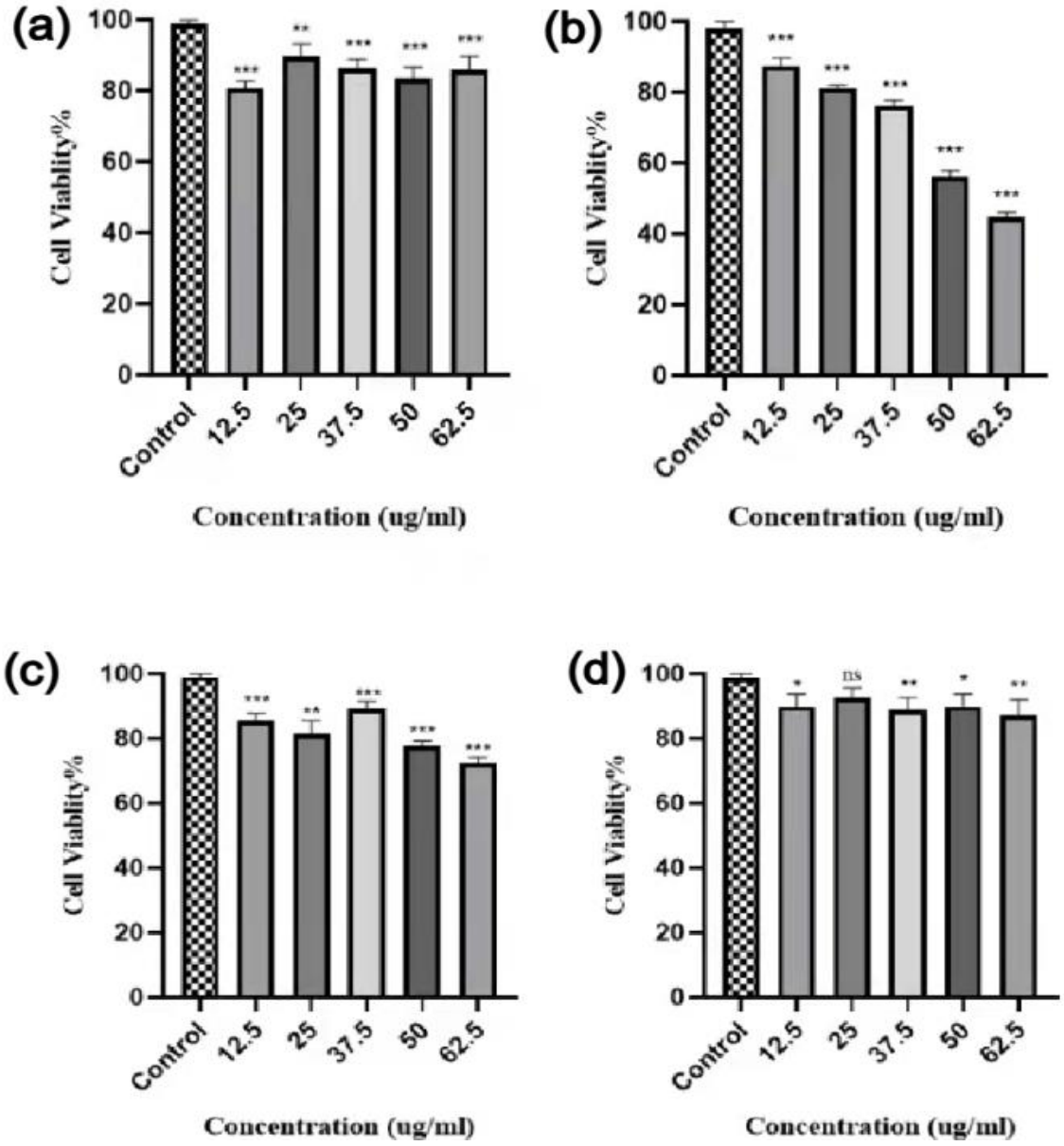


Figure 2.8 – The effects of 4 polar crude extracts on A375 cells. (a) is a crude extract of petroleum ether polarity; (b) is a crude extract of ethyl acetate polarity; (c) is a crude extract of n-butanol polarity; (d) is a crude extract of water polarity

To verify which crude tumour extract of the ethyl acetate polar fraction inhibited the most, we again performed three sets of MTT cytotoxicity experiments that set the concentration gradient to 12.5、25、37.5、62.5、50、75、100 (μg/ml). The other experimental conditions remained unchanged, determining the IC_{50} of 5 tumour cells

as shown in Figure 2.9.

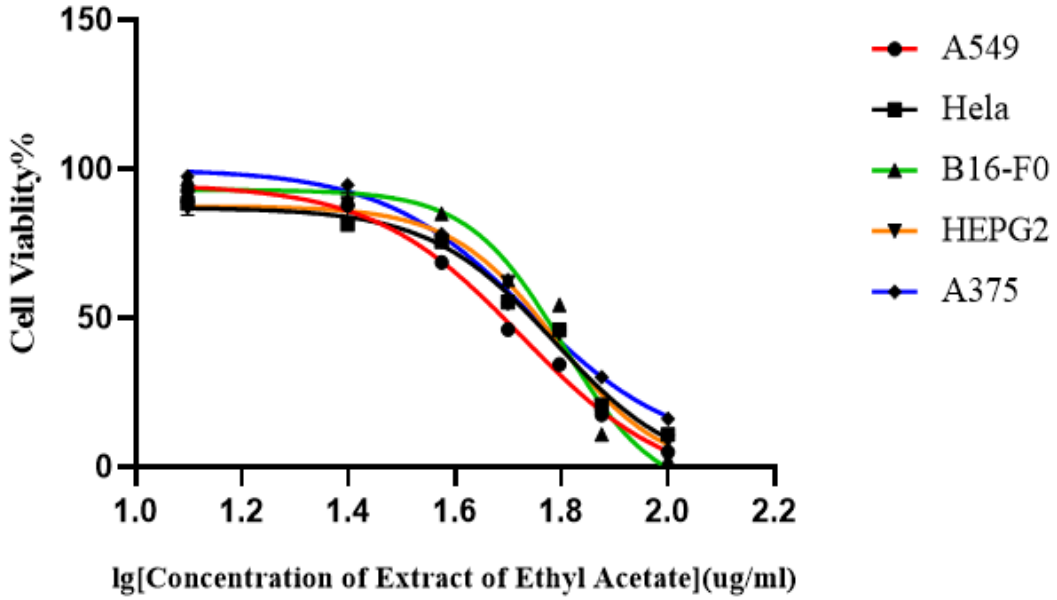


Figure 2.9 – IC_{50} of ethyl acetate extracts on 5 different tumour cells

The obtained data were analysed using GraphPad Prism software. The calculated average IC_{50} values for ethyl acetate extracts and standard deviations are shown in Table 2.6 for five different tumour cells. As is seen, the crude extracts of ethyl acetate polarity had the lowest IC_{50} in A549 cells. Therefore, among these 5 cells, A549 cells are most sensitive to stimulating crude extracts with ethyl acetate polarity and exhibit the best inhibition of A549 cells.

Table 2.6 – The average values of IC_{50} for 5 different tumour cells in ethyl acetate extracts

Cells	**A549**	**Hela**	**B16-F0**	**HEPG2**	**A375**
IC_{50}	53.09±	61.11±	63.21±	64.41±	54.61±

2.3.3 Analysis of cell scratch test

The scratch method studied the effects of different ethyl acetate extracts on the cell migration of tumour cells. The experimental results were used to calculate and analyse the cell healing rate after 24 h of administration and determine whether the tested extracts promoted cell migration or repair. The healing rate can be calculated to

reflect the migration or repairability of the selected test sample to 4 tumour cells. The measured experimental data are shown in Table 2.7 and Figure 2.9 to 2.13.

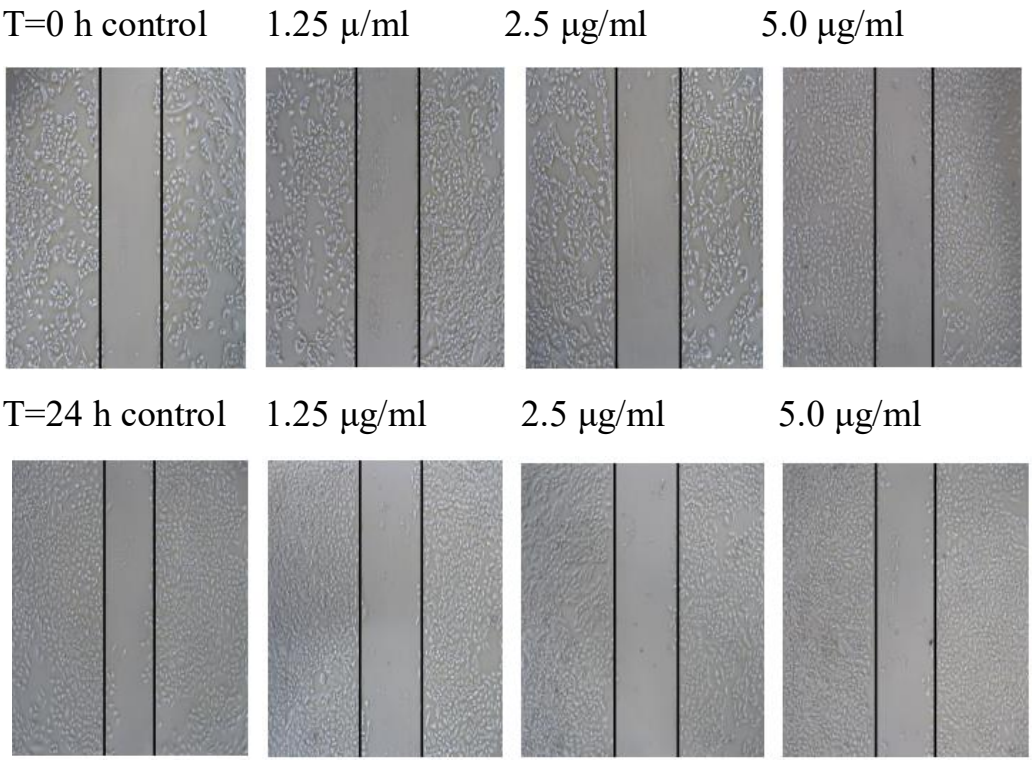
Table 2.7 – The healing rates and inhibition rates of 5 types of tumour cells

Types of Cells	Concentration (μg/mL)	Healing Rate (%) (mean±SEM）	Inhibition Rate (%)
Ctr	-	32.84±0.26	-
A549	1.25	28.27±0.57	4.57
	2.5	26.35±0.56	6.49
	5.0	15.95±0.19	16.89
Ctr	-	42.61±0.15	-
Hela	1.25	39.18±0.26	3.43
	2.5	32.62±0.43	9.99
	5.0	25.76±0.22	16.85
Ctr	-	38.89±0.63	-
B16-F0	1.25	37.14±1.40	1.75
	2.5	29.19±0.36	9.70
	5.0	25.82±0.39	13.07
Ctr	-	35.74±0.26	-
HepG2	1.25	32.27±0.38	3.47
	2.5	25.29±0.42	10.45
	5.0	19.17±0.33	16.57
Ctr	-	30.99±0.37	-
A375	1.25	26.93±0.41	4.06
	2.5	22.51±0.78	8.48
	5.0	15.76±0.22	15.23

When the crude extract of the polar fraction of ethyl acetate was added at a concentration of 1.25 μg/mL, it did not practically affect the healing ability of all these four cells, with a healing rate of 28.27% (A549 cells), 39.18% (Hela cells), 37.14% (B16-F0 cells), 32.27% (HepG2) and 26.93 % (A375 cells). The inhibition of the healing rate is only 4.57%, 3.43%, 1.75%, 3.47% and 4.06%, respectively, compared to the control sample.

When the crude extract was added at a concentration of 2.5 μg/mL, it had a more apparent inhibitory effect on the healing of all tumour cells, with a healing rate of 26.35%, 32.62%, 29.19% and 22.51%, respectively. And inhibitory healing rates of Hela cells, B16-F0 cells and A375 cells were around 10%.

When the concentration of the crude extract was 5 μg/mL, the healing rate of these tumour cells was significantly inhibited, with a healing rate of only 15.95%, 25.76%, 25.82%, and 15.76%, respectively. All inhibited healing rates exceeded 15%, and A549 cells were the highest in the concentration of 5 μg/mL, which was 16.89%.



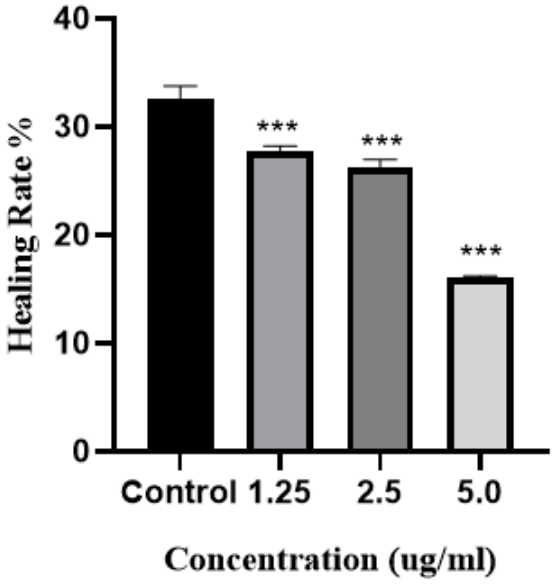
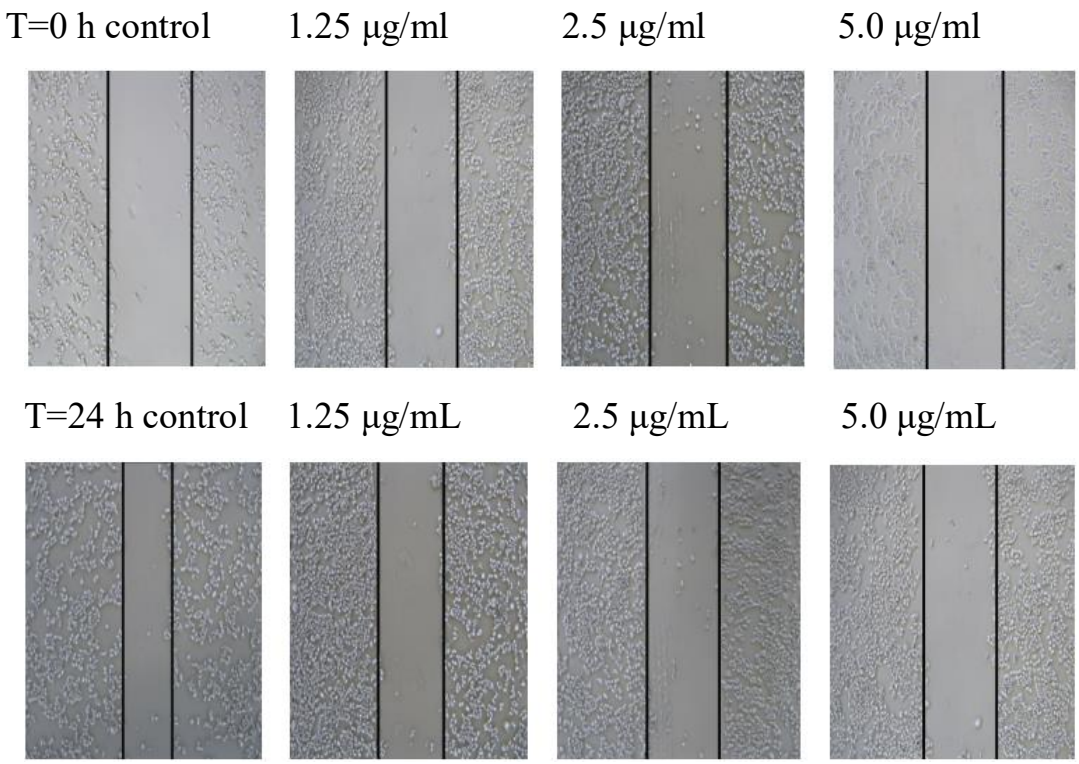


Figure 2.9 – The results of the A549 Cell Scratch Test of the crude extract of ethyl acetate polarity.

The t-test was used for comparison among groups,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

all of the concentration groups were compared with the control group



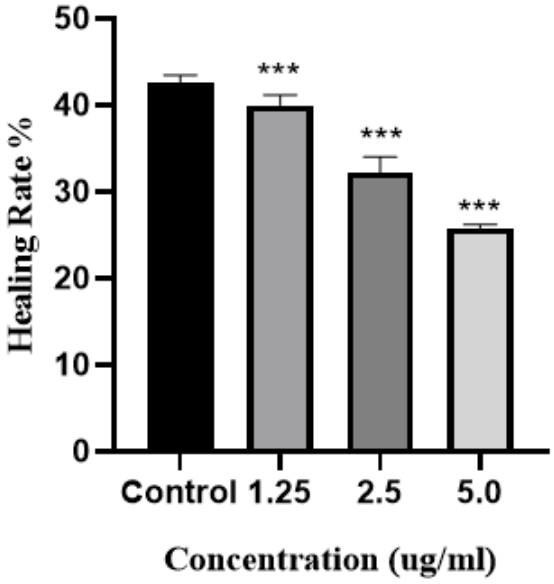
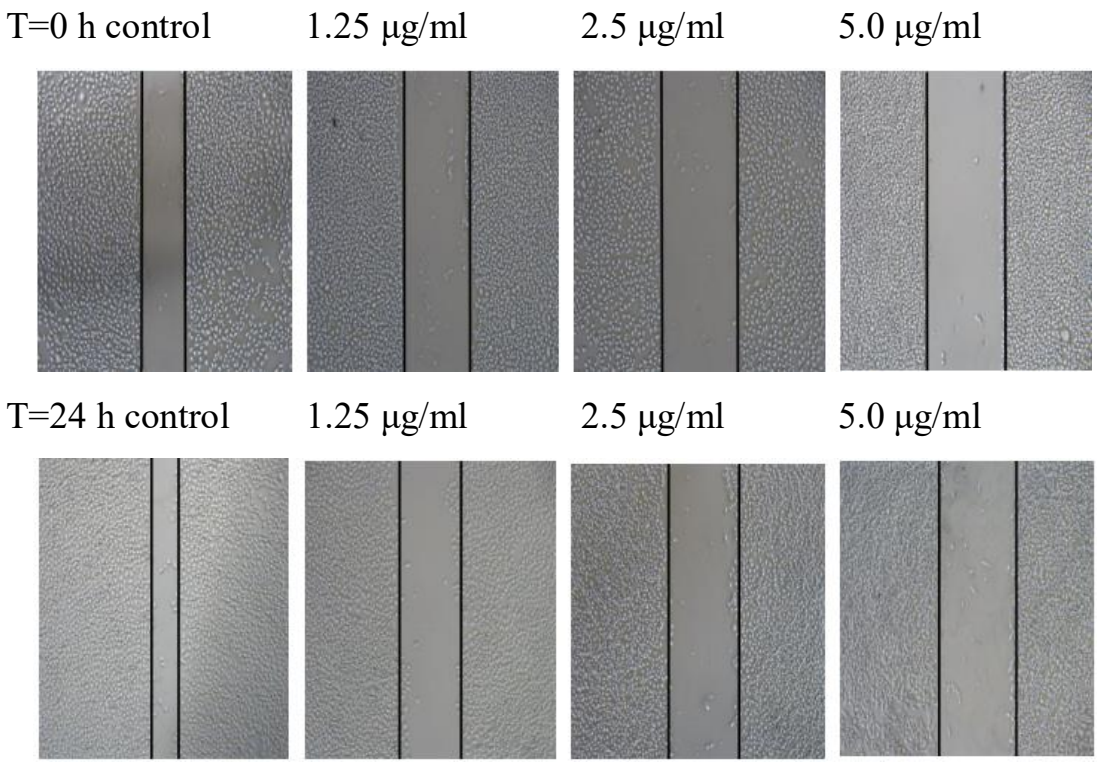


Figure 2.10 – The results of Hela Cell Scratch Test of the crude extract of ethyl acetate polarity

The t-test was used for comparison among groups,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

all of the concentration groups were compared with the control group



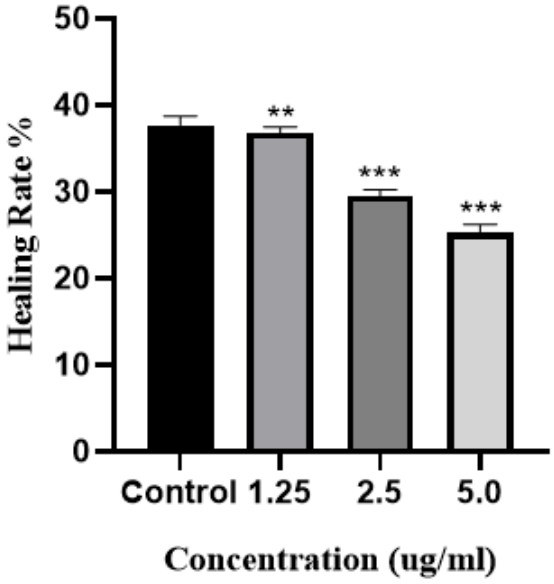
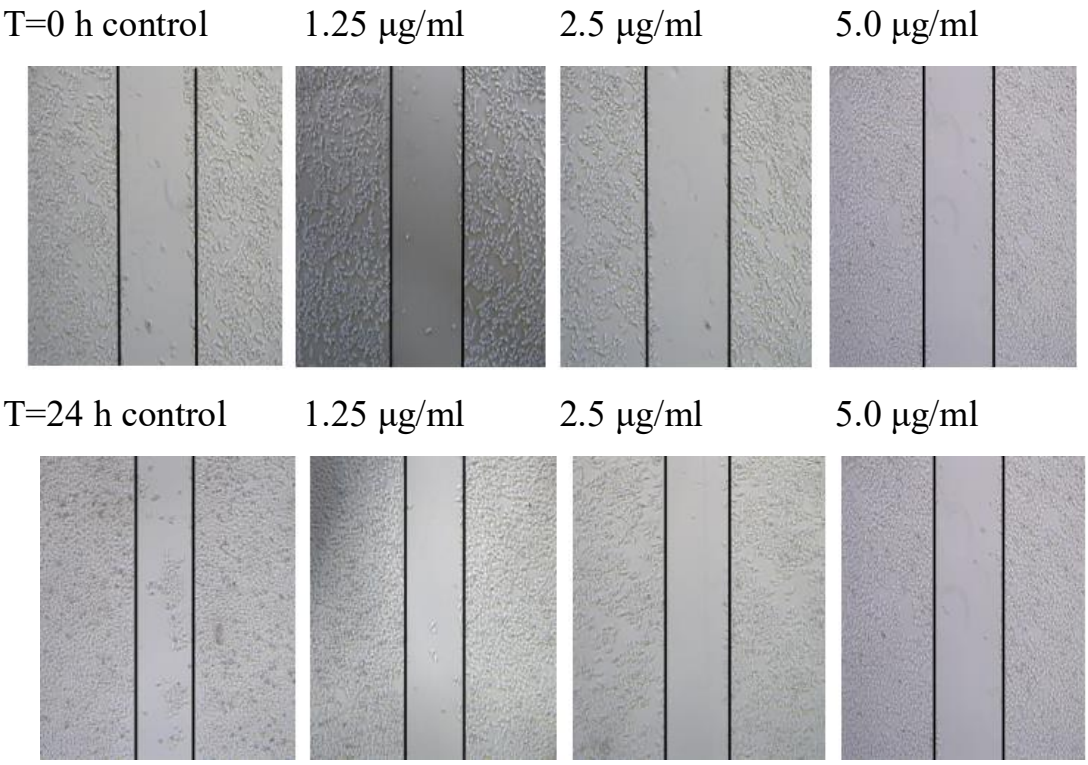


Figure 2.11 – The results of the B16-F0 Cell Scratch Test of the crude extract of ethyl acetate polarity

The t-test was used for comparison among groups,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

all of the concentration groups were compared with the control group



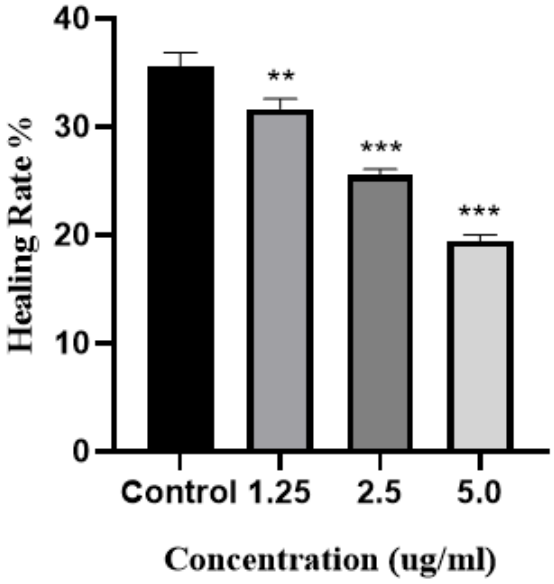
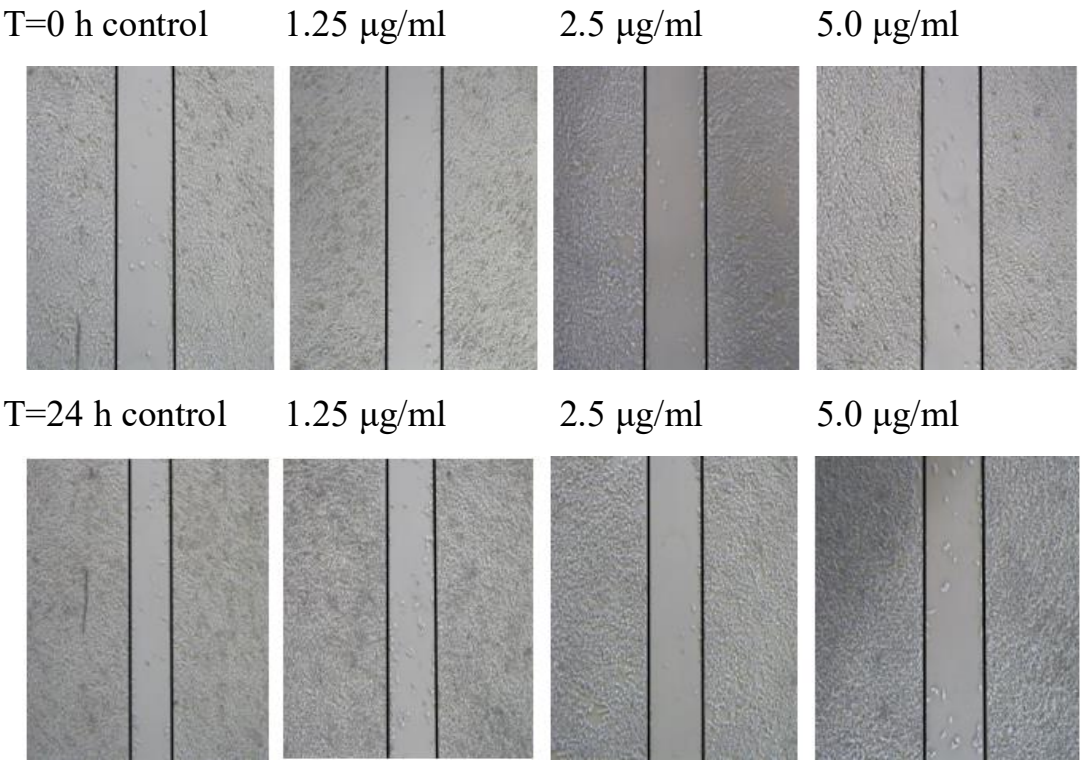


Figure 2.12 – The results of HepG2 Cell Scratch Test of the crude extract of ethyl acetate polarity

The t-test was used for comparison among groups,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

all of the concentration groups were compared with the control group



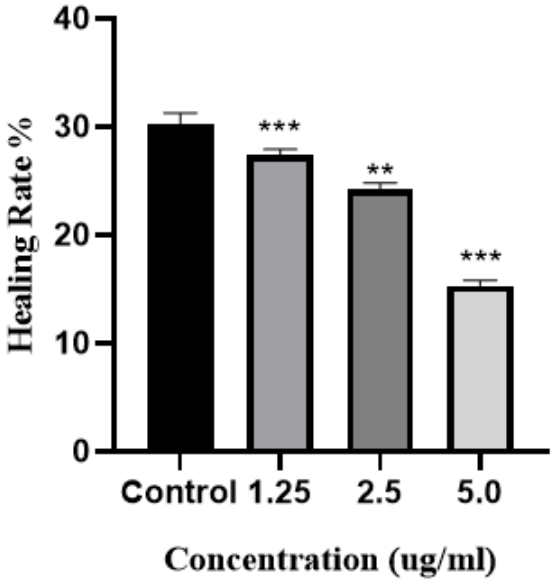


Figure 2.13 – The results of the A375 Cell Scratch Test of the crude extract of ethyl acetate polarity

The t-test was used for comparison among groups,

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

all of the concentration groups were compared with the control group

Conclusions to Chapter 2

1. The crude extracts of different polar parts of the Speranskia tuberculata (Bunge) Baill were extracted and separated.
2. Four crude extracts with different polarities, petroleum ether, ethyl acetate, n-butanol and water, were obtained and screened for anticancer activity using the MTT cytotoxicity and cell scratch tests.
3. The crude extracts of ethyl acetate had a significant ability to kill five types of tumour cells.
4. It exerted the most significant impact on A549 cells.
5. In contrast, the three polar crude extracts of petroleum ether, n-butanol and water did not have the powerful inhibitory ability on these cells.

CHAPTER 3. ANTI-ATHEROSCLEROSIS EFFECT OF BIO-ACTIVE COMPONENTS OF SPERANSKIA TUBERCULATA (BUNGE) BAILL

Atherosclerosis (AS) is a chronic progressive disease. The main feature is narrowing the arterial vessel lumen under the deposition of atherosclerotic plaques, causing poor blood circulation. Eventually, this leads to ischemic damage to downstream tissues. AS not only directly endangers human life and health, it can also cause a series of high-risk diseases such as aneurysm, aortic dissection and coronary artery disease. It has a high morbidity and fatality rate, and it is difficult to treat, and the treatment cost is high. With the gradual improvement of people's living standards and the excessive intake of high-nutrient and high-fat foods by middle-aged and older people, AS has become one of the major diseases that middle-aged and older people are prone to suffering from. Approximately more than 24 million people die every year worldwide. Because of AS and the related diseases caused by it, it is increasing year by year [67].

In recent years, after countless scientific researchers have continuously explored the mechanism of AS, many pathogenic mechanisms have been proposed one after another. Due to the complexity and diversity of the pathogenic mechanism of AS, various hypotheses are supported by some scholars. Also, there are many controversies and doubts. At present, the most accepted pathogenic factors of AS are mainly related to high blood pressure, hyperlipidemia, smoking, drinking, obesity, genetics and other factors. The well-recognized mechanism theory includes cholesterol metabolism imbalance and oxidative stress leading to endothelial dysfunction. , Inflammation and so on.

The main characteristics of AS are an abnormal accumulation of lipids in blood vessels and activation of inflammatory response. Abnormal lipid metabolism leads to the accumulation of cholesterol and other lipid substances in cells, forming foam cells and lesions forming plaques. The plaque ruptures due to inflammation, creating a thrombus, squeezing the arterial cavity and narrowing the vessel lumen [68]. The factors that cause AS disease are very complex, including the interaction and

influence between various cells, the interaction between activated free radicals and low-density lipoprotein, and the secretion and induction of various cytokines. Under the combined action of multiple mechanisms of macrophage differentiation, monocyte migration, endothelial cell damage and proliferation, foam cell formation, and apoptosis, it promotes the aggregation of lipid particles on the arterial intima to form lipid fragments [69]. The appearance of lipid fragments is the beginning of the AS process. At the same time, endothelial cells will proliferate and migrate after being injured and interact with smooth muscle cells to form a fibrous network, enveloping a large number of lipid fragments and forming early plaques in the arteries. Plaque in the arteries accumulates continuously, and fibrosis develops a convex bulge, which compresses the space in the blood vessel, causing blood circulation to be blocked, causing downstream tissue damage, and in severe cases, tissue necrosis. Inflammation destroys the stability of the plaque. Under the induction of inflammatory factors, the macrophages infiltrate the lipid plaque. They secrete many proteolytic enzymes, which destroys the fibrous surface of the plaque and causes the plaque to rupture, form thrombus, and reduce atherosclerosis. The hardening process is pushed to a high-risk period [70].

At present, the clinical treatment of AS is mainly to reduce the blood lipid level in the body by adjusting the body's response. There are fewer types of drugs, mainly divided into the following types: 1. Statins, mainly by inhibiting hydroxymethylglutaric acid monoacyl. The role of coenzyme A reductase (HMG-CoA) reduces the production of own cholesterol. For example, lovastatin and simvastatin have better effects; 2. Nicotinic acid drugs have the function of increasing the enzyme activity of lipoprotein esterase. To accelerate the decomposition of cholesterol, for example, asimimus has a better effect; third, fibrate drugs, similar to niacin drugs, can accelerate the deterioration of cholesterol but at the same time have the function of increasing HDL-c in the serum. Drugs mainly include bezafibrate, phenafibrate, etc.; four bile acid chelator drugs can inhibit bile acid circulation in the liver and reduce cholesterol intake by cells. The representative drugs are mainly

cholesterol, Acid, colestipol, etc. There are relatively few drug treatments at present. They can only participate in the treatment of AS by directly or indirectly inhibiting the synthesis of one's cholesterol or promoting cholesterol decomposition. Although these drugs have a certain therapeutic effect on AS, they also have many side effects, such as loss of liver function, causing gastrointestinal dyspepsia, allergic reactions, etc., which cause certain damage to the human body.

Traditional Chinese herbal medicine has significant comprehensive treatment effects and low side effects. It can be directly used as a medicine or food additive to participate in the prevention or treatment of AS. Domestic and foreign research on Chinese herbal extracts and their active ingredients in treating AS has been remarkable in recent years. Studies have proven that Chinese herbal extracts such as Ginkgo biloba [71], Phu Huanglian [72], Panax notoginseng [73], Usnea Alcohol [74] have good curative effects on the prevention and treatment of atherosclerosis. Many active compounds have also been reported to have the effect of preventing and treating atherosclerosis. Wang [75] et al. found that E3317 can increase the expression of ABCA1 and promote cholesterol efflux, thereby treating AS. Yu [76] et al. found that ApoE mice fed with a high-fat diet can effectively prevent AS after consuming quercetin and further studied that quercetin has the function of inhibiting oxidative stress and protecting endothelial damage. At the same time, Zhang [77] found that quercetin has the function of regulating the secretion of ROS and cytokines IL-8, NF-κB, etc., inhibiting the progress of inflammation, controlling cell apoptosis, and playing an anti-AS effect. Zhou [78] et al. found that retinoic acid can effectively reduce arterial plaque in ApoE mice by regulating ABCA1 and ABCG1 to promote cholesterol efflux.

This chapter mainly studied the anti-atherosclerosis effects of the crude extracts of Speranskia Tuberculata (Bunge) Baill of petroleum ether fraction (PE), mainly divided into the following six parts:

1. MTT cytotoxicity test verifies that whether the PE has a killing effect on macrophages (RAW 264.7 cells) or not;

2. Oil red O staining and detection of oxidized low-density lipoprotein content;

3. PE inhibits the absorption of cholesterol by cells and promotes macrophages Cholesterol efflux experiment;

4. Determination of cholesterol homeostasis in macrophages;

5. QTOF-LC/MS characterization of the PE;

6. Determination of total polyphenol and flavonoid content of PE.

3.1 Experimental methods

3.1.1 Experimental Apparatus

Table 3.1 – Experimental Apparatus

Apparatus	**Model**	**Manufacturer**
Multifunctional Microplate Reader	Spectra Max M5	Molecular Device
High-speed Freezing Centrifuge	Neofuge 1600R	Shanghai Lishen Scientific Instrument Co., Ltd
Cell Ultra Clean Bench	ZHJH-C115B	Shanghai Zhicheng Instrument Analysis and Manufacturing Co., Ltd.
Ultralow Temperature Freezer	DW-86L728J	Haier Biomedical Co., Ltd.
Pure Water Producer	STU4100	Shanghai Suitian Environmental Protection Technology Co., Ltd.
Vortex Mixer	Vortex-2	Shanghai Hushi Industrial Co., Ltd.
Decoloration Shaker	TS-1	Qilinbeier Instrument Co., Ltd.
BD Flow Cytometer	BD FACS Calibur	Bidi Medical Equipment Co., Ltd

Apparatus	Model	Manufacturer
Cell Culture Box	311	Semerfeld Technology Co., Ltd
HH Series Digital Display Constant Temperature Water Bath Pot	XMTD203	Jiangsu Science and Technology Instrument Co., Ltd
Rotating Evaporator / Governor	RE-52AA/RE-201D	Shanghai Yarong Biochemical Instrument Factory
Collector Constant Temperature Heating Magnetic Stirrer	DF-101S	Gongyi Yingyu High-tech Instrument Factory
KQ-300DE ultrasonic instrument	KQ-300E	Kunshan Ultrasonic Instrument Co., Ltd
DM IL LED Inverted Microscope	Leica DM IL LED	Leica

3.1.2 Experiment reagent

Table 3.2 – Experimental reagents

Experimental drugs and reagents	Specification	Manufacturer
4%neutral formaldehyde	biotechnology level	MACKLIN
23-(dipyrrometheneboron difluoride)-24-norcholesterol	99%	Avanti
methyl-cyclodextrin	99%	SIGMA
Cholate solution	98%	MACKLIN
8-(4-Chlorophenylthio)adenosine 3’5’-cyclic monophosphate	99%	SIGMA

Experimental drugs and reagents	Specification	Manufacturer
sodium salt		
Trizol	reagent level	Solarbio
Tween 20	reagent level	Solarbio
HDL	biotechnology level	Yiyuan Biotechnologies
TC Content Detection Kit		Solarbio
FC Content Detection Kit		Solarbio
HiFiScript cDNA Synthesis Kit		CWBIO
Dil-oxidize LDL		Yiyuan Biotechnologies
oil redo	analytically pure	Ron Reagent
4% polyformaldehyde	biotechnology level	SIGMA
Oxidize LDL		Yiyuan Biotechnologies
22-hydroxycholesterol	≥98%	SIGMA
cis-retinoic acid	≥98%	SIGMA
isopropanol	analytically pure	Tianjin Fuyu Fine Chemical Co., Ltd.
hematoxylin	biotechnology level	MACKLIN

3.1.3 MTT cytotoxicity test

Experimental steps:

1) RAW 264.7 cells were spread in a 96-cell plate, diluted to 0.5-1×10^4 / well with DMEM + 10 % FBS, and cultured overnight (37 °C, 5 % CO_2).

2) When the cells are entirely adherent, the sample is added to stimulate the cells. 12.5, 25, 37.5, 50 and 62.5 (μg/ml) of PE were added, and the culture system was

100 μL per well. Three replicate wells were set in each group, with the control wells (only cells and RPMI1640+10%FBS). 100μl PBS solution was added to the outermost hole, and the cells were cultured for 24 h.

3) Remove the medium containing the sample, add the appropriate amount of PBS to clean two times, then add 100 μL medium containing 0.5 % MTT (5 mg/mL) per well, continue to culture for 3-4 h.

4) Remove the medium containing MTT and buckle the 96-well plate on clean paper three times. 100 μL DMSO was added to each well for 10 min, and the absorbance of the cell lysate was measured at 492 nm. The types of tumour cells are detected following this step.

5) Cell viability was calculated according to the following formula:

Cell viability % = A_X / A_0 × 100 %; A_X is the absorbance of the experimental group, and A_0 is the absorbance of the blank control.

3.1.4 Oil Red O Staining

Experimental Principle: Oil Red O is a fat-soluble dye that dissolves well within fats and can specifically stain neutral fats (such as triglycerides). This property can be used to distinguish foam cells by colouration. After Oil Red O stains, the fat inside the foam cells, the red substance dissolves in isopropyl alcohol, and the solution has a strong absorption at 518 nm.

Experimental Steps:

1) The experiment uses cells RAW264.7 planted in 12-well plates, the cell density controlled at 2~3×10^4 cells/well, at 37℃, 5% CO_2, it is incubated until the cell state is stable and fully adhered to the wall.

2) After the cells were stabilized, ox-LDL was added to each well at a concentration of 50 μg/mL and blank control (0.1% DMSO) was set, and the sample group was PE, with a concentration of 10 μg/ml and 20 μg/ml, and incubated for 24 h.

3) After the sample incubation was completed, the medium containing the samples was removed, the cells were washed three times with PBS, 1 mL of 4%

polyformaldehyde was added to each well for 10 min of fixation, rinsed three times with PBS, washed with an appropriate amount of 60% isopropanol, 1 mL of 0.3% oil red O dye was added to each well for 15 min of staining, and then washed twice with 1 mL of 50% isopropanol, and IX 73 inverted microscope to observe and take pictures.

4) Finally, 500 μL of isopropanol was added to each well and shaken at room temperature for 10 min to fully dissolve the oil red O in the cells, and the absorbance was measured at 518 nm.

3.1.5 Detection of ox-LDL content

1) Macrophages RAW264.7 were spread in 6-well plates with a cell density of 2×10^4 cells/well and cultured until the cells were in a stable state and completely adhered to the wall.

2) After the cells were stabilized, a control group (0.1% DMSO) was set up, and 50 μg/mL PE was added to the experimental group, and the cells were incubated for 24h. Then 50 μg Dil-ox-LDL was added to each well and removed after 6 h of incubation.

3) After the incubation of Dil was completed, the medium containing Dil was removed and washed three times with PBS, the cells were digested with trypsin and blown up to the suspension, the cytosol was collected, centrifuged for 5 min at 2500 RPM at room temperature, then washed two times with PBS, the centrifugation step was repeated, and the cell precipitate was collected.

4) Blow up the cell precipitate to suspension with 300 μL PBS, mix upside down, and flow cytometry collects 104 cells to analyse the fluorescence intensity in each cell (flow cytometer FL2, excitation wavelength: 514 / 549 nm, emission wavelength: 565 nm).

3.1.6 Measurement of macrophage cholesterol dynamic homeostasis

Inhibition of Cellular Uptake of Cholesterol.

BODIPY-labelled cholesterol was added to macrophages after co-incubation by

the action of PE and other drugs. After the action, the cells are lysed, and the fluorescence intensity in the cells is measured. The fluorescence intensity in the cells is proportional to the amount of cholesterol they have ingested.

1) Fluorescent dye BODIPY-cholesterol: Prepare 20 mg/mL cholesterol solution, weigh 100 mg of cholesterol dissolved in 5 mL of 75 ℃ ethanol, pipette 0.135 mL of 20 mg/mL cholesterol solution in a conical flask, add 1 mL of 1 mg/mL of 23-(dipyrromethene boron difluoride)-24-norcholesterol, mix gently, rotate and evaporate until dry, add 35 mL RPMI1640 medium to dissolve, add 0.92 g methyl-cyclodextrin and rotate and stir until completely dissolved, sonicate for 30 min at 37°C, store in a water bath for 3 h at 4°C, and sonicate for 30 min before use.

2) The cell line used for the cholesterol efflux experiment was mouse macrophage RAW 264.7, grown in 24-well plates at a cell density of $3\sim5\times10^4$ cells/well, 37℃, 5% CO2, and incubated overnight until the cells have completely adhered to the wall.

3) Each well was added 500 μL of RPMI1640 + 0.2% BSA medium mixed with the samples to be tested, with a drug concentration of 20 μg/ml for PE, a blank control (0.1% DMSO) and positive control of caffeic acid (CA) 20 μM. It was incubated for 18 h at 37°C in a 5% CO_2 incubator.

4) After the samples were incubated, 1 mL of BODIPY-Cholesterol (BODIPY-Cholesterol: RPMI1640 = 1:1) fluorescent dye was added to each well for 2 h. The fluorescent dye was carefully aspirated away, 500 μL of PBS was added to each well for 2 washes, and 500 μL of RPMI1640 medium was washed for 2 washes.

5) Add 0.5 mL Cholate solution sodium cholate solution to each well to lyse the cells, shake to lyse for 4 h. Add 200 μL cell lysate to each well of the enzyme labelling plate and measure the fluorescence intensity of the cell lysate at an excitation wavelength of 482 nm and an emission wavelength of 515 nm on the enzyme labelling instrument. Intracellular cholesterol content = (A×2.5)/cellular protein concentration

Pro-cholesterol efflux assay in macrophages.

TOPFluence is a fluorescent probe that binds to cholesterol and does not affect cell physiology. The fluorescent cholesterol accumulates in the cells after being engulfed by macrophages by binding to cholesterol. Then the apolipoprotein that promotes intracellular cholesterol efflux is added, and the intracellular fluorescent cholesterol is discharged into the cell culture medium. After adding the sample reagent to stimulate the cells, we determine whether the addition of the sample reagent has, to some extent, promoted the efflux of cholesterol from the cells by testing the fluorescence intensity remaining in the final cell culture medium and the cells.

1) The experiment used cells RAW 264.7 grown in two 24-well plates, one for sample experimental assay, the other as T=0 for fluorescence intensity control (only fluorescence staining after DMSO stimulation for 18 h), cells diluted with RPMI1640 then grown in 24-well plates, incubated until cells completely spread to the bottom of 24-well plates.

2) Add 1 mL BODIPY-Cholesterol (BODIPY-Cholesterol: RPMI1640 = 1:1) per well to stain for 1.5 h. Carefully aspirate the fluorescent dye away, add 500 μL PBS per well to wash two times and 500 μL serum-free RPMI1640 (SF RPMI1640) to wash two times.

3) Then 500 μL of medium mixed with the drug to be tested (SF RPMI1640+0.2% BSA) at PE 10 μg/ml and 20 μg/ml, positive control at CA 20 μM and blank control (0.1% DMSO) was added to each well and incubated for 18 h at 37°C in a 5% CO2 incubator.

4) After 18 h of sample stimulation, the medium was carefully aspirated, 500 μL SF RPMI1640 was added to each well and washed carefully for two times, 500 μL SF RPMI1640, high-density lipoprotein (HDL) (100 μg) of SF RPMI1640 was added to act for 18 h. Meanwhile, the cell medium of the T=0 plate was carefully aspirated, 1 mL sodium cholate solution was added to each well, and the fluorescence intensity of T0 cells was measured on a shaker for 4 h. The excitation wavelength: 482 nm, emission wavelength: 515 nm.

5) After promoting cholesterol efflux for 18 h, the medium of the cells was carefully transferred to a 1.5 mL centrifuge tube, centrifuged at 4000 RPM, 5°C, for 10 min. In contrast, the cells were lysed by adding 1 mL of sodium cholate solution per well, protected from light for 4 h. The fluorescence intensity of the cell supernatant and cell lysate was measured by enzyme marker (excitation wavelength: 482 nm, emission wavelength: 515 nm). Cholesterol efflux %=A(sample cell supernatant)/A[(sample cell supernatant)+T0 cells]

Determination of Total Cholesterol Content in Macrophages

Esterases hydrolyse cholesteryl esters to produce free cholesterol (FC) and fatty acids (FFA), and FC is catalysed by cholesterol oxidase to produce 4-Cholestenone and H_2O_2. H_2O_2 and 4-aminoantipyrine and phenol generate red quinones by the action of peroxidase, with a specific absorption peak at 500 nm.

1) Sample Preparation: Experiments were performed using RAW264.7 cells grown in 6-well plates, and after the cells were plastered entirely, 50 μg/mL ox-LDL was added to incubate the cells for 12 h, followed by the addition of PE concentration of 20 μg/ml to co-incubate the cells for 12 h. Add 500 μL isopropanol to each well, scrape the cells from the 6-well plate with a spatula, pipette and blow several times, transfer into a centrifuge tube, sonicate and crush for 2 min at 12000 RPM, centrifuge for 10 min, and collect the supernatant.

2) Standard Solution Preparation: 5 μM of the standard solution was diluted with isopropyl alcohol into 2, 1.25, 0.625, 0.3125, 0.15625, 0.078 μM of standard solution and set aside.

3) Sample Test: The reagents were added sequentially by order in Table 3.3.

Table 3.3 – Sample addition order of Total Cholesterol (TC) Content Test Kit

Reagent	**Standard Group**	**Blank Group**
Standard Solution (μL)	20	/
Isopropyl Alcohol (μL)	/	20
Working Solution (μL)	180	180

The solution was mixed thoroughly and left to stand at 37°C for 15 min, and the absorbance of the sample at 500 nm was measured.

4) Standard Curve Plotting: The concentration of the standard solution was used as the horizontal coordinate, and A (standard - blank) was used as the vertical coordinate to plot the equation of the standard curve: y=0.5016x-0.0583. TC Calculation Formula: TC (μmol/dL) = x×100

Determination of Free Cholesterol Content in Macrophages

1) Sample Preparation: Experiments were performed using cells RAW264.7 grown in 6-well plates, and after waiting for the cells to adhere to the wall completely, 50 μg/mL ox-LDL was added incubate the cells for 12 h. After that, a PE concentration of 20 μg/ml was added to co-incubate the cells for 12 h. Add 500 μL isopropanol to each well, scrape the cells off the plate wall with a spatula, pipette and blow several times, transfer to a 1.5 mL centrifuge tube, sonicate and crush for 10 min at 12000 RPM, centrifuge for 10 min, and collect the supernatant as a sample.

2) Cellular Protein Concentration Determination: Bradford measured the sample protein concentration.

3) Sample measurement: The working solution of the kit, preheated at 37°C for 30 min in advance, and reagents were added sequentially by the order in Table 3.4.

Table 3.4 – Free Cholesterol (FC) Determination of the Order of Solvent Addition

Reagent Name	**Blank Group**	**Standard Group**	**Sample Group**
Anhydrous Ethanol	10	/	/
50 μmol/ml Cholesterol	/	10	/
Sample to be tested	/	/	10
Working Solution	190	190	190

After adding reagents was completed, mix well and let stand at room temperature for 15 min, then measure the absorbance at 500 nm.

FC relative content calculation: FC content (μmol/mg prot) = [A (sample group - blank group))/A (standard group - blank group)]/sample protein concentration

3.2 Experimental results and analysis

All experiments were repeated three times, and the statistical analysis was carried out with the help of GraphPad Prism software. The comparison between the groups was performed by t-test. *p＜0.05, **p＜0.01 mean statistical difference, ***p＜0.001 means significant statistics difference.

3.2.1 The results and analysis of the MTT cytotoxicity test

The results of the MTT cytotoxicity test are shown in Figure 3.1. The PE has no significant killing effect on macrophages (RAW 264.7).

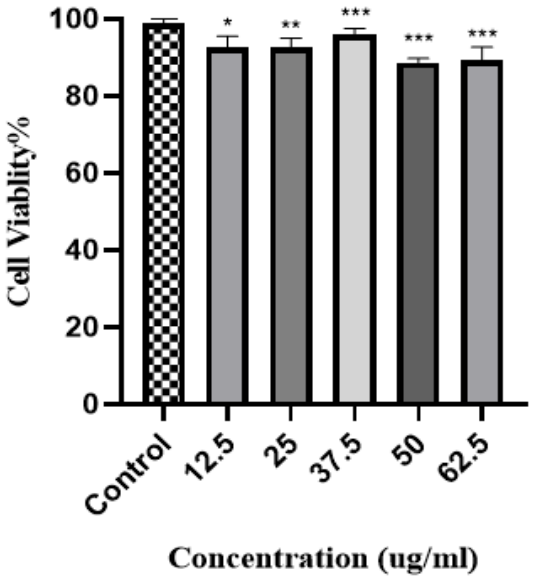


Figure 3.1 – The effects of PE on RAW 264.7 cells.

3.2.2 The results and analysis of Oil red O staining

The Oil red O staining experiment was performed on macrophages with different concentrations of PE. Under the incubation of ox-LDL, macrophages swallow many cells and convert them into fat in the cells. Oil red O dye stains the lipid-rich cells, as shown in Figure 3.2. PE incubation of the Oil red O staining area after the cells was significantly less than the DMSO group, which proved that petroleum ether polar extracts could reduce the accumulation of lipids in the cells to a certain extent, protect macrophages and reduce the formation of foam cells.

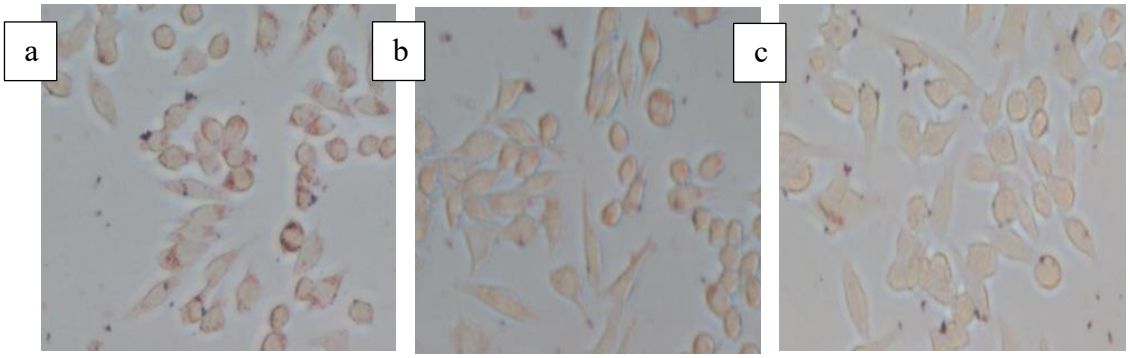


Figure 3.2 – Graphic representation of oil red O staining results. (a) DMSO +50 μg/mL ox-LDL; (b) 10μg/ml PE + 50 μg/mL ox-LDL; (c) 20μg/ml PE + 50 μg/mL ox-LDL

After isopropyl alcohol dissolves the cells, measure the absorbance at 518 nm. As shown in Figure 3.3, the absorbance value is directly proportional to the Oil red O content in the cells to determine the fat content in the cells.

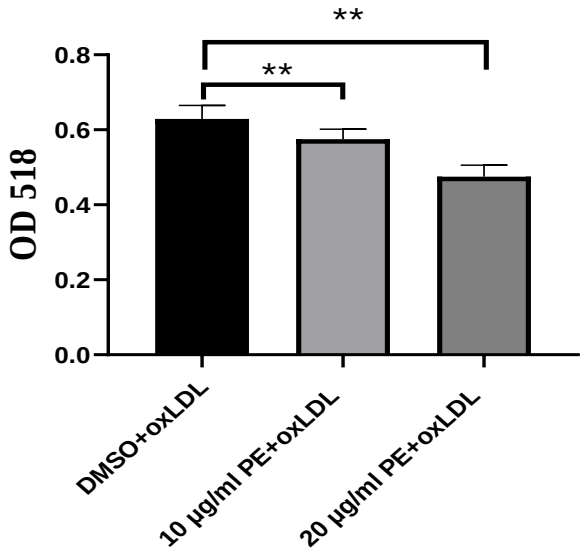


Figure 3.3 – Isopropanol dissolves the cells stained with Oil Red O and measures the absorbance at 518nm

The average absorbance at 518 nm at a PE concentration of 10 μg/mL was 0.56, the average absorbance at 518 nm at a 20 μg/mL concentration was 0.48, and the DMSO group was 0.62. The value of the PE group was significantly lower than that of the DMSO group, which proved that the Oil red O content in the cells was significantly less than that of the DMSO group. Therefore, it can indicate that the

lipids in the macrophages of the PE group were considerably less than in the DMSO group.

3.2.3 The results and analysis of the content determination of ox-LDL

Dil fluorescence detection of oxidized low-density lipoprotein content in cells is shown in Figure 3.4. Using flow cytometry to detect Dil-oxLDL, analyse the content of ox-LDL in cells. Use fluorescently labelled ox-LDL to measure the Dil fluorescence intensity in the cells after DMSO or PE is incubated with the cells. The fluorescence intensity of Dil is directly proportional to the content of ox-LDL. The stronger the fluorescence intensity, the higher the content of ox-LDL. The stronger the fluorescence intensity of the Dil label, the higher the peak shift to the right, the higher the ox-LDL content in the cell.

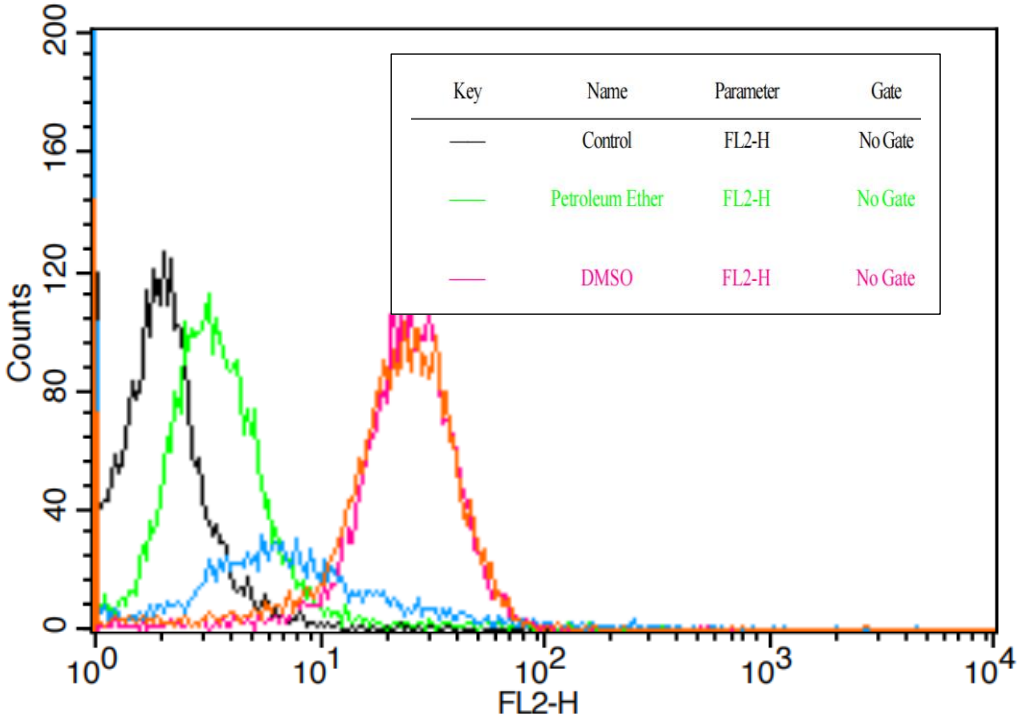


Figure 3.4 – The effect of PE and CA on the uptake of ox-LDL on macrophages

Figure 3.4 shows that the abscissa represents the increased fluorescence intensity from left to right. Control is the blank group without Dil-oxLDL. Since there is no fluorescence in the cells, it is located on the leftmost side of the abscissa. The Dil fluorescence intensity in the cells is relatively weak. When PE and ox-LDL were

added to incubate the cells, the fluorescence curve shifted significantly to the left relative to the group of cells incubated with DMSO and ox-LDL, the fluorescence intensity decreased, and the ox-LDL content in the cells decreased, indicating the PE could effectively reduce the ox-LDL in the cells. In the group which added ox-LDL, the curve shifted significantly to the right.

3.2.4 The results and analysis of inhibiting the absorption of cholesterol of macrophages

The beginning of atherosclerosis disease is the deposition of lipids in macrophages, which significantly affects cholesterol balance. Therefore, controlling the balance of intracellular cholesterol content is particularly important for preventing and treating atherosclerosis.

Measure the effect of PE on cholesterol uptake by macrophages using fluorescently labelled cholesterol (Figure 3.5).

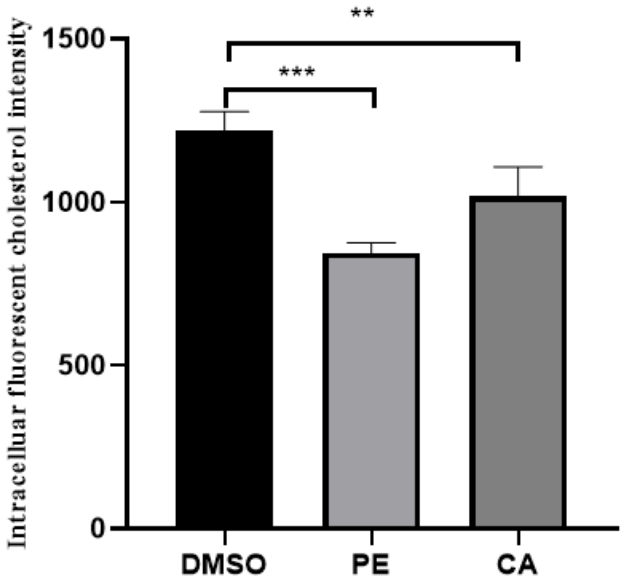


Figure 3.5 – PE and CA acted on macrophages and phagocytosis of fluorescent cholesterol, the fluorescence intensity of fluorescent cholesterol in the cell

As shown in Figure 3.5, at the excitation wavelength of 482 nm and the emission wavelength of 515 nm, the fluorescence intensity of the PE group was 843, while the average fluorescence intensity of cells in the DMSO group was 1218, and the average fluorescence intensity of cells in the CA group was 1016. The average fluorescence

intensity in the cells of the PE group was lower than that of the DMSO group. It is proved that PE can effectively inhibit the uptake of cholesterol by the cells and reduce the cholesterol content in the cells.

3.2.5 The results and analysis of pro-cholesterol efflux assay in macrophages

The experimental results of measuring the ability of PE to promote cholesterol efflux in cells are shown in Figure 3.6. When intracellular cholesterol uses HDL as a cholesterol carrier to flow to the liver, the outflow rate of cholesterol in the DMSO group was 12.5%. The outflow rate of the PE low-dose group (10 μg/ml) increased to 14.7%, when the concentration of PE increased to 20 μg/ml, the outflow rate of cholesterol increased to 18.2%, which was obviously dose-dependent. After the CA (20 μM) treatment, the cholesterol efflux rate can also significantly increase to 16.5%.

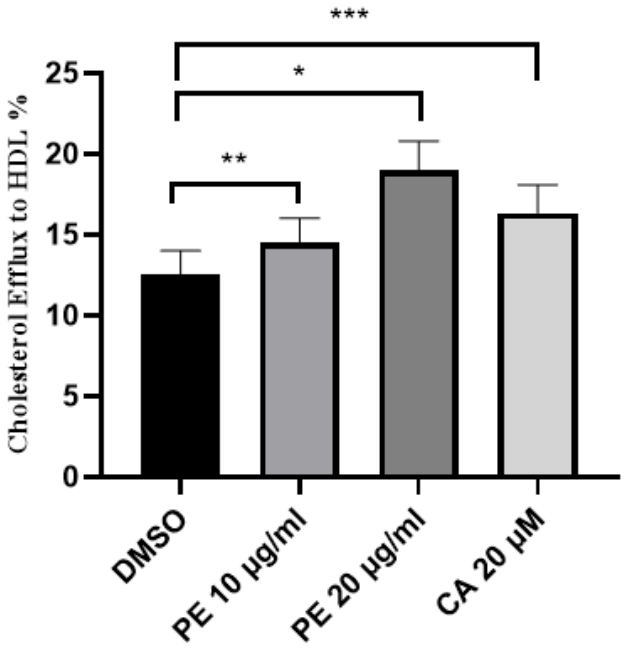


Figure 3.6 – The percentage of cholesterol flowing to HDL

3.2.6 Determination of Total Cholesterol (TC), Free Cholesterol (FC) and Cholesterol Ester (CE) contents in macrophages

In the presence of ox-LDL, macrophages will take up a large amount of ox-LDL. ox-LDL is broken down into free cholesterol by lysosomes in cells, distributed in

cells, and converted into cholesterol particles under the action of cholesterol esterase (CE). PE can effectively reduce the intracellular TC and FC. As shown in Figure 3.7, in the Control group, without the co-incubation of ox-LDL, the content of FC and TC in macrophages is lower. In the presence of ox-LDL, macrophages swallow a large amount of it and convert it into cholesterol, and the overall FC and TC are greatly improved. Under the action of PE, the content of TC was 135 nmol/mg, significantly less than 155 nmol/mg in the DMSO group. Also, under the action of PE, the intracellular FC content was 110 nmol/mg, and the DMSO group was 122 nmol/mg, and the FC content also decreased to a certain extent.

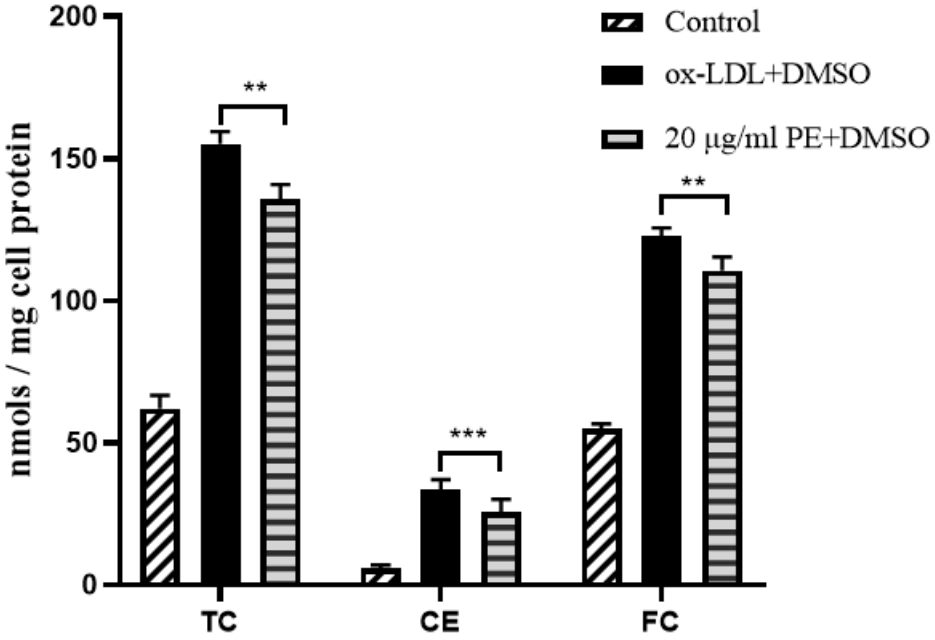


Figure 3.7 – Determination of cholesterol content in macrophages induced by ox-LDL under the action of PE.

FC: Free Cholesterol; CE: Cholesterol Ester; TC: Total Cholesterol

3.3 Determination of total polyphenol and flavonoid content of PE

The entire polyphenol content of different extracts was examined with the Folin-Ciocalteu reagent, and the resulting blue complex was measured at 680 nm using a SpectraMax M5 spectrophotometer. PE was used as a standard for the calibration curve, and the total polyphenol content was expressed as mg PE

equivalents per gram of dry weight of extracts.

A commercial enzymatic kit examined the flavonoid content. The absorbance of the resulting complex was measured at 430 nm. Rutin was used as a standard for the calibration curve, and total flavonoid data were expressed in mg of rutin equivalents (RE) per gram of dry weight. The results are shown in Table 3.5.

Table 3.5 – Percentage of the PE in 1g Speranskia Tuberculata (Bunge) Baill powder and the content of phenolic and ketones substances in 1g of PE

Samples	**Yield (% of original powder)**	**Total Phenolic Content (mg PE/g)**	**Total Flavonoid Content (mg RE/g)**
Petroleum ether extracts (PE)	0.36 ± 0.03	21.53 ± 1.15	39.27 ± 1.46

3.4 QTOF-LC/MS characterization of the PE

The characterization of PE was performed using quadrupole time-of-flight tandem liquid-chromatograph/mass-spectrometer (QTOF-LC/MS, Agilent Technologies, USA). The separation of compounds was achieved on water cortex C18 2.1*50mm 1.7µm column in gradient mode. Mobile phase A (water with 0.1% formic acid) and mobile phase B (methanol) were set as follows: 70% A – 30% B (0-7 min), 60% A - 40% B (7-17 min), 20% A - 80% B (17-26 min), 10% A - 90% B (26-31min), with 4 min balance back to 90% A - 10% B. The injection volume was 20 µL, and the flow rate was 0.3 ml/min. The mass spectra were acquired in ESI negative mode (100-1500 m/z). The parameters were as follows: drying gas (nitrogen) with a flow rate of 15L/min; sheath gas temperature 350°C, flow rate 12 L/min; voltage 3200V. The results are shown in Table 3.6.

Table 3.6 – Top ten abundant compounds characterized by QTOF-LC/MS in PE

Name	**Formula**	**M wt (g/mol)**
Sesamin	C20 H18 O6	354.1103
Glabrone	C20 H16 O5	336.0998
11-Eicosenoic acid	C20 H38 O2	310.2872
Tylophorinidine	C22 H23 N O4	365.1627
Corynoxeine	C22 H26 N2 O4	382.1893
Anti-isorhynchophylline N-oxide	C22 H28 N2 O5	400.1998
Myrtenyl acetate	C12 H18 O2	194.1307
Annotine	C16 H21 N O3	275.1521
Codamine	C20 H25 N O4	343.1784
Cephalofortuneine	C20 H27 N O5	361.1889

Conclusion to Chapter 3

This chapter focused on the role of PE in regulating the balance of cholesterol in macrophages and in inhibiting the transformation of macrophages to foam cells and explores its possible mechanisms.

It was determined that PE effectively inhibited the transition from macrophages to foam cells by labelling foam cells with neutral fat by Oil red O staining. The uptake and efflux of cholesterol by macrophages were characterized by fluorescently labelling cholesterol flow. The results showed that PE minimized cholesterol deposition in macrophages by inhibiting macrophage phagocytosis of cholesterol while promoting the dual effect of intracellular cholesterol efflux.

Finally, we measured the contents of phenolic substances and flavonoids in PE and the monomeric compounds with high contents, which laid a good foundation for further research.

GENERAL CONCLUSIONS

This manuscript mainly focused on crude extracts of different polarities in Speranskia tuberculata (Bunge) Baill, exploring its role in preventing and treating tumour and atherosclerotic diseases.

At the beginning of Chapter 1, we introduced the classification of Speranskia tuberculata (Bunge) Baill, which includes Herba Speranskiae Tuberculatae， Incarrillea sinensis Lam and Vicia amoena Fisch. They are mainly found in Gansu, Heilongjiang, Jilin, etc. The whole herb can be used as medicine, and the main effects include antioxidant, anti-inflammatory, anti-tumour, heat-clearing and blood-activating. After that, the traditional and modern extraction methods, separation and purification of chemical components in Chinese herbal medicines are listed.

According to the different physiological characteristics of Chinese herbal medicine, choosing the proper treatment method is very important. We used the solvent extraction method to extract the chemical compounds. Solvent extraction is a method of dissolving the active ingredients from the medicinal tissues by selecting solvents with high solubility for active ingredients and low solubility for impurity components according to the different solubility of various components in Chinese herbal medicines in different solvents. According to the nature of the components to be extracted, select the appropriate solvent and add it to the appropriately crushed Chinese herbal medicine materials. The solvent will gradually enter the cell through the cell wall due to diffusion and osmosis, dissolving soluble substances, resulting in poor concentration inside and outside the cell.

As a result, the concentrated solution inside the cell continues to diffuse outward, and the solvent outside the cell continues to enter the medicinal tissue cells. This operation will repeat many times until the solution concentration inside and outside the cell reaches a dynamic equilibrium. The saturated solution is filtered out, concentrated, and then filtered. A new solvent is added to the later dregs, and the

above process will repeat. The required ingredients can be almost wholly dissolved or basically dissolved, and then all the concentrated liquids are combined, which will be a mixed solution containing the required active ingredients.

Chapter 2 tested the anti-tumour effect of the extract of Speranskia tuberculata (Bunge) Baill. The MTT Cytotoxicity Test and Cell Scratch Test were mainly used in this Chapter. We also used five types of tumour cells: human alveolar adenocarcinoma A549 cells, human cervical carcinoma Hela cells, Hepatocellular carcinoma HepG2 cells, mouse melanoma B16-F0 cells, and human malignant melanoma A375 cells. The experimental principle is that MTT can react with succinate dehydrogenase in living cells mitochondria to form blue-purple crystalline formazan, insoluble in water deposited in living cells. The blue-violet formazan has a particular absorption peak at 492 nm after dissolved in DMSO. When the number of collected cells was within a specific range, the number of living cells was proportional to the absorbance at 570 nm after the dissolution of formazan.

Apart from that, the scratch method studied the effects of different ethyl acetate extracts on the cell migration of five tumour cells. The experimental results were used to calculate and analyse the cell healing rate after 24 h of administration and determine whether the tested compounds promoted cell migration or repair. Cell scratch tests can reflect cell repair or migration ability to some extent. After administration, until the end of the experiment, the growth and migration of the surrounding cells before and after the scratch can be observed and compared. The healing rate can be calculated to reflect the migration or repairability of the selected test sample to five tumour cells.

Firstly, the crude extracts of different polar parts of the Speranskia tuberculata (Bunge) Baill were extracted and separated. Four crude extracts with different polarities, petroleum ether, ethyl acetate, n-butanol and water, were obtained and screened for anticancer effect using the MTT cytotoxicity and cell scratch tests. It was determined that the crude extracts of ethyl acetate significantly impacted killing five types of tumour cells, and it exerted the most significant impact on A549 cells. In

contrast, the three polar crude extracts of petroleum ether, n-butanol and water did not have the powerful inhibitory effects on these cells.

Chapter 3 focused on the role of crude extracts of petroleum ether (PE) in regulating the balance of cholesterol in macrophages, inhibiting macrophages' transformation to foam cells, and exploring its possible mechanisms.

It was determined that PE effectively inhibited the transition from macrophages to foam cells by labelling foam cells with neutral fat by Oil red O staining. The uptake and efflux of cholesterol by macrophages were characterized by fluorescently labelling cholesterol flow. The results showed that PE minimized cholesterol deposition in macrophages by inhibiting macrophage phagocytosis of cholesterol while promoting the dual effect of intracellular cholesterol efflux.

Finally, we measured the contents of phenolic substances and flavonoids in PE and the monomeric compounds with high contents, which laid a good foundation for further research.

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