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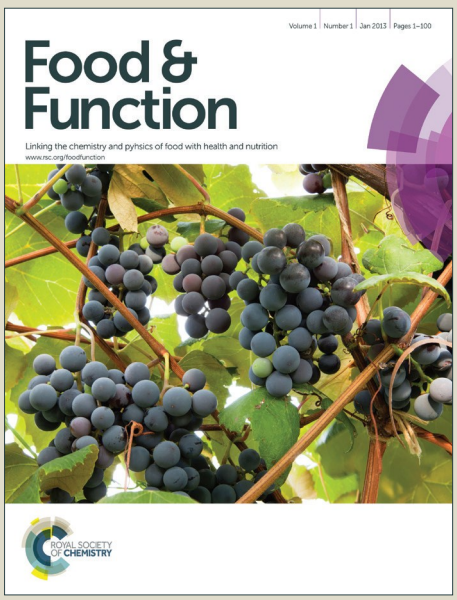

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Separation and purification of anti-tumor peptide from rapeseed (Brassica campestris L.) and the effect on cell apoptosis

Lifeng Wang *， Jing Zhang，Qiang Yuan，Huihui Xie，Jiayi Shi，Xingrong Ju

(College of Food Science and Engineering/Collaborative Innovation Center for Modern Grain Circulation and Safety/Key Laboratory of Grains and Oils Quality Control and Processing, Nanjing University of Finance and Economics, Nanjing 210023, China)

Correspondence author: Dr. Lifeng Wang

***Corresponding author. Address: School of Food Science and Engineering, Nanjing University of Finance and Economics, Nanjing, Jiangsu, PR China 210023.**

Phone: 86-025-86718569

Fax: 86-025-86718572

E-mail: wanglifeng_8@163.com

ABSTRACT

The rapeseed peptides were prepared by means of the combined methods of laboratory bacteria enzyme synergy of solid-state fermentation on the rapeseed meal. The rapeseed peptides were separated and purified with the tumor cells in vitro anti-proliferative activity as an index. Moreover, a kind of rapeseed peptide component RSP-4-3-3 (rapeseed anti-tumor peptide RSP-4-3-3) with high activity was selected. Furthermore, by using the reversed-phase high performance liquid chromatography (RP-HPLC) coupled with the electrospray ionization mass spectrometry (ESI-MS/MS), the analysis result of its possible amino acid sequence showed it was Trp-Thr-Pro (408.2Da). Inverted microscope observation technology and Western Blot experiments were applied to explore the antitumor impact of the rapeseed peptide RSP-4-3-3 on tumor cell. The results showed that rapeseed antitumor peptide RSP-4-3-3 could significantly change the morphological features of the HepG2 cells *in vitro* and cause apoptosis, thus inhibiting the proliferation of the HepG2 cells.

KEYWORDS：Anti-tumor rapeseed peptides; Separation and Purification; Amino Acid Sequence; Cell Apoptosis

INTRODUCTION

Numerous studies have found that hydrolysis of rapeseed protein can generate relatively small molecular mass peptides with biological activities, such as hypotension activity [1-3], anti-oxidant activity [4-6], anti-tumor activity [7, 8], and anti AIDS activity [9, 10]. Therefore, rapeseed protein hydrolysis has great potential and broad market prospects. Rapeseed peptides are the hydrolysate of rapeseed proteins, and have relatively complicated constituents, including small amount of free amino acids and some macromolecular substances that cannot be completely hydrolyzed. The bioactive activity of the peptides is not only related to its structure, amino acid composition and the sequence, but also to the molecular mass [11, 12]. The peptides featuring stronger antitumor activity generally have smaller molecular weight. Tumor is a kind of disease when anomaly occurs in the level of cell proliferation and death [13]. Cell apoptosis is a form of cell death, which is accompanied by a series of physiological characteristics, including cell shrinkage, chromatin aggregation and DNA degradation between nucleosomes. The abnormal proliferation and the declining ability of apoptosis of tumor cells are the two main factors of tumorigenesis and development. Therefore, for the purpose of chemotherapy and biological treatment, the induction of apoptosis is of great significance [14].

In this experiment, human HepG2 hepatoma carcinoma cells, MCF-7 breast cancer cells and HeLa cervical cancer cells were taken as research objects, and methylene blue colorumetric method were used to evaluate the anti-proliferative activity of the rapeseed peptides (RSP) towards the tumor cells, so as to select the sensitive cell stains. Based on the proliferation inhibition rate of human HepG2 hepatoma carcinoma cells *in vitro*, RSP was separated and purified by ultrafiltration, Sephadex G-15 gel filtration and reverse phase High-performance liquid

chromatography (RP-HPLC) to obtain the rapeseed peptides fraction with high anti-proliferative activity (anti-tumor rapeseed peptides). And the possible amino acid sequence of anti-tumor rapeseed peptide was analyzed. On the basis, HepG2 cell was treated with the anti-tumor rapeseed peptide. Then the cellular morphological changes were observed under inverted microscope. Besides, as revealed by the Western Blot experiments, the expression of proteins related tumor cell apoptosis is detected and the antitumor mechanism of the rapeseed peptide was preliminarily identified. It is thus expected that our research can provide an experimental basis for efficient utilization of the abundant rapeseed protein resources in China, for the theoretical research of the anti-tumor activity of the rapeseed peptide, as well as for the development and application.

Experimental

Chemicals and Reagents

Rapeseed peptides (RSP) were prepared according to our previous studies [15] and stored at -20 °C. Human HepG2 hepatoma carcinoma cells, human MCF-7 breast cancer cells and human HeLa cervical cancer cells were purchased from Cell bank of Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin-Streptomycin Solution, 0.25 % Trypsin were purchased from Gibco Biotechnology Company (Carlsbad, CA, USA). 5-Fluorouracil (5-Fu), Thiazolyl Blue Tetrazolium Bromide (MTT), Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, trifluoroacetic acid (TFA) were purchased from Merck Chemicals Co., Inc (Darmstadt, Germany). Sephadex G-15, RIPA Lysis Buffer, Tween-20, Phenylmethyl sulfonyl fluoride (PMSF), Aprotinin, Leupeptin, Pepstatin, Protein Marker(14.4-97 kDa), 30% Acrylamide, Tris, Ammonium Persulphate, Sodium

dodecyl sulfate, sodium salt (SDS), N, N, N, N-Tetramethylethylenediamine (TEMED), Bromophenol Blue, 2-Mercaptoethanol MCH, Skimmed Milk Powder were purchased from Solarbio Biological Technology Co., Ltd (Beijing, China). P53 Antibody, Bax Antibody, Bcl-2 Antibody, β-actin Antibody, HRP-labeled Goat Anti-Rabbit IgG (H+L), HRP-labeled Goat Anti-Mouse IgG (H+L) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Other reagents used in this experiment were analytical grade.

Separation and purification of anti-tumor rapeseed peptide

The RSP (1 g) was dissolved in 1000 mL of distilled water and fractionated according to molecular size through an ultrafiltration (UF) membrane bioreactor system (Millipore Pellicon XL, Millipore, Bedford, MA) with a molecular weight cut-off 10 kDa, 5 kDa and 3 kDa. RSP-1, RSP-2, RSP-3 and RSP-4 represent the fractions with M_W distribution of >10 kDa, 5–10 kDa, 3–5 kDa and <3 kDa, respectively. Control pressure was set between 0.10 to 0.22 Mpa in the ultrafiltration process. Collect various molecular weight of rapeseed peptides fluid, freeze drying, weighing the quality of different molecular weight peptides and the anti-proliferative activity of each fraction was determined. The sample with the highest anti-proliferative activity after ultrafiltration was further separated using Sephadex G-15 and a gel filtration column (15.0×700 mm) eluted with distilled water, at a flow rate of 30 mL/h. The sample dissolved in distilled water at a concentration of 10 mg/mL and filtered through micropore films (0.22 μm pore size). Each fraction was measured at 280 nm. Fractions were automatically collected every minute by the fraction collector, the same elution peak was pooled, and the anti-proliferative activity of fractions was determined. The fraction with the highest anti-proliferative activity after gel filtration was further separated using RP-HPLC (Water Corporation, Milford, MA, USA). The

sample was dissolved in distilled water at a concentration of 10 mg/mL and filtered through micropore films (0.22 μm pore size). XBridge^{TM} Prep C18 (10×150 mm, 5 μm) was used as the column (Water Corporation, Milford, MA, USA). The injection volume was 100 μL, and the flow rate was 2.36 mL/min. The mobile phase for the chromatography analysis were: solvent A 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile in distilled water (v/v); and solvent B, 0.05% trifluoroacetic acid (TFA) in 80% acetonitrile. Elution was carried out using a linear gradient with in 20 min. The column was operated at 30 °C. The sample was detected at 280 nm. The chromatogram was calculated with the Empower 2 integral software (Water Corporation, Milford, MA, USA). The fractions eluted under the same elution peak were pooled, vacuum concentration and then freeze drying. The anti-proliferative activity of each fraction was determined.

Cell proliferation inhibition tests

The HepG2 cells, HeLa cells and MCF-7 cells were cultured at 37 °C with 5% CO_2 using DMEM medium supplemented with 5% FBS and 10% penicillin–streptomycin mixture. Anti-proliferative activity of rapeseed peptides towards the tumor cells were detected by MTT assay.

Briefly, the tumor cells were seeded at a density of 2×10^3 cells per well on a 96-well microplate with 100 μL of growth medium per well. 100 μL DMEM with different concentrations of samples (50, 100, 200, 400, 800, 1600 μg/mL) were added to each well, and the 50 μg/mL 5-Fluorouracil (5-Fu) served as the control. Cell-free medium (100 μL) was added to the peripheral wells of a 96-well microplate as the blank. Removed the growth medium after incubation 24 h and the wells were washed with PBS. Added 20 μL of MTT (5 mg/mL) to every well and incubated for 4 h at

37 °C. Removed the medium after the incubation and add 50 μL of DMSO to each well. The 96-well microplate was placed on a tablet oscillator for 20 min. Measured the absorbance at 570 nm in the SpectraMax M2e Microplate System (Molecular Devices, CA, USA). The different concentrations of samples were compared to the control according to the following equation:

$$Cell\ \ viability(\%) = \left(\frac{OD_1 - OD_0}{OD_2 - OD_0} \right) \times 100$$

Where OD_1 is the optical density value of sample; OD_2 is the optical density value of the control; OD_0 is the optical density value of the blank. Every sample was measured in three replicates, and the results were averaged.

The amino acid sequence analysis of anti-tumor rapeseed peptide

The anti-tumor rapeseed peptide obtained though separation and purification was dissolved in distilled water and filtered through micropore films (0.22 μm pore size). And the purity was analyzed by HPLC. XBridge™ Prep C18 (4.6×250 mm, 5 μm) was used as chromatographic column (Water Corporation, Milford, MA, USA). The elution was performed for 30 min with the mobile phase containing solvent A (5% acetonitrile with 0.05% trifluoroacetic acid) and solvent B (80% acetonitrile with 0.05% trifluoroacetic acid). The column was operated at 30 °C. The injection volume was 20 μL and the rate of flow was 0.5 mL/min. Chromatogram was recorded at 280 nm. The chromatogram was calculated with the Empower 2 integral software (Water Corporation, Milford, MA, USA).

The possible amino acid sequence of anti-tumor rapeseed peptides that obtained through a series of separation and purification technologies was analyzed by RP-HPLC in combination with ESI-MS/MS. The LC condition adopted in this assay

was as followed: Agilent C18 Eclipse XD8-18 (4.6×250 mm, 5 μm) was used as chromatographic column. The elution was performed for 50 min with the mobile phase containing solvent A (5% acetonitrile with 0.1% acetic acid) and solvent B (80% acetonitrile with 0.1% acetic acid). Gradient elution was carried out according to the following process: 0-40 min, linear gradient 95-50% A; 40-50 min, linear gradient 50-95% A. The column was operated at 30 °C. The injection volume was 20 μL and the rate of flow was 0.6 mL/min. The MS analysis was performed as followed: Detection was under positive ion mode with scanning range of 50~1000 m/z; The assay was carried out at the capillary temperature of 350 ºC and 3.50 volts; The detection wavelength was 280 nm; 25.0 Psi was used as the pressure of Atomizing nitrogen and 9.0 mL/min was used as the velocity of dry nitrogen.

Observe the cell morphology by inverted microscope

The tumor cells were seeded at a density of 2×10^5 cells /mL on a 96-well microplate with 100 μL of growth medium per well. The cells were incubated under the condition of 37 °C, 5% CO_2 for 24 h. Then, 100 μL of media with different concentrations of anti-tumor rapeseed peptides (0 μg/mL, 50 μg/mL, 200 μg/mL, 800 μg/mL) were added to each well. And the cells were incubated under the condition of 37 °C for 48 h. General morphology of HepG2 cells treated with different concentrations of RSP-4-3-3 was observed under inverted microscope.

Western Blot test

The extraction of proteins from the cytoplasm using Yoon H[16] reported methods, with some modifications. Briefly, the HepG2 cell was seeded at a density of 5×10^5 cells per well. The cells were incubated under the condition of 37 °C, 5% CO_2 for 24 h. Removed the growth medium, and add 100μL of media with different

197 concentrations of anti-tumor rapeseed peptides (0 μg/mL, 50 μg/mL, 200 μg/mL, 800
198 μg/mL). Removed the growth medium after incubation 48 h and the wells were
199 washed twice with pre cooling PBS (4 °C). The cells were collected and centrifuged 5
200 min (1000 g, 4 °C). Removed the supernatant and put it in ice bath for future research.
201 Appropriate amount of cell radio immuno precipitation assay (RIPA) Lysis Buffer that
202 contained protease inhibitors (0.2 mmol/L of phenylmethy sulfonyl fluoride, 15
203 μg/mL of aprotinin, 5 μg/mL of Leupeptin and 1 μg/mL of pepstatin) was added to the
204 collected cells. Then, blow suspension and split in the ice was conducted for 15~20
205 min. In the process of spliting, it was frequently shocked to ensure full pyrolysis of
206 cells. The above cell lysis solution was centrifuged for 10 min under the condition of
207 12000 g (4 °C). The supernatant fluid was the required protein samples. After the
208 protein in lysis solution was quantitated by means of
209 BCA(2,2-Biquinoline-4,4-dicarboxylic acid disodium salt) protein quantitation
210 kit(Solarbio ,Beijing, China), SDS-PAGE loading buffer was added to various protein
211 samples and boiled in boiling water for 5 min, centrifuged for 5 min under the
212 condition of 1000 g and kept at -20 °C for later use.

213 Western Blot analysis using Yoon H[17] reported methods, with some
214 modifications. 12 % of SDS-PAGE separation gel was adopted to separate p53, Bax,
215 Bcl-2 and β-actin. The protein after the electrophoresis was transferred to PVDF
216 membrane by wet process. Then, PBST solution (1×PBS buffer containing 0.1 % of
217 Tween-20) containing 5 % skimmed milk powder was added and enclosed for 1h at
218 room temperature. Next, the PBST solution containing primary antibodies was added
219 and kept in swing bed at 4 °C for one night. Primary antibodies were collected and
220 cleaned the membrane with PBST solution for 3 times, with each time lasting for 10

min. Then, secondary antibody PBST solution containing horse radish peroxidase (HRP) labeling was added and incubated for 1 hour at room temperature. Afterwards, the membrane was washed with PBST solution for 3 times, with each time lasting for 10 min. Membrane developing used Beyo ECL Plus (Beyotime, Shanghai, China). Next, by using Image Lab gel-imaging and analysis software, the corresponding area in the band and optical density were analyzed to determine the strength of the membrane protein bands and analyze the expression quantity change of protein that was treated with different concentrations of samples.

Statistical analysis

All the tests for antioxidant activities of the samples were conducted in triplicate and the data were expressed as the mean ± the standard deviation (SD). The statistical analysis was performed using SPSS (version 17.0, Statistics for Social Science, Chicago, IL, USA) and the significant difference was determined with 95% confidence interval ($P < 0.05$). All graphical representations were composed using Sigmaplot, version 12.5.

Results and discussion

In vitro anti-proliferative activity of RSP against three kinds of tumor cells

Human HepG2 hepatoma carcinoma cells are a class of cell lines, which are widely used in cancer research, genetics and nutrition studies[18]. Human Hela cervical cancer cell is a kind of cell which is often used in biological and medical research; it has been widely used in tumor research because it has the characteristic continuous serial passage, unlimited division, rapid proliferation and strong infectious; the Human Hela cervical cancer cell has become one of the most important tools in medical research[19]. Human MCF-7 breast cancer cells are more commonly used as a model cell for the

research of breast cancer metastasis[20].

As shown in Figure 1, in the dose range that cancer cells can grow, RSP had a relatively dose-dependent inhibition on the proliferation of human HepG2 hepatoma carcinoma cells, MCF-7 breast cancer cells and HeLa cervical cancer cells. For the same cell specie, the one treated with higher RSP concentration demonstrated stronger inhibitory effect. Moreover, the anti-proliferative effect of the RSP at the same concentration became increasingly obvious as time went by. Noticeably, HepG2 cells were the most sensitive to the inhibitory efffect of RSP. At 1600 μg/mL RSP, the inhibition of proliferation reached 36.97±4.06% at 72 h (Figure 1A**)**, which was higher than that of the MCF-7 cells (30.62±2.79 %, Figure 1B) and HeLa cells (25.23±1.49 %**,** Figure 1C).

Xue Zhaohui[19] took three fractions of rapeseed peptides obtained by enzymolysis of rapeseed protein after gel column separation as the samples and measured the inhibitory effect on the human HeLa cervical cancer cell by the MTT colorimetric method. Their research results showed that fraction 2 had the best anti-proliferative effect and its inhibition rate reached 22.51 % under the concentration of 640 μg/mL. The value was higher than the experimental results of this study possibly because different preparation methods of rapeseed peptides cause different types, contents and sequences of amino acids in the sample, and the sample used in experment was the crude rapeseed peptides after fermentation with low purity.

Ultrafiltration

Separation membrane takes the molecular weight cutoff as the indicators and physically screens the solute in the liquid by means of natural or synthetic polymer

thin film so as to reach the purpose of separation; it includes ultrafiltration, nanofiltration and microfiltration; usually, the three ones are used together to realize the multilevel separation of peptide and improve product quality; Membrane separation possesses many advantages, such as low energy consumption, simple equipment, convenient operation, easy recycling and no secondary pollution [21]. Among them, ultrafiltration membrane separation technology is the most widely used[22]. Rapeseed meal after the bacteria enzyme coordination treatment did generate the rapeseed peptide with different molecular weight sizes, but the majority was small molecular peptides. Though the separation, the rapeseed peptide with molecular weight RSP-1(>10 kDa), RSP-2(5-10 kDa), RSP-3(3-5 kDa) and RSP-4(<3 kDa) accounted for respectively about 26.46±0.93 % (mass fraction), 10.78±0.67 % (mass fraction), 12.67±0.24 % (mass fraction) and 41.38±1.04 % (mass fraction).

The anti-proliferative effect results of different molecular weight components of RSP obtained by ultrafiltration membrane separation on HepG2 cells in vitro are shown in Figure 2. As is shown above, except the fraction RSP-4(>10 kDa), the other different molecular weights, especially the small molecular weight fractions of rapeseed peptides had a certain inhibiting effect on human HepG2 hepatoma carcinoma cells; the inhibitory effect of different molecular weights of rapeseed peptides fractions under the same concentration also existed certain differences. Among the four kinds of RSP different molecular weight fractions obtained by ultrafiltration membrane separation, the tumor cells in vitro anti-proliferative activity of rapeseed peptides fractions RSP-4(<3 kDa) was the strongest. Besides, the inhibition rate on HepG2 under different concentrations was higher than that of other components. When it was proceeded by the concentration of 1600 μg/mL for 48h, the HepG2 inhibition rate

292 could reach 37.32 ±1.54 %.

293 **Gel filtration chromatography (GFC)**

294 Gel filtration chromatography separates materials based on different molecular sizes
295 and shapes and it is featured by simple operation, rapid separation, little product loss
296 and no affect on the biological activity of materials to be separated [23]. The column
297 chromatography separation diagram of RSP-4 by Sephadex G-15 is shown in Figure 3.
298 The corresponding eluents of four elution peaks were respectively collected and
299 named as RSP-4-1, RSP-4-2, RSP-4-3 and RSP-4-4 according to the order of elution.
300 Based on the principle of glucan gel chromatography separation, the molecular weight
301 of four elution peaks decreased gradually.

302 Figure 4 illustrates the elution peaks obtained by Sephadex G-15 column had
303 anti-proliferative effect on human HepG2 hepatoma carcinoma cells in vitro. RSP-4-1
304 nearly had no inhibiting effect on HepG2 cells in vitro proliferation, while it had
305 relatively obvious inhibiting effect on the rest of three fractions, and presented a
306 certain dose-effect relationship. The anti-proliferative effect of RSP-4-3 and RSP-4-4
307 on HepG2 cell in different concentrations were slightly higher than the effect before
308 chromatography separation. HepG2 cells anti-proliferative activity in vitro of RSP-
309 4-3 in various concentrations was superior to other fractions.

310 **Semi-Preparative high performance liquid chromatography (Semi-Preparative**
311 **HPLC)**

312 High performance liquid chromatography (HPLC) can separate single peptide
313 according to the molecular weight size and structure of polypeptides; it is
314 characterized by a high degree of purification and high resolution, and is one of the
315 main means of separation and purification of polypeptides, especially in the
316 separation of small molecular peptide researches [24, 25]. The study further separated

and purified RSP-4-3 with semi-preparative RP-HPLC, collected four major fractions (Figure 5) and named them as RSP-4-3-1, RSP-4-3-2, RSP-4-3-3 and RSP-4-3-4 according to the order of elution. In addition, the four fractions showed positive reaction to ninhydrin and biuret, which indicated the four fractions were peptides. The anti-proliferative effect of the four fractions on HepG2 cells in vitro was respectively measured and the results were shown in Figure 6.

According to the Figure 6, within concentration range of 50~800μg/mL, except fraction RSP-4-3-1, the rest fractions of rapeseed peptide obtained by means of semi-preparation RP-HPLC had relatively obvious anti-proliferative effect in vivo on human HepG2 hepatoma carcinoma cells. RSP-4-3-3 showed comparatively strong inhibiting ability and its inhibition rate of tumor cell increased gradually with the increase of concentration; under the different concentrations of the experiment, the anti-proliferation of RSP-4-3-3 on HepG2 cell was significantly higher than the rapeseed peptide component before the separation. Under the 800 μg/mL concentration of RSP-4-3-3, the inhibition of proliferation reached 58.86±2.87% for HepG2 cells. The results showed that, after a series of separation and purification, rapeseed peptide fractions RSP-4-3-3 had a stronger anti-proliferative activity towards HepG2 cells.

Identification of peptide by ESI-MS/MS

Analytical High performance liquid chromatography was applied to conduct purity identification of anti-tumor rapeseed peptide RSP-4-3-3 (Figure 7). The results showed that the rapeseed peptide obtained by the prep-HPLC separation was a single component peak, suggesting that it might contain only one target peptide fragment.

When RP-HPLC-ESI-MS/MS is applied to analyze antitumor rapeseed peptide, RP-HPLC first separates antitumor rapeseed peptide. Then, after the ESI source

ionization, the first-level MS selects specific m/z parent ions and enters the collision activation room. Next, peptide ions have collisions with inert gas and generate fragment ions. As a result, the second-level MS analyzes the fragment ions pyrolysis generates (MS/MS). In the experiment, the molecular weight of RSP-4-3-3 was measured by means of the electrospray ionization mass spectrometry (ESI-MS) of positive ion mode and the amino acid sequence was measured with the help of tandem mass spectrometry.

The corresponding peak of molecular ion in the mass spectrum is called molecular ion peak or parent ion peak. It is generally located at the top of the mass-to-charge ratio (m/z) and its mass number is the molecular weight of the compound. ESI-MS spectrum of RSP-4-3-3 is shown in Figure 8. After removing impurity peak, the main component of RSP-4-3-3 with m/z was 409.2 Da. Besides, RSP-4-3-3 carried single charge ([M+H]+), so the relative molecular mass was 408.2 Da. Such results are consistent with the conclusions that the relative molecular weight of food protein source bioactive peptides is usually lower than 1500 Da [26, 27]. According to the average relative molecular weight of amino acids (128), the rapeseed peptide was tripeptides. The parent ion 409.2 Da was further selected for MS/MS secondary mass spectrum analysis (Figure 9).

As can be seen from Figure 9, there is Trp imino ion characteristic spectral lines in area of m/z< 200, suggesting that the peptide might contain Trp. According to the counter-pair difference value of b series ions m/z value (b1:185.1, b2:294.1 and b3:409.2) in the Figure, it can be concluded that the three fragments bombarded from C terminal of peptide chain in the secondary mass spectrum were 115.1 (b3-b2), 109 (b2-b1) and 185.1 (b1) respectively and their corresponding amino acid residues were

366 respectively Pro, Thr and Trp. Thereby, amino acid sequence of the peptide with the
367 m/z a value of 409.2 Da was Trp-Thr-Pro. Studies show that the tripeptide at
368 C-terminal containing Tyr and Trp has relatively strong ability of scavenging free
369 radicals[27], and some polypeptide containing Pro also can generate synergies with
370 polyphenols[28, 29], prevent the body from being in the oxidative stress state and play a
371 role of anti-tumor. Hydrophilic peptide (containing Arg, Asp, His, Lys, Thr and other
372 hydrophilic amino acids) by way of electrostatic attraction, specific effect on tumor
373 cells, quickly lead to the cell membrane rupture, leakage of cell contents and
374 ultimately cause cell death[30].

375 **Effects of anti-tumor rapeseed peptides on the morphology of HepG2 cells**

376 Cell necrosis and cell apoptosis are two completely different forms of cell death [31].
377 Cell necrosis refers to the passive reactions of cells to external harm such as
378 biological invasion, ischemia, hyperpyrexia, and physical chemical trauma and it is
379 featured by cell swelling, membranolysis or cell membrane dissolution [32]. Cell
380 apoptosis, also called programmed cell death (PCD) is a process in which a kind of
381 cell death mechanism inside the cell under the effect of apoptosis stimulating signals,
382 and results in the programmed cell death through a series of signal transduction and
383 the process is highly ordered and decided by gene [33].They can be distinguished from
384 each other through observing the cell morphology under microscope.

385 Figure 10 shows the morphology of human HepG2 hepatoma carcinoma cells
386 processed by RSP-4-3-3. From it, people can see the control group cells had good
387 adherence to wall and refraction, grew vigorously in the shape of paving stone, and
388 were closely packed, with clear outline. After being processed by RSP-4-3-3 at
389 different concentrations for 48 h, the number and morphology of cells showed

different degrees of changes. To be specific, the cells processed by low concentration of RSP-4-3-3(50 μg/mL) became round and lost three-dimensional structure, and their edge blurred, or adhered to in the culture medium plate bottom surface in the flat shape, or lost adherence to wall and floated in the culture medium; cell surface formed a lot of bubble or bud protrusions. Moreover, the cells processed by high concentrations of RSP-4-3-3 (800 μg/mL) showed more obvious change and decreased significantly; the adherent cells significantly reduced and a large number of cells died and fell. Cell morphology became quite irregular, and there was almost no connection between cells. Such results showed that anti-tumor rapeseed peptide could significantly change the morphological features of HepG2 cell in vitro, make cells present typical apoptosis form, and have obvious anti-proliferative effects [34].

Effects of anti-tumor rapeseed peptides on the expression of apoptosis protein in HepG2 cells

Mitochondrion is the regulation center of cell apoptosis. The apoptosis pathway mediated by mitochondria is one of the most typical apoptotic pathways [35]. With the in-depth study of cell apoptosis, it is proved that Bcl-2 family in the mitochondrial pathway plays important roles in the process of cell apoptosis signal transduction and its expression and regulation are the key factors influencing cell apoptosis [31]. It can be divided into two categories, namely the protein promoting and inhibiting apoptosis. In Bcl-2 family, Bcl-2 and Bax respectively are the most representative and most widely researched inhibiting apoptosis protein and promoting apoptosis protein [36]. Moreover, Bcl-2/Bax ratio is the “molecular switch” to initiate cell apoptosis [37]. Down-regulation of Bcl-2 or up-regulation of Bax expression can induce and promote tumor cell apoptosis. Besides, Bax and Bcl-2 can form the polymer that can adjust mitochondrial

membrane permeability, and cause cytochrome C release, Caspase activation and the final cell apoptosis. P53 is a kind of tumor profilin and plays a key role in the process of response to DNA damage or genome abnormality in cells. Besides, the activation of p53 protein can cause cell cycle arrest, DNA repair and cell apoptosis.

Western Blot was used to detect the expression level of the related proteins (p53, Bcl-2 and Bax) to HepG2 cells proliferation and apoptosis of anti-tumor rapeseed peptide (RSP-4-3-3) after processing. The result is shown in Figure 11. According to the figure, the expression of p53 protein in HepG2 cells increased with the increase of RSP-4-3-3 concentration, and had significant dose-effect dependency. When it was processed in the concentration of 800 μg/mL for 48 h, the protein expression quantity of p53 in cells was about three times of that in the control group (0 μg/mL). Besides, with the increase of RSP-4-3-3 concentration, the expression of anti-apoptosis protein Bcl-2 in HepG2 cells gradually decreased, while the expression of pro-apoptosis proteins Bax gradually increased. All these indicated that rapeseed anti-tumor peptide RSP-4-3-3 inhibited the proliferation of human HepG2 hepatoma carcinoma cells in vitro through participating in mitochondria mediated cell apoptosis.

Conclusion

In the present study, RSP was separated and purified by ultrafiltration, Sephadex G-15 gel filtration and RP-HPLC to obtain rapeseed peptides fraction RSP-4-3-3 with high anti-proliferative activity. The amino acid sequence of RSP-4-3-3 was identified to be Trp-Thr-Pro (408.2 Da) by using ESI-MS/MS. Human HepG2 liver cancer cells were treated with different concentrations of RSP-4-3-3. General morphology of HepG2 cells was observed under inverted microscope. The results showed that, cell number and cell morphology were changed in different degrees and showed typical apoptotic morphology. All these indicated that, anti-tumor rapeseed peptides could

dramatically alter the morphological characteristics of HepG2 cells and caused them to apoptosis in vitro. Effects of RSP-4-3-3 on the expression of tumor suppressor protein p53, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax in HepG2 cells were also analyzed by Western Blot test. The results showed that, RSP-4-3-3 significantly up-regulated the expression of p53 and Bax, while down-regulated that of Bcl-2 in HepG2 cells. And the expression of each protein showed a significant dose-dependent relationship with the concentration of RSP-4-3-3. All these indicated that, anti-tumor rapeseed peptides RSP-4-3-3 inhibited the proliferation of human HepG2 liver cancer cells in vitro by participating in mitochondrial mediated cell apoptosis.

Competing interests

The authors declare no competing financial interest.

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644 **Figure captions**

645 **Figure 1.** Percent inhibitions of the proliferation of HepG2 (**A**), MCF-7 (**B**) and HeLa
646 (**C**) cells in vitro by different concentrations of RSP for different time (mean ±SD,
647 n=6).

648 **Figure 2.** Percent inhibitions of the proliferation of HepG2 cells in vitro by rapeseed
649 peptides fractions obtained from ultrafiltration (mean ±SD, n=6).

650 **Figure 3.** Separation of rapeseed peptides fraction (RSP-4) by Sephadex G-15 gel
651 filtration

652 **Figure 4.** Percent inhibitions of the proliferation of HepG2 cells in vitro by rapeseed
653 peptides fractions obtained from gel filtration chromatography (mean ± SD, n=6).

654 **Figure 5.** Separation of rapeseed peptides fraction RSP-4-3 by RP-HPLC column

655 **Figure 6.** Percent inhibitions of the proliferation of HepG2 cells in vitro by rapeseed
656 peptides fractions obtained from semi-preparative RP-HPLC column (mean ± SD,
657 n=6).

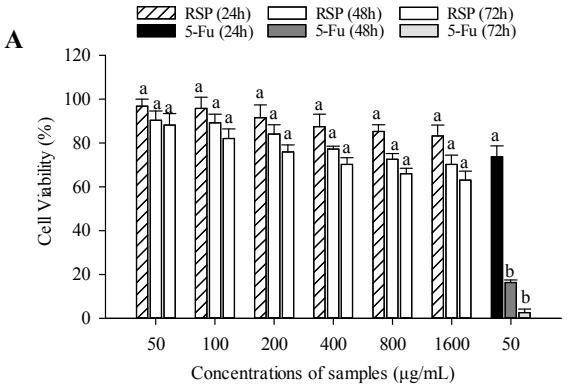
658 **Figure7.**Separation of anti-tumor rapeseed peptides RSP-4-3-3 by analytical
659 RP-HPLC column

660 **Figure 8.** ESI-MS spectrum of RSP-4-3-3

661 **Figure 9.** ESI-MS/MS spectrum of RSP-4-3-3

662 **Figure 10.** Effects of morphological changes of HepG2 cells treated with different
663 concentrations (A: Control; B: 50 μg/mL; C: 200 μg/mL; D: 800 μg/mL) of anti-tumor
664 rapeseed peptides RSP-4-3-3 (20×object)

665 **Figure 11.** Effects of different concentrations of anti-tumor rapeseed peptides
666 RSP-4-3-3 on expressions of p53, Bcl-2 and Bax protein in HepG2 cells (mean ±SD,
667 n=3).



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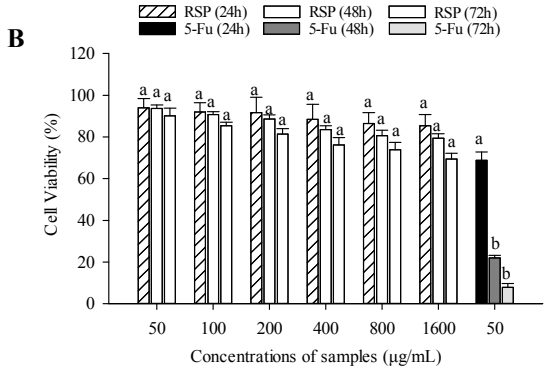

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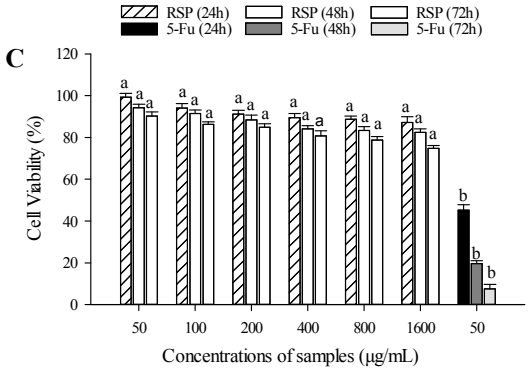

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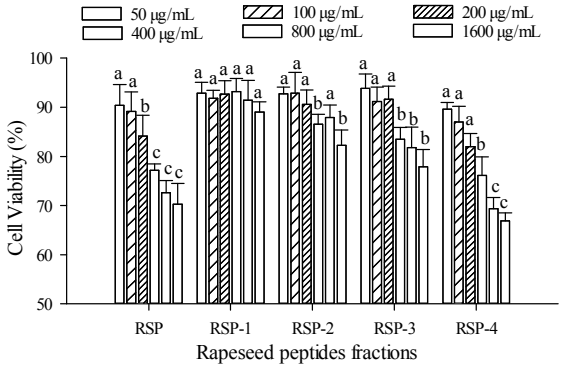
Figure 1



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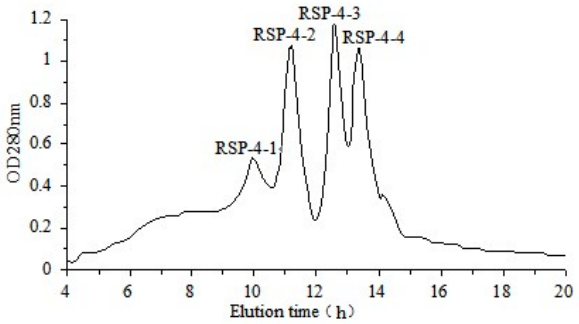

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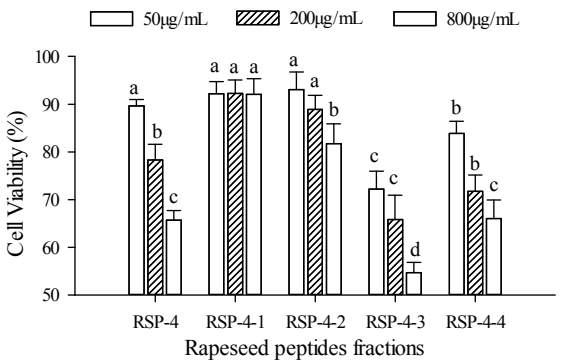

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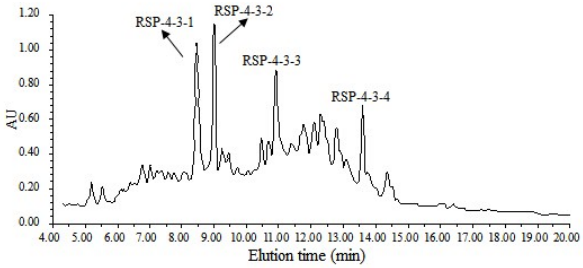
Figure 4



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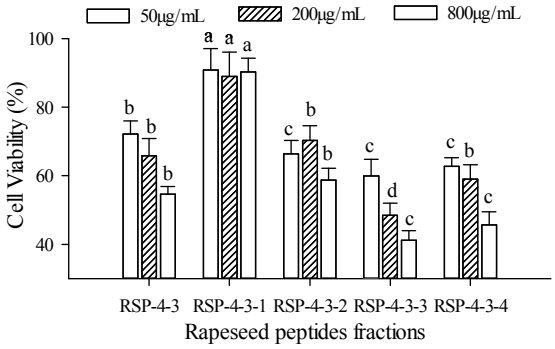

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Figure 6

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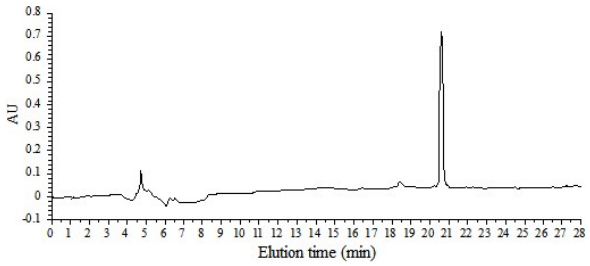

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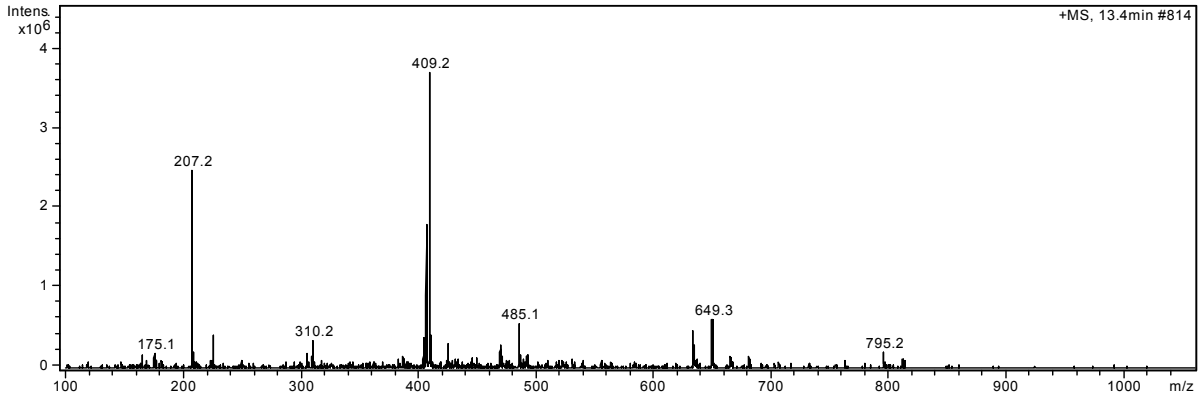

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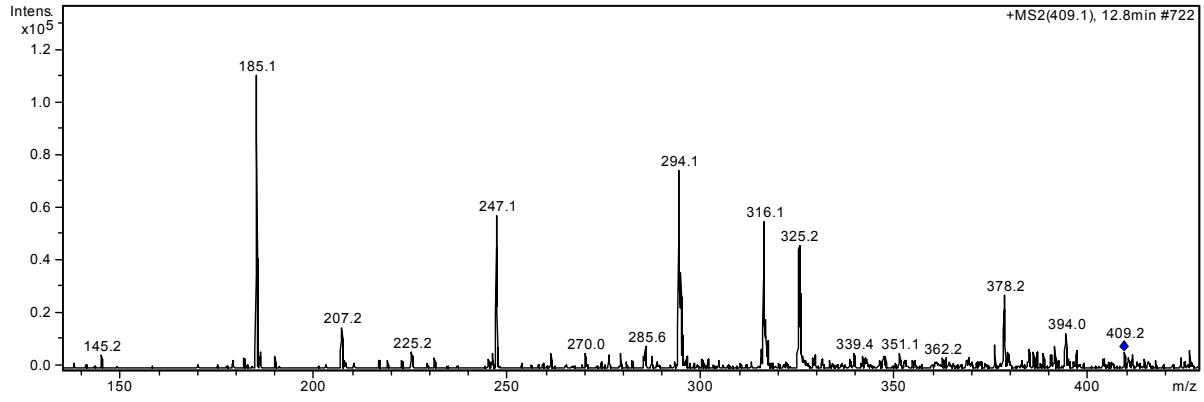

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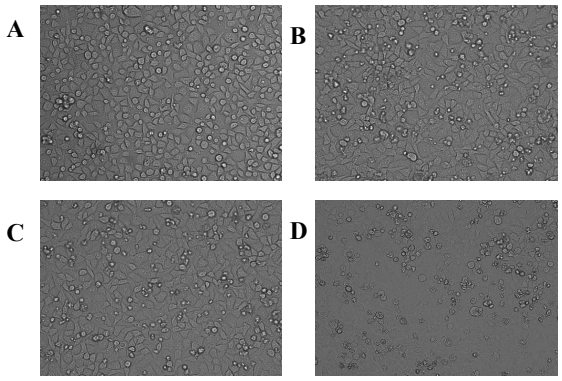

690 **.Figure 10**

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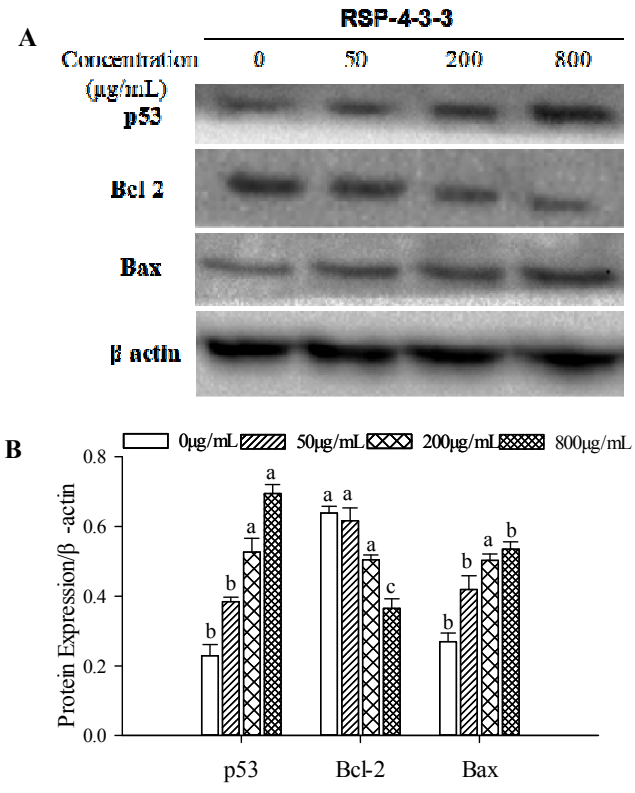

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Figure 11