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ANTI-ATHEROSCLEROSIS EFFECT OF THE HERB *SPERANSKIA TUBERCULATA (BUNGE) BAILL*

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The article describes the inhibitory effect of extracts of the herb Speranskia Tuberculata (Bunge) Baill on atherosclerosis of vessels. Crude extracts were prepared using petroleum ether (PE extracts). Subsequently, the dried extracts were dissolved in dimethyl sulfoxide to prepare working solutions with 12.5 to 75 μg/mL concentrations. RAW 264.7 cells, a macrophage cell line, were used as the research object. Atherosclerosis was induced in the cells by incubating oxidised low-density lipoproteins. The study of the influence of PE extract concentration was carried out in two experiments. The first included oil red O staining on macrophages with different concentrations of PE extract added, followed by a spectrophotometric examination of the solutions. Thus, the effect of PE extract concentration on lipid content in cells was investigated. The second method was based on flow cytometry and ended by measuring the intensity of fluorescent cholesterol in the cells of various samples. Cell samples additionally containing only oxidised low-density lipoproteins, as well as cells treated with PE extract and caffeic acid, were compared. PE extracts of Speranskia Tuberculata (Bunge) Baill effectively inhibit the transition from macrophages to foam cells, as shown by labelling foam cells with neutral fat using Oil red O staining. The processes of cholesterol uptake by macrophages were investigated by flow cytometry using fluorescently labelled cholesterol. PE extract minimises cholesterol deposition in macrophages by inhibiting macrophage phagocytosis of cholesterol while promoting the effect of intracellular cholesterol outflux. Thus, the role of PE extracts in regulating the cholesterol balance in macrophages and inhibiting the transformation of macrophages into foam cells has been experimentally proven. Possible mechanisms for realising these effects are proposed.

Key words: *Speranskia Tuberculata, atherosclerosis, RAW 264.7, oil red O staining, oxidised low-density lipoprotein, flow cytometry.*

Цзеюань Сунь, Тетяна Деркач. Антиатеросклерозна дія трави Speranskia Tuberculata (Bunge) Baill

У статті описано інгібуючий вплив екстрактів трави Speranskia Tuberculata (Bunge) Baill на атеросклероз судин. Сирі екстракти отримано за допомогою петролейного ефіру (ПЕ екстракти). Надалі висушені екстракти були розчинені в диметилсульфоксиді для приготування робочих розчинів із концентраціями від 12,5 до 75 мкг/мл. Як дослідження об'єкта використано клітини RAW 264.7, лінія клітин макрофагів. Атеросклероз у клітини було внесено шляхом інкубації окислених ліпопротеїнів низької щільності. Дослідження впливу концентрації ПЕ-екстракту проводилося у двох експериментах. Перший з них включав спочатку фарбування масляним червоним О макрофагів із різними концентраціями ПЕ-екстракту з подальшим спектрофотометричним дослідженням розчинів. Таким чином, досліджено вплив концентрації ПЕ-екстракту на вміст ліпідів у клітинах. Другий метод базувався на використанні проточної цитометрії та завершувався вимірюванням інтенсивності флюорисцентного холестеролу в клітинах різних зразків. Разом зі зразками клітин, які додатково вмістили лише окислені ліпопротеїни низької щільності, досліджено клітини, оброблені екстрактом ПЕ та кафеїновою кислотою. ПЕ-екстракти Speranskia Tuberculata (Bunge) Baill ефективно інгібують перехід від макрофагів до пінистих клітин, як показано міченням пінистих клітин нейтральним жиром за допомогою фарбування масляним червоним О. Процеси поглинання холестерину макрофагами досліджено проточною цитометрією із застосуванням холестерину з флуоресцентним міченням. ПЕ-екстракт мінімізує відкладення холестерину в макрофагах шляхом інгібування макрофагального фагоцитозу холестерину, одночасно сприяючи ефекту внутрішньоклітинного відтоку холестерину. Таким чином, експериментально доведено роль ПЕ-екстрактів у регуляції балансу холестерину в макрофагах і в інгібуванні трансформації макрофагів у пінисті клітини. Запропоновано можливі механізми реалізації цих ефектів.

Ключові слова: *Speranskia Tuberculata, атеросклероз, клітини RAW 264.7, фарбування масляним червоним О, окислені ліпопротеїни низької щільності, проточна цитометрія.*

Introduction. Atherosclerosis (AS) is a chronic progressive disease. The main symptom is the narrowing of the lumen of the arterial vessel due to the deposition of atherosclerotic plaques, which leads to impaired blood circulation [1, 2]. Eventually, this leads to ischemic damage in downstream tissues. AS not only directly threatens human life and health but can also cause many high-risk diseases such as aneurysm, aortic dissection, and coronary heart disease. It has a high morbidity and mortality rate, is difficult to treat, and the death rate is increasing every year.

In recent years, many pathogenic mechanisms of AS have been proposed. However, the debate is still ongoing. The most accepted pathogenic factors of AS are related to high blood pressure, hyperlipidemia, smoking, alcohol consumption, obesity, genetics and other factors. A well-known theory of the mechanism involves the imbalance of cholesterol metabolism and oxidative stress, which leads to endothelial dysfunction, inflammation, and so on [1].

Violation of lipid metabolism in AS leads to abnormal accumulation of cholesterol and other lipid substances in blood vessels and activation of the inflammatory response. It creates foam cells and plaque-forming lesions. As a result of inflammation, the plaque ruptures, forming a thrombus, compressing the artery cavity and narrowing the vessel's lumen [2].

The appearance of lipid fragments gives evidence of the beginning of the AS process. At the same time, endothelial cells proliferate and migrate after injury. They interact with smooth muscle cells, forming a fibrous network that covers a large number of lipid fragments and forms early plaques in the arteries. Plaque in the arteries builds up continuously, compressing the space in the blood vessel, causing blockage of blood flow, causing tissue damage and, in severe cases, tissue necrosis. Inflammation destroys the stability of plaque. Under the influence of inflammatory factors, macrophages infiltrate the lipid plaque. They secrete many proteolytic enzymes that destroy the fibrous surface of the plaque and cause plaque rupture and blood clot formation.

Currently, the clinical treatment of AS consists mainly of lowering the level of blood lipids in the body by adjusting the body's response. At the moment, there are relatively few medicines. They may participate in treating AS only by directly or indirectly inhibiting cholesterol synthesis or promoting cholesterol breakdown. They also have many side effects, such as loss of liver function, gastrointestinal dyspepsia, allergic reactions, etc., which cause some harm to the human body.

In Traditional Chinese Medicine (TCM), many AS treatments with low side effects are known [3–6]. The use of such drugs is possible as a medicine or food supplement to participate in the prevention or treatment of AS. At the same time, the thousand-year experience of TCM is a natural reason for the fact that not all traditional drugs are accepted and evaluated by scientific pharmacy. Many well-known medicinal plants are still not included in the pharmacopoeia of even the People's Republic of China and other countries. Such plants include the endemic Chinese herb *Speranskia Tuberculata (Bunge) Baill*. Scientific medical information about this plant is quite limited [7; 8], while its use for medical purposes dates back many years.

The aim of the work was to study the effect of different concentrations of *Speranskia Tuberculata (Bunge) Baill* herb extracts, produced by dissolution with petroleum ether at the stage of crude extract production, on atherosclerosis developing in RAW 264.7 cells.

Experimental. RAW 264.7 cells were used as the research object. RAW 264.7 is a macrophage cell line established from a male mouse tumour induced with the Abelson murine leukaemia virus. Cells of this type were studied in different conditions, namely, in the absence of signs of atherosclerosis, the presence of such signs caused by the incubation of oxidised low-density lipoprotein (oxLDL), as well as after treatment of cells with extracts of the herb *Speranskia Tuberculata (Bunge) Baill* of different concentrations prepared with the help of petroleum ether (from now on PE extracts).

The study of antiatherosclerotic properties was preceded by an assessment of the effect of PE extracts of different concentrations ranging from 12.5 to 75 μg/mL on the viability of RAW 264.7 cells using the MTT test. The goal was to identify a possible significant killing effect of PE extract on macrophages. Methods of making raw and working solutions of PE extracts, sample preparation of cancer cells of various origins, and carrying out cytotoxic MTT tests were described in detail in [9].

Studies of antiatherosclerotic properties were carried out using two methods. Raw RAW264.7 cells without atherosclerosis were plated in 12-well plates in both cases. Cell density was controlled at $2\sim3\times10^4$ cells per well at 37°C, 5% CO_2. The sample was incubated until the cell became stable and completely adhered to the wall. The presence of atherosclerosis was ensured by adding oxLDL at a 50 μg/mL concentration to each well after stabilising the cells.

The first method was aimed to determine the possible effect of PE extract on the content of oxLDL in cells. Accordingly, samples of RAW264.7 cells with added lipids (from now on, DMSO+exLDL samples) were prepared, as well as samples treated with either 10 μg/mL or 20 μg/mL PE extract (DMSO+exLDL+PE). The purpose of the second method was to compare the effect of PE extract of 50 μg/mL concentration and caffeic acid (CA) on the behaviour of lipids in DMSO+50 μg/mL exLDL. As is known [10–12], caffeic acid protects against atherosclerotic lesions.

The first experiment included Oil red O staining on macrophages with different PE extract concentrations (0–20 μg/mL). A fat-soluble dye, Oil Red O, dyes neutral fats (e.g., triglycerides). After staining the fat inside the foam cells, the red substance was dissolved in isopropyl alcohol. The solution has a strong absorbance at 518 nm, which is then measured for each sample on a spectrophotometer.

In more detail, the method of the first experiment included the following steps. After incubating the sample for 24 hours, the medium containing the samples was removed, and the cells were washed three times with Dulbecco's phosphate-buffered saline (PBS). A volume of 1 ml of 4% polyformaldehyde was added to each well for 10 min of fixation, three times washed with PBS and 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, followed by two washes with 1 mL of 50% isopropanol. Finally, 500 μL of isopropanol was added to each well and shaken at room temperature for 10 min to completely dissolve the Oil red O in the cells. Absorbances of each sample were measured at 518 nm, obtaining relative absorbance values compared with the absorbance of blank controls to minimise noise's influence.

The second experiment focused on determining the content of oxLDL in the DMSO+exLDL sample compared with the DMSO+exLDL+PE and DMSO+exLDL+CA samples. Blank control samples with 0.1% DMSO were also used. That is, the goal was to investigate a possible decrease in the accumulation of lipids in cells under the influence of PE extract, a change in the content of oxLDL in cells, as well as the possibility of inhibiting the absorption of cholesterol by macrophages.

The preparation of the samples began in the same way as described above. Then, 50 μg of fluorescently labelled oxLDL (Dil-oxLDL) was added to each well and removed after 6 hours of incubation. After Dil incubation, the medium containing Dil was removed and washed three times with PBS. Cells were split with trypsin and blown into a suspension. The cytosol was collected and centrifuged for 5 min at 2500 rpm at room temperature. It was then washed twice with PBS. The centrifugation step was repeated, and the cell pellet was collected. The cell pellet was resuspended in 300 μL of PBS, vortexed, and the cells were collected for flow cytometry to analyse the fluorescence intensity in each cell.

The effect of PE extract on macrophage cholesterol uptake was also measured using fluorescently labelled cholesterol in the flow cytometry method. Fluorescently labelled oxLDL was used to measure the fluorescence intensity of Dil in cells after incubation of samples with oxLDL-only cells or after PE treatment.

All experiments were repeated three times, followed by the statistical analysis with the GraphPad Prism software. A comparison between the samples was evaluated using a t-test. Conventional designations of significance levels reached, * corresponds to $p<0.05$, ** – $p<0.01$, *** – $p<0.001$, were given in the illustrations where appropriate.

Results and Discussion. MTT cytotoxicity test results are shown as a function of cell viability versus PE extract concentration in Fig. 1. PE extracts do not demonstrate a significant effect in destroying macrophages (RAW 264.7). Thus, if detected, the main effect of PE extracts is reduced to the impact on atherosclerotic structures.

The experiment with Oil red O staining was performed on macrophages with added PE extract concentrations of 0–20 μg/mL. During oxLDL incubation, macrophages swallow many cells and turn them into fat in the cells. The Oil red O dye stains the lipid-rich cells (Fig. 2a).

The resulting pattern of dye distribution in the DMSO+oxLDL sample was compared with similar patterns after the addition of 10 and 20 μg/mL PE extract (Fig. 2b and Fig. 2c). Stained fragments after incubation with PE extract were significantly smaller than in the DMSO+oxLDL sample. Such observation proves that PE extracts can reduce the accumulation of lipids in cells. They protect macrophages to some extent and reduce the formation of foam cells.

After observing the Oil Red O staining results, the red substance was dissolved in isopropyl alcohol, and the absorbance was measured at 518 nm for all samples (Fig. 3).

The relative absorption was directly proportional to the Oil red O content in the cells, which allowed one to estimate the fat content in the cells. It is also evident that, compared to the DMSO+oxLDL sample, the content of oil red O in the cells was significantly

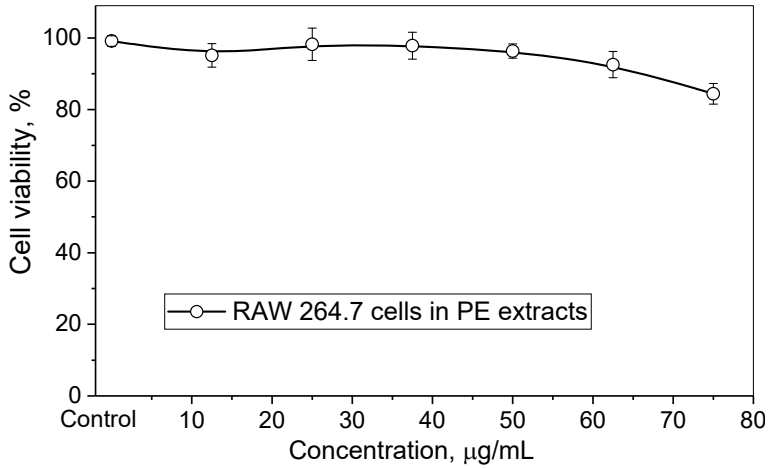

Fig. 1. The effects of PE extract concentrations on the viability of RAW 264.7 cells

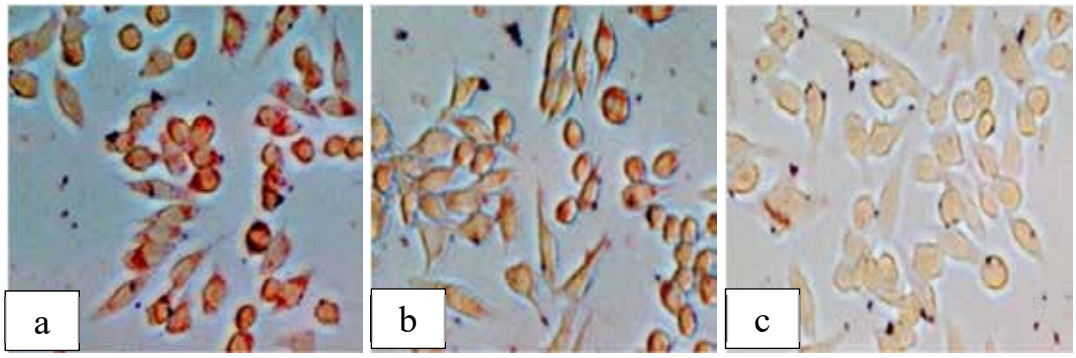

Fig. 2. Oil red O staining results: a – DMSO +50 μg/mL oxLDL;
b – 10 μg/mL PE + 50 μg/mL oxLDL; c – 20 μg/mL PE + 50 μg/mL oxLDL

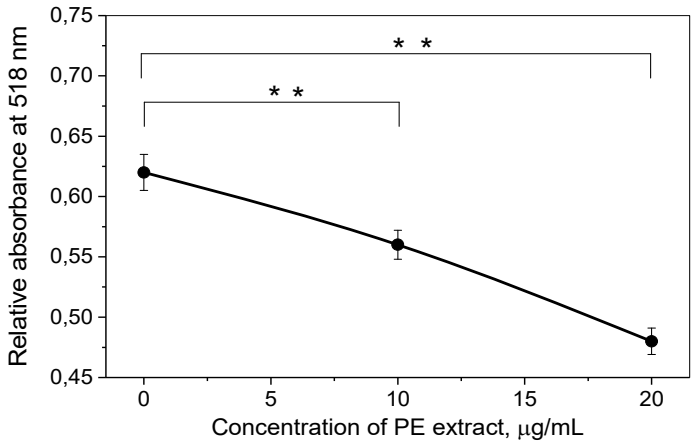

Fig. 3. Relative absorbance at 518 nm as a function of the concentration of added PE extract

lower in the samples with PE extract, but it fell with the increasing concentration of the extract. This observation made it possible to conclude that PE extract reduces the lipid content in macrophages compared to the sample without extract.

The results of experiments using the flow cytometry method confirm the above conclusion. In this method, the state of the cell population is analysed by fluorescent labelling of cell nuclei in suspension, followed by analysis of the fluorescence property of each cell in the population. The fluorescently labelled Dil-OxLDL can be detected in cells of various origins. In turn, the fluorescence intensity of Dil is directly proportional to the content of oxLDL. The stronger

the fluorescence intensity, the higher the content of oxLDL. Blank control, DMSO+oxLDL samples, and DMSO+oxLDL samples with PE extract or caffeic acid after incubation were analysed. Dil fluorescent detection of the content of oxidised low-density lipoproteins in cells is illustrated in Fig. 4.

Blank control is an empty group without Dil-oxLDL. Accordingly, there is no fluorescence, and the curve is located to the left on the abscissa axis. The fluorescence of cells incubated with DMSO+oxLDL is maximal; that is, it is in the extreme right position. When PE extract was added, the fluorescence curve shifted significantly to the left relative to the group of cells incubated with DMSO+oxLDL. Thus, the fluorescence intensity decreased, and the content of oxLDL in cells decreased, indicating that PE extract can effectively reduce the level of oxLDL in cells.

Atherosclerosis begins with the deposition of lipids in macrophages, which significantly affects the cholesterol balance. Therefore, controlling the balance of intracellular cholesterol is especially important for preventing and treating atherosclerosis. The effect of PE extract on macrophage cholesterol absorption is measured using fluorescently labelled cholesterol (Fig. 5).

The average fluorescence intensity in the cells of the PE extract group was lower than that of DMSO+oxLDL. In other words, PE extract and CA affect macrophages and phagocytosis of fluorescent cholesterol. They can effectively inhibit cholesterol absorption by cells and reduce the cholesterol content in cells.

Conclusions. This article focuses on the role of petroleum ether extracts of Speranskia Tuberculata (Bunge) Baill in regulating cholesterol balance in macrophages and inhibiting the transformation of macrophages into foam cells and explores possible mechanisms for this.

PE extracts of *Speranskia Tuberculata (Bunge) Baill* effectively inhibit the transition from

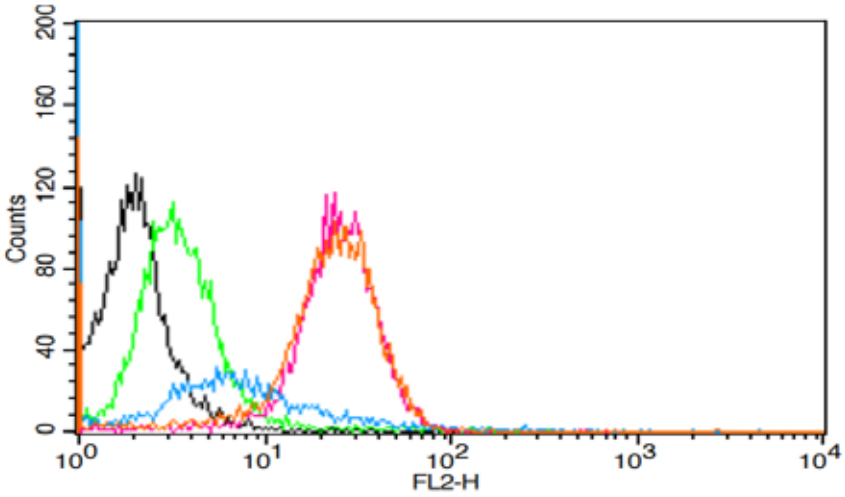

Fig. 4. Effect of PE and CA on the absorption of oxLDL by macrophages: black curve – blank control, red – DMSO+oxLDL, green – DMSO+oxLDL+PE, blue – DMSO+oxLDL+CA

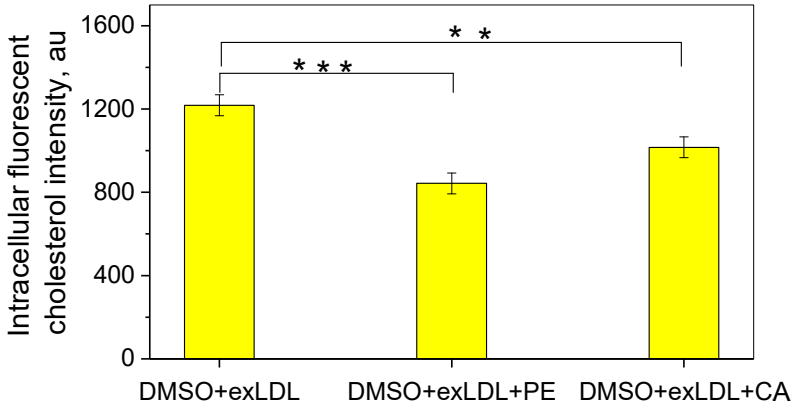

Fig. 5. The intensity of fluorescent cholesterol in the cells of different samples (excitation wavelength 482 nm and emission wavelength 515 nm)

macrophages to foam cells, as shown by labelling foam cells with neutral fat using Oil red O staining. The processes of cholesterol uptake by macrophages were investigated by flow cytometry using fluorescently labelled cholesterol. PE extract minimises cholesterol deposition in macrophages by inhibiting macrophage phagocytosis of cholesterol while promoting the effect of intracellular cholesterol outflux.

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