HOKKAIDO UNIVERSITY

Title	Antitumour activity of EPA-enriched phospholipids liposomes against S180 ascitic tumour-bearing mice
Author(s)	Du, Lei; Yang, Yu-Hong; Wang, Yu-Ming et al.
Citation	Journal of functional foods, 19, 970-982 https://doi.org/10.1016/j.jff.2015.06.061
Issue Date	2015-12
Doc URL	https://hdl.handle.net/2115/63704
Rights	© 2015, Elsevier. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International http://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	JFF-Lei Du-1.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Elsevier Editorial System(tm) for Journal of Functional Foods
Manuscript Draft

Manuscript Number: JFF-D-15-01043R2

Title: Antitumour activity of EPA-enriched phospholipids liposomes against S180 ascitic tumour-bearing mice

Article Type: Full Length Article

Keywords: EPA-enriched phospholipids, liposomes, S180 ascitic tumour, antioxidant, mitochondrial apoptotic pathways.

Corresponding Author: Dr. Lei Du, Ph.D.

Corresponding Author's Institution:

First Author: Lei Du, Ph.D.

Order of Authors: Lei Du, Ph.D.; Yu-Hong Yang, Ph.D.; Yu-Ming Wang, Dr.; Chang-Hu Xue, Dr.; Hideyuki Kurihara, Dr.; Koretaro Takahashi, Dr.

Abstract: The health benefits of eicosapentaenoic acid (EPA)-enriched phospholipids (PL) have witnessed a recent upsurge. In the present study, PL from starfish Asterias amurensis (SFP) and sea cucumber Cucumaria frondosa (SCP) were extracted and the liposomes were prepared. Both SFP and SCP liposomes showed antitumour effects in vitro and exhibited high transport and uptake effects in small intestinal epithelial cell models. The results also demonstrated that dietary SFP and SCP liposomes prolonged the life span and ameliorated oxidative stress status of S180 ascitic tumour-bearing mice. Results also showed that dietary SFP and SCP liposomes up-regulated the expression of Bax and down-regulated Bcl-2 level in tumour cells, then induced release of cytochrome c from mitochondria to cytosol, thus activation of caspase 9 and caspase 3. Taken together, these findings suggest that SFP and SCP liposomes exert antitumour effects mainly via activation of mitochondrial apoptotic pathways in tumour-bearing mice.

Dear Dr. Seiber

On behalf of my co-authors, we thank you so much for giving us an opportunity to revise our manuscript. We really appreciate your comments and suggestions on our manuscript entitled “Antitumour activity of EPA-enriched phospholipids liposomes against S180 ascitic tumour-bearing mice” (ID: JFF-D-15-01043). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our research.

We have studied your comments carefully and have tried our best to revise our manuscript according to the comments. We have made revision which marked in red in the paper.

We would like to express our great appreciation to you for comments on our paper.

Thank you and best regards.

Yours sincerely,

Yu-Ming Wang
wangyuming@ouc.edu.cn

Koretaro Takahashi
kore@fish.hokudai.ac.jp

List of changes and responses

Responds to the Editor's comments:

Editor: There are changes that must be made before I can pass it on to the production people. It is essential that all marked points be addressed in order to avoid delays in the publication.

Response: We have made correction according to the Editor's comments. In order to highlight the change what we have done, the modified parts have been marked in red in the revised manuscript.

About the questions in P7 and P15, analysis of molecular species of phospholipids in starfish and sea cucumber used in this study shows that EPA is often located in the sn-2 position of phospholipids. In addition, other polyunsaturated fatty acids such as DHA and AA are also incorporated into the sn-2 position. And the saturated chain such as C18:0 (stearic acid) is always in the sn-1 position (data not shown).

Finally, about the question in P11, we are so sorry that we failed to find the approved number. But we carried out the guidelines of Ethical Committee of Experimental Animal Care strictly.

We really appreciate your comments and suggestions.

Highlights

- Phospholipids derived from *Asterias amurensis* (SFP) and sea cucumber *Cucumaria frondosa* (SCP) are rich in eicosapentaenoic acid (EPA).
- SFP and SCP liposomes inhibited cell proliferation of various kinds of cancer cells.
- SFP and SCP liposomes showed high transport and uptake effects in small intestinal epithelial cell models.
- Dietary SFP and SCP liposomes prolonged the life span of S180 ascitic tumour bearing-mice.
- SFP and SCP liposomes exerted antitumour effects via activation of mitochondrial apoptotic pathways.

Manuscript
Click here to view linked References

Antitumour activity of EPA-enriched phospholipids liposomes against S180 ascitic tumour-bearing mice

Lei Du [a,b#], Yu-Hong Yang [a,b#], Yu-Ming Wang [b*], Chang-Hu Xue [b], Hideyuki Kurihara [a], Koretaro Takahashi [a*].

[a] Faculty of Fisheries Sciences, Hokkaido University (3-1-1 Minato-cho, Hakodate, Hokkaido, 041-8611, Japan)

[b] College of Food Science and Engineering, Ocean University of China, No.5 Yu-Shan Road, Qingdao, Shandong Province, 266003, People's Republic of China

[#] These authors contributed equally to this work.

[*] To whom correspondence should be addressed:

Yu-Ming Wang

College of Food Science and Engineering, Ocean University of China, No.5, Yu Shan Road, Qingdao, Shandong Province 266003, People's Republic of China

Tel: +86-532-82032597, Fax: +86-532-82032468. E-mail address: wangyuming@ouc.edu.cn

Koretaro Takahashi

Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

Tel: +81-138-40-5560, Fax: +81-138-40-5560. E-mail address: kore@fish.hokudai.ac.jp

Abbreviations

AO, acridine orange; AP, apical; BL, basolateral; BW, body weight; CAT, catalase; DHA, docosahexaenoic acid; EB, ethidium bromide; EPA, eicosapentaenoic acid; GSH, glutathione; PL, phospholipids; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SCP, sea cucumber phospholipids; SFP, starfish phospholipids; SOD, superoxide dismutase; TEER, transepithelial electrical resistance.

Abstract

The health benefits of eicosapentaenoic acid (EPA)-enriched phospholipids (PL) have witnessed a recent upsurge. In the present study, PL from starfish *Asterias amurensis* (SFP) and sea cucumber *Cucumaria frondosa* (SCP) were extracted and the liposomes were prepared. Both SFP and SCP liposomes showed antitumour effects *in vitro* and exhibited high transport and uptake effects in small intestinal epithelial cell models. The results also demonstrated that dietary SFP and SCP liposomes prolonged the life span and ameliorated oxidative stress status of S180 ascitic tumour-bearing mice. Results also showed that dietary SFP and SCP liposomes up-regulated the expression of Bax and down-regulated Bcl-2 level in tumour cells, then induced release of cytochrome c from mitochondria to cytosol, thus activation of caspase 9 and caspase 3. Taken together, these findings suggest that SFP and SCP liposomes exert antitumour effects mainly via activation of mitochondrial apoptotic pathways in tumour-bearing mice.

Key words: EPA-enriched phospholipids, liposomes, S180 ascitic tumour, antioxidant, mitochondrial apoptotic pathways.

1. Introduction

Cancer is now the second leading cause of death in the world. Many factors influence cancer initiation and growth, including various kinds of cytokines, growth factors and oxidative stress (Colomer et al., 2007). Oxidative stress is defined as an imbalance between production of free radicals such as reactive oxygen species (ROS) and antioxidant defenses (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). It has been suggested that cancer initiation and progression is related to continuous oxidative stress by increasing DNA mutations and damage, genome instability and cell proliferation (Bartsch & Nair, 2006; Klaunig, Kamendulis, & Hocevar, 2010; Visconti & Grieco, 2009). Apoptosis, a process of programmed cell death, plays a crucial role in controlling cell proliferation and prevention of tumour development, which is characterized by morphological changes including cytoplasmic shrinkage, membrane blebbing, nuclear chromatin condensation, chromosomal DNA cleavage and nuclear fragmentation (Elmore, 2007). Recent knowledge on apoptosis has provided the basis for novel targeted therapies that exploit apoptosis for therapeutic benefit in cancer (Rahman, Sultan, Islam, & Biomol, 2012). Thus, agents for ameliorating oxidative stress and/or for triggering the apoptosis in cancer cells may be effective in cancer therapy.

Although conventional cancer therapies such as chemotherapy and radiotherapy can kill the cancer cells effectively, it also damages the normal cells, which often impair cancer patients` quality of life and affects the survival (Rein & Kurbacher, 2001). Nowadays, increasing evidence from cell and animal studies indicate that dietary n-3 polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) can prevent carcinogenesis

(Larsson, Kumlin, Ingelman-Sundberg, & Wolk, 2004). In addition, EPA supplement has been reported to be beneficial for cancer patients and without adverse effects at the recommended doses (no more than 3 grams per day) (Colomer et al., 2007). Moreover, it has also been observed that EPA administrated in combination with different antineoplastic agents and radiotherapy can increase cancer treatment efficacy without additional side effects (Calviello, Serini, Piccioni, & Pessina, 2009). However, most of the studies have been carried out using EPA bound to triacylglycerol or ethyl esters while few studies report the antitumour effects of EPA bound to phospholipids (PL). Marine resources such as starfish *Asterias amurensis* and sea cucumber *Cucumaria frondosa* are rich in EPA-enriched PL. On the other hand, use of liposomes as a carrier system can improve the therapeutic efficacy of a wide range of drugs. PL are the main constituent of liposomes that is due to its typical amphiphilic properties and the advantages of biodegradability, lack of antigenicity, and protection of encapsulated functional compounds against degradative enzymes to some extent (Hossain, Kurihara, Hosokawa, & Takahashi, 2006; Nag & Awasthi, 2013).

Hence, in the present study, PL from starfish *Asterias amurensis* (SFP) and sea cucumber *Cucumaria frondosa* (SCP) were isolated and the liposomes composed of these PL were prepared. We determined the antitumour activity of SFP and SCP liposomes on several kinds of cancer cells. To assess the bioavailability of EPA-enriched PL, we evaluated the transport and uptake effects of SFP and SCP liposomes in small intestinal epithelial cell models. To explore the possible mechanism, we investigated the mechanisms of SFP and SCP liposomes inhibiting the tumour growth in S180 ascitic tumour-bearing mice.

2. Materials and Methods

2.1. Materials

The starfish *Asterias amurensis* were collected at the coast of Nemuro, Hokkaido, Japan. The sea cucumber *Cucumaria frondosa* were purchased from aquatic product market, Qingdao, China. Soy-PL was supplied from NOF Corporation (Tokyo, Japan). Dulbecco's modified Eagle medium (DMEM), Roswell Park Memorial Institute-1640 medium (RPMI-1640), fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA), non-essential amino acids (NEAA) and penicillin-streptomycin were purchased from GIBCO (Grand Island, NY, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT), acridine orange (AO) and ethidium bromide (EB) were purchased from Sigma (St. Louis, MO, USA). Rabbit anti-Bax, Bcl-2, cytochrome c, cleaved-caspase 9, cleaved-caspase 3 and β-actin polyclonal antibodies, and goat-anti rabbit antibody IgG-horseradish peroxidase (HRP) were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Preparation of phospholipids from starfish and sea cucumber

The phospholipids were extracted from the gonad and viscera of starfish as well as the body wall of sea cucumber following the modified method of Liu et al. (2014). Just in brief, extracted total lipids were applied to a silica gel column chromatography and eluted with chloroform, acetone and then methanol sequentially. Finally, the methanol eluent was collected and evaporated to obtain the phospholipids. The purity of phospholipids were confirmed to be 94.5 % (SFP) and 93.7 % (SCP) conducted by normal phase high

performance liquid chromatography coupled with an evaporative light scattering detector (HPLC-ELSD) analysis.

2.3. Fatty acid composition analysis of phospholipids

The phospholipids were converted to methyl ester derivatives following the method of Prevot and Mordret (Prevot & Mordret, 1976) with slight modifications. The dried sample was dissolved in *n*-hexane and 0.2 mL 2 M methanolic-NaOH was added. Then, the mixture was shaken, kept at 50 °C for 30 seconds and 0.2 mL 2 M HCl in methanol was added and shaken to neutralize. The mixture was separated by centrifugation at 700 ×g for 5 minutes. The upper *n*-hexane layer was collected, concentrated, and subjected to gas chromatographic analysis with 0.5 μm thickness PEG-20M liquid phase-coated 40 m × 1.2 mm diameter G-300 column (Chemicals Evaluation and Research Institute, Saitama, Japan) connected to Hitachi 263 gas chromatograph (Hitachi Co. Ltd., Ibaraki, Japan) that was equipped with flame ionization detector. The temperatures of the column, detector, and injection port were 190, 240 and 250 °C, respectively.

2.4. Preparation of liposomes

The liposomes were prepared following the method of Bangham (Bangham, Standish, & Watkins, 1965). The mixing molar ratios of the composite lipid classes were SFP/cholesterol = 1:1 (mol/mol), SCP/cholesterol = 1:1 (mol/mol) and soy-PL/cholesterol = 1:1 (mol/mol). The mixtures were dissolved in chloroform and dried to thin film under reduced pressure in a rotary evaporator. The lipid films were hydrated with water to exfoliate lipid bilayers by

vigorous vortex mixing for 5 min and were then extruded 21 times through polycarbonate membrane filter (Whatman Inc., Newton, MA, USA) with pore size of 200 nm. The size distribution of liposomes were determined using a dynamic light scattering particle size analyzer LB-500 (HORIBA, Kyoto, Japan).

2.5. Cell Culture

Human colon carcinoma Caco-2 cells were obtained from American Type Culture Collection (Rockville, CT, USA). Raji cells (B-cell lymphoma line) were a generous gift from Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan. Murine sarcoma S180 cells, mouse leukaemia P388 cells, human hepatoma HepG2 cells and human cervical carcinoma HeLa cells were obtained from Shanghai Cell Bank (Shanghai, China).

The S180, HepG2 and HeLa cells were grown in RPMI-1640 medium, supplemented with 10 % heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2. P388 cells were cultured in DMEM medium with 100 U/mL penicillin, 100 μg/mL streptomycin and 10 % heat-incubated FBS.

The Caco-2 cells were grown in DMEM with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 % NEAA, and 10 % heat-incubated FBS at 37 °C in a 5 % CO_2-humidified incubation. Raji cells were cultured in RPMI 1640 medium with 100 U/mL penicillin, 100 μg/mL streptomycin, 1 % NEAA and 10 % heat-incubated FBS.

2.6. Cell viability assay

Cell viability was determined by the colorimetric MTT assay. Cancer cells (2×10^4 cells/well) were seeded in 96-well plates. After 24 h, the cells were treated with different concentrations of SFP or SCP liposomes for 24, 48 and 72 h, respectively. After incubation for indicated time, MTT solution (0.5 mg/mL in RPMI-1640 medium) was added and further incubated for 4 h. The absorbance was subsequently measured at 570 nm in each well by using Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Triplicate experiments were performed in a parallel manner for each concentration. Cell viability ratio was calculated by the following formula: cell viability ratio (%) = $(A_t / A_c) \times 100\%$, where A_t and A_c are the average absorbance of three parallel experiments from the treated and control groups, respectively.

2.7. Establishment and validation the small intestinal epithelial cell models

For Caco-2 cell monolayer model, caco-2 cells were seeded at a density of 3×10^4 cells/mL onto a 12 mm polycarbonate transwell filter insert with 3.0 μm pores and a surface area of 0.6 cm^2 (Millipore Co. Ltd., Billerica, MA, USA). The transwell filter inserts were placed in 24-well cell culture plates. The cells were grown with 400 μL DMEM in the apical (AP) chamber and 600 μL DMEM in the basolateral (BL) chamber. The AP and BL chamber were given fresh DMEM at two-day intervals until use. Caco-2 cells were grown for 20 days (postconfluence) before the experiments.

For microfold cell (M cell) monolayer model, the incubaton of M cells from caco-2 cells was performed accoding to the methods of Gullberg et al. (2000). Briefly, 5×10^5 cells/mL raji

cells were resuspended in RPMI/DMEM (1:2, v/v) mixture medium and added to the BL chamber of 14-day-old Caco-2 cell monolayer. The cocultures were maintained for another 6 days.

To verify the monolayer integrity, the transepithelial electrical resistance (TEER) was measured using a Millicell® ERS (Billerica, MA, USA). The full differentiation of M cell monolayer was confirmed by checking the amount of engulfed fluorescent latex beads (Murakawa et al., 2007).

2.8. Transport and uptake studies of liposomes

Before each experiment, the AP chamber and BL chamber were washed twice and pre-equilibrated for 30 min in Hanks balanced salt solution (HBSS) containing 10 mM MES and 1 % FBS at pH 6.0 in the AP chamber and HBSS containing 10 mM HEPES and 1 % FBS at pH 7.4 in the BL chamber. One mM calcein encapsulated liposomes in 400 µL HBSS (pH 6.0) was added to the AP chamber while the BL chamber contained 600 µL HBSS at pH 7.4. After incubation at 37 °C for 2 h, the HBSS in the BL chamber was collected and 10 % Triton X-100 was added to disrupt the transported liposomes. Then, cells were washed by PBS, harvested and lysed. The fluorescent intensity was determined using Hitachi F-2500 fluorescence spectrophotometer (Hitachi Co. Ltd., Ibaraki, Japan). The excitation and emission wavelengths were 490 and 520 nm, respectively.

2.9. Animals

All animal experimental procedures were approved according to the guidelines of Ethical Committee of Experimental Animal Care at Ocean University of China. Kunming (KM) male mice (18-22 g, 6-8 weeks) were purchased from Vital River Laboratory Animal Technology Co. (Beijing, China). The animals were provided with standard chow diet and tap water, and were kept at a constant temperature of 24 °C, relative humidity of 65 ± 15 % and in a 12 h light-dark cycle.

2.10. Animal protocols

2.10.1 Experiment 1

S180 cells (1×10^6 cells in 0.2 mL PBS) were injected into the KM mice via abdominal cavity. The mice were then randomly divided into three groups (8 mice in each group): model control group, SFP-treated and SCP-treated group. After 24 h, the animals in each group were given intragastrically of normal saline, SFP or SCP liposomes (100 mg/kg BW) once per day for totally 14 consecutive days. After the final drug administration, the mice were allowed free access to water and food until death and then calculated the survival rate.

2.10.2 Experiment 2

S180 ascitic tumour-bearing mice model was established as *Experiment 1* described. After inoculated tumour cells, the mice were randomly divided into four groups (8 mice in each group): normal control group, model control group, SFP-treated and SCP-treated group. After 24 h, the mice were given intragastrically of normal saline, SFP or SCP liposomes (100

mg/kg BW) once per day for 14 days. At day 15, the mice of each group were sacrificed. The ascitic fluid in each group were collected and weighed. A portion of ascitic tumour in each group was placed in nuclease-free tubes and dissolved in Trizol reagent to extract total RNA and the other portion was used for AO and EB staining. The viable tumour cells counts were measured. Liver were quickly excised, rinsed in ice-cold normal saline, then were snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

2.11. Analysis of biochemical parameters in liver

Prior to biochemical analysis, each liver sample (100 mg/mL buffer) was homogenized in 50 mM phosphate buffer (pH 7.0) under ice bath condition. The homogenate was then centrifuged at 7,770 ×g for 15 min at 4 °C. Glutathione (GSH) content, superoxide dismutase (SOD) and catalase (CAT) activity in liver were measured by commercial kits (Nanjing Jiancheng Biotechnology Institute, Jiangsu, China) according to the manufacturer instructions. All liver parameters were expressed as content or activity per mg protein. The protein concentration in each fraction was determined by Pierce™ BCA protein assay kit (Thermo Scientific, Waltham, MA, USA).

2.12. Cellular morphological analysis of apoptosis

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by fluorescence microscopy using AO and EB staining. The ascitic tumour cells in each group were harvested, centrifuged, washed and appropriately diluted with phosphate buffered saline (PBS). The 20 μL of diluent cell suspension was stained with 100 μg/mL AO

and 100 μg/mL EB for 10 min at room temperature. Cells were then observed by fluorescent microscope (IX-51, Olympus, Tokyo, Japan) equipped with an automatic photomicrograph system.

2.13. Real-time polymerase chain reaction analysis

Total RNA was extracted from the S180 ascitic tumour cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's recommended procedures. One microgram RNA was converted to first strand cDNA using MMLV reverse transcriptase (Promega, Madison, WI, USA) and random primers. Real-time PCR was performed in the Bio-Rad iCycler iQ5 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Twenty five microlitres of the final reaction volume were used for the quantitative real-time PCR assay that consisted of 12.5 μL Maxima SYBR Green qPCR Master mix (Fermentas, Glen Burnie, MD, USA), 10 μM of primers (0.3 μL each of forward and reverse primer), 5.9 μL nuclease-free water, and 6 μL of template. The thermal conditions consisted of an initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 20 s and extension at 72 °C for 30 s, for a final step, a melting curve began at 65 °C and increased 0.5 °C after each 10 s by 61 cycles. Data normalization was accomplished using an endogenous reference β-actin. A dilution curve from one cDNA source using dilutions of 1:2, 1:4, 1:8 and a no-template control was run for each gene. The gene expression level was analyzed by relative quantification using the standard curve method. Primer sequences are described in **Table 1**.

2.14. Western blotting analysis

For western blotting analysis, S180 ascitic tumour cells were lysed in ice-cold RIPA lysis buffer to obtain cellular protein. For isolation of mitochondrial and cytosolic fractions, the Mitochondria/Cytosol Fractionation Kit (BioVision Inc., Milpitas, CA, USA) was used. The proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blotted with Tris-Hcl buffered buffer saline (TBS) containing 1 % Tween 20 (TBST) and 5 % non-fat milk for 2 h, and then incubated with Bax, Bcl-2, cytochrome c, cleaved-caspase 9, cleaved-caspase 3 or β-actin primary antibodies (diluted 1:1000) at 4 °C overnight. After washing with TBST for 5 times, membranes were incubated with IgG-HRP secondary antibodies at room temperature for 1 h. The bands were detected by using an enhanced chemiluminesence kit (Applygen, Beijing, China). Normalization of protein expression was carried out using β-actin as control.

2.15. Statistical analysis

All the values in this study are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using SPSS software. Data were analyzed using Tukey's post hoc test. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Fatty acid composition of phospholipids

Fatty acid compositions of phospholipids extracted from starfish, sea cucumber and soy are shown in **Table 2**. SFP contained 42 % EPA (C20:5), 8.8 % arachidonic acid (AA) and 6.8 % docosahexaenoic acid (DHA, C22:6) while SCP included 47.9 % EPA, 11.5 % stearic acid (C18:0) and 6.1 % oleic acid (C18:1). The major fatty acids in soy-PL were linoleic acid (C18:2, 62.7 %), palmitic acid (C16:0, 18.9 %) and oleic acid (9.7 %).

3.2. Effects of SFP and SCP liposomes on proliferation of cancer cells *in vitro*

The inhibitory effects of SFP and SCP liposomes against several kinds of cancer cells were measured by colorimetric MTT assay. As shown in **Fig. 1**, both SFP and SCP liposomes showed a dose-dependent inhibitory effects on the viability of S180, P388, HepG2 and HeLa cells. The IC_{50} value of SFP and SCP liposomes on S180 cells at 72 h were 198.5 μM and 187.5 μM, respectively. These results indicate that SFP and SCP liposomes exert anti-proliferative activity against several kinds of cancer cells.

3.3. Effects of phospholipids liposomes on transport and uptake in small intestinal epithelial cell models.

To assess the absorption of SFP and SCP liposomes, we established the small intestinal epithelial cell models and evaluated the transport and uptake effects of SFP and SCP liposomes. Prior to transport and uptake studies, the monolayer integrity of small intestinal epithelial cell models (Caco-2 cell monolayer model and M cell monolayer model) were

ensured by TEER values and the amount of engulfed latex beads. The average TEER value of Caco-2 cell monolayer after 20 days was 593 ± 32 $\Omega \cdot cm^2$. The result of number of the engulfed fluorescent latex beads also indicated that the M cell monolayer model was well-established (data not shown).

The phospholipids liposomes transport through the Caco-2 or M cell monolayer model was measured by the transported calcein amount encapsulated in the liposomes. The average particle sizes of calcein encapsulated liposomes of SFP, SCP and soy-PL were 168.9 ± 34.4, 174.2 ± 38.6 and 169.1 ± 34.8 nm, respectively. As shown in **Fig. 2**, the phospholipids liposomes exhibited significantly higher transport in M cell monolayer model. In addition, the transport and uptake of SFP and SCP liposomes were potently higher than soy-PL liposomes in both Caco-2 and M cell monolayer models. These data suggest that the liposomes composed of n-3 PUFAs largely promote the absorption of those liposomes through small intestinal epithelial cells.

3.4. Antitumour effects of SFP and SCP liposomes in S180 ascitic tumour-bearing mice

To better understand the antitumour activity of SFP and SCP liposomes *in vivo*, we employed the S180 ascitic tumour-bearing mice model and evaluated the mean survival time of tumour-bearing mice after treated with SFP or SCP liposomes. As shown in **Fig. 3 & Table 3,** the mean survival time of model control group was 14.8 ± 0.88 days, while the mice treated with SFP and SCP liposomes (100 mg/kg BW) prolonged the survival time to 19.9 ± 1.33 days ($P < 0.01$) and 20.1 ± 0.74 days ($P < 0.01$).

To quantify the antitumour effects of SFP and SCP liposomes on S180 ascitic tumour-bearing mice, the ascitic fluid weight and tumour cells viability in mice were measured and calculated at day 15. As shown in **Fig. 4**, significant reductions in S180 ascitic fluid weight were observed in SFP-treated and SCP-treated mice as compared to the tumour model group. Moreover, the ascitic tumour cell viability in mice after dietary SFP and SCP liposomes for 14 days also decreased notably (**Fig. 4**). These findings suggest that SFP and SCP liposomes also exhibit potent antitumour activity *in vivo*.

3.5. Effects of SFP and SCP liposomes on hepatic biochemical parameters in mice

Continuous oxidative stress can lead to chronic inflammation and cause most chronic diseases such as cancer. To investigate whether SFP and SCP liposomes ameliorate the oxidative stress status in S180 ascitic tumour-bearing mice, GSH content and the activities of SOD and CAT in liver were determined. Inoculation of S180 tumour cells in mice drastically decreased hepatic GSH content and inhibited hepatic SOD and CAT activities. Oral administration of SFP and SCP liposomes at 100 mg/kg BW could partly recover GSH content, SOD and CAT activities in the liver of S180 ascitic tumour-bearing mice (**Fig. 5**). These results reveal that the antitumour activity of SFP and SCP liposomes may be partly attributed to their antioxidant properties.

3.6. Effects of SFP and SCP liposomes on induction of ascitic tumour cells apoptosis

To ascertain whether SFP and SCP liposomes inhibit cell proliferation of S180 ascitic tumour cells by inducting apoptosis, the cellular morphological changes of S180 ascitic tumour cells treated with SFP and SCP liposomes were evaluated using fluorescence microscopy after staining S180 ascitic tumour cells with AO and EB. Images manifested that the viable tumour cells with normal morphology stained bright green (arrow 1) were observed in model control group (**Fig. 6A**), while the later apoptotic tumour cells with condensed and fragmented chromatin as well as apoptotic bodies (arrow 2 and 3) stained bright orange were exhibited after treatment of SFP or SCP liposomes (**Fig. 6B** and **6C**). These results suggest that SFP and SCP liposomes would induce apoptosis of S180 ascitic tumour cells.

3.7. Effects of SFP and SCP liposomes on expression of apoptosis-related factors

Finally, to further clarify whether the mitochondrial apoptotic pathways are involved in SFP and SCP-induced apoptosis, the mRNA or protein expression of pro-apoptotic factors such as Bax, cytochrome c, caspase 9, cleaved-caspase 9, caspase 3 and cleaved-caspase 3 as well as anti-apoptotic factor Bcl-2 were investigated. As shown in **Fig. 7** and **Fig. 8**, the mRNA expression of Bax in S180 ascitic tumour cells were increased significantly with SFP and SCP liposomes treatment (by 2.3 and 2.1 fold) as compared to the tumour model group. Moreover, SFP and SCP liposomes also elevated the Bax protein expression by 2.1 and 2.9 fold ($P < 0.01$). On the other hand, after treatment with SFP and SCP liposomes at 100 mg/kg BW in tumour-bearing mice, the mRNA expression of Bcl-2 were highly down-regulated by

45.7 % and 48.1 % in comparison to the model control group. Furthermore, western blotting assay showed that SFP and SCP liposomes also significantly decreased the Bcl-2 protein expression by 29.5 % and 39.3 % ($P < 0.01$). The imbalance of Bax/Bcl-2 ratio leads to the damage of mitochondrial membrane and the release of cytochrome c from the mitochondrial intermembrane into the cytosol. Therefore, we also determined the cytochrome c protein level in mitochondria and cytosol of S180 ascitic tumour cells. As we expected, SFP and SCP liposomes decreased the protein levels of cytochrome c in mitochondria notably, whereas increased the cytochrome c protein expression in cytosol as compared with model control group. Our findings also found that dietary SFP and SCP liposome increased the mRNA levels of caspase 9 (by 1.8 and 1.7 fold) and caspase 3 (by 2 and 1.8 fold) remarkably ($P < 0.01$). In addition, the protein levels of cleaved-caspase 9 and cleaved-caspase 3 in S180 ascitic tumour cells were also elevated significantly after treated with SFP and SCP liposomes. These findings reveal that SFP and SCP-induced apoptosis may be mediated via activation of mitochondrial apoptotic pathways.

4. Discussion

In the present study, our findings manifested that liposomes composed of EPA-enriched phospholipids extracted from starfish and sea cucumber exhibited potent antitumour effects on several kinds of cancer cells and S180 ascitic tumour-bearing mice.

It is now well known that liposomes which are mainly composed of PL have biological and technological advantages as optimal delivery systems for biological substances (Bozzuto & Molinari, 2015). PL is poorly soluble in water or culture medium, therefore the liposomes

composed of SFP and SCP must be the reasonable form used in this study. Moreover, some trials demonstrate that liposomes composed of n-3 PUFAs such as EPA alter the membrane fluidity, intestinal morphology and increase the transport and uptake of nutrients (Perin, Jarocka-Cyrta, Keelan, Clandinin, & Thomson, 1999; Stillwell, Ehringer, & Jenski, 1993). Furthermore, it has also been suggested that n-3 PUFAs bound to phospholipids are more efficient in delivering n-3 PUFAs to blood and desired tissues (Goustard-Langelier, Guesnet, Durand, Antoine, & Alessandri, 1999; Lu, Nielsen, Timm-Heinrich, & Jacobsen, 2011; Wijendran et al., 2002).

Human colon carcinoma Caco-2 cells are most commonly used as a model of human intestinal absorption of drugs and other compounds. When cultured as a monolayer, Caco-2 cells differentiate to form tight junctions, microvilli and a number of enzymes and transporters (van Breemen & Li, 2005). M cells are known as specialized epithelial cells of the follicle-associated epithelium of the gastrointestinal tract and have a high capacity for transcytosis of a wide range of microorganisms and macromolecules (Kucharzik et al., 2000). And it has been suggested that M cell is well established by cocultures of Caco-2 cells and B-cell lymphoma Raji cells (Gullberg et al., 2000). Our present data revealed that the transport and uptake effects of liposomes composed of n-3 PUFAs enriched PL such as SFP and SCP were significantly higher than soy-PL liposomes composed of mainly n-6 fatty acids which is commonly used in liposomal membrane materials in both Caco-2 and M cell monolayer models, suggesting the higher absorption of SFP and SCP liposomes in small intestine epithelial cells.

In S180 ascitic tumour-bearing mice, a rapid increase in ascitic fluid with tumour cells growth and high early mortality rate were observed. Dietary SFP and SCP liposomes prolonged the life span of tumour-bearing mice. It has been suggested that the agent whether prolongs the life span of tumour-bearing animals is considered as a major indicator to evaluate its antitumour activity (Gupta et al., 2004). In addition, we also found that oral treatment of SFP and SCP liposomes decreased the ascitic fluid volume and the ascitic tumour cells viability in tumour-bearing mice. It may be concluded that SFP and SCP liposomes increase the life span of S180 ascitic tumour-bearing mice via decreasing ascitic fluid volume and killing ascitic tumour cells.

A wealth of studies report that excessive production of free radicals will result in oxidative stress and continuous oxidative stress can lead to chronic inflammation, which is associated with an increased risk of several kinds of cancer (Gupta et al., 2004; Klaunig et al., 2010). High concentration of GSH is found in the liver and it is considered to play an important role in the endogenous antioxidant system. In addition, enzymatic antioxidant defense mechanisms are considered to be responsible for the elimination of free radicals such as superoxide and hydrogen peroxide. SOD catalyzes the dismutation of superoxide into hydrogen peroxide, which has to be eliminated by CAT (Gupta et al., 2004). It has been reported that a decrease in GSH content as well as SOD and CAT activities are observed in the liver of tumour-bearing mice (Alam, Majumder, Akter, & Lee, 2015; Ray et al., 2014). And inhibition of antioxidant enzyme activities also occur in cancer patients (Tu et al., 2014). Previous studies have shown that dietary fish oil up-regulates the genes coding of some

antioxidant enzymes such as SOD in mice, suggesting the protective effect against the production of ROS and thus against tumour growth (Takahashi et al., 2002). Moreover, it has also been proved that the continual consumption of EPA plus DHA significantly decreased the ROS levels in the plasma of patients with advanced inoperable non-small cell lung cancer (Finocchiaro et al., 2012). Consistent with this, similar findings were observed in the present study. Oral administration of SFP and SCP liposomes significantly restored GSH levels as well as SOD and CAT activities in the liver of S180 ascitic tumour-bearing mice as compared to the model control group, suggesting that antitumour properties of SFP and SCP liposomes may also be partly attributed to their potent antioxidant activities. However, others have reported that EPA was found to suppress the cancer cell growth by its formation of oxidation products, which leads to apoptosis and cell growth (Larsson et al., 2004). These disparities could be explained because lipid peroxidation of EPA *in vivo* may not correspond with that *in vitro*. On the other hand, inflammation has been hypothesized to increase the production of free radicals and ROS, which leads to carcinogenesis. However, n-3 fatty acids such as EPA can ameliorate inflammation and thus decrease the production of free radicals *in vivo*.

The observation of AO and EB staining indicated that SFP and SCP liposomes killed S180 ascitic tumour cells via induction of apoptosis. Apoptosis is a complex biological process involving many pathways, which has been a potential target for novel therapeutic molecules. The members of Bcl-2 family are considered as one of the most important regulators of the mitochondrial pathways of apoptosis (Wang et al., 2006). Bcl-2 family contains pro-apoptotic genes such as Bax and anti-apoptotic genes such as Bcl-2.

Pro-apoptotic gene Bax acts as a promoter of apoptosis while anti-apoptotic gene Bcl-2 serves as a repressor (Elmore, 2007). The Bax/Bcl-2 ratio regulates the apoptosis by controlling the integrity of the outer mitochondrial membrane. As mentioned above, the imbalance of Bax/Bcl-2 ratio leads to the damage of mitochondrial membrane and the release of cytochrome c from the mitochondrial intermembrane into the cytosol. The released cytochrome c in cytosol then activates initiator caspases, which cleave a number of cellular protein to cause apoptotic changes. Caspase 9, one of the upstream caspases in apoptosis signaling cascades and its activation is normally required for executioner caspases activation. Caspase 3, the critical executioner caspases of apoptosis, is activated by initiator caspases, ultimately resulting in apoptosis (Elmore, 2007; Lowe, 2000; Rahman et al., 2012; Zhang et al., 2015). Studies have suggested that EPA induced apoptosis in esophageal squamous cell carcinoma and HepG2 cells through mitochondrial pathways (Mizoguchi et al., 2014; Zhang et al., 2015). In this study, our data revealed that the mRNA and protein expression of pro-apoptotic gene Bax was up-regulated, whereas the anti-apoptotic factor Bcl-2 was down-regulated in S180 ascitic tumour cells after treated with SFP or SCP liposomes. As a consequence of these changes, the Bax/Bcl-2 ratio was significantly increased, which induced the decrease of cytochrome c protein level in mitochondria and increase of the cytochrome c protein expression in cytosol. And the up-regulations of caspase 9 and caspase 3 mRNA levels as well as cleaved-caspase 9 and cleaved-caspase 3 protein expression in tumour cells were also observed in the SFP and SCP liposomes-treated group. These findings imply that

the mechanism of SFP and SCP liposomes in inducing apoptosis of S180 ascitic tumour cells must involve activation of mitochondrial apoptotic pathways.

In summary, our findings in this study demonstrated that liposomes composed of EPA-enriched PL inhibited cell proliferation of several kinds of cancer cells. SFP and SCP liposomes also exhibited high transport and uptake effects in small intestinal epithelial cell models. In addition, our present data also revealed that oral administration of SFP and SCP liposomes prolonged the life span of S180 ascitic tumour-bearing mice through decreasing ascitic fluid volume and killing ascitic tumour cells. Moreover, oral intake of SFP and SCP liposomes could restore GSH content and activities of endogenous antioxidant enzymes in the liver of tumour-bearing mice. Furthermore, our data also provided evidence that SFP and SCP liposomes exerted their antitumour effects via inducing apoptosis of tumour cells. The mechanism of SFP and SCP induced apoptosis of tumour cells must be responsible for activation of mitochondrial apoptotic pathways. These present findings may provide the basis for a promising prospect of EPA-enriched PL supplementation in cancer therapy.

Acknowledgments

This work was supported by the grants form National Natural Science Foundation of China (NSFC)-Shandong Joint Fund for Marine Science Research Centers (U1406402), NSFC (31371757, 31201329), the Program for New Century Excellent Talents in University (NCET-13-0534) and the Towa Foundation for Food Science & Research. The author Lei Du also thanks China Scholarship Council (No. 201206330082) for supporting his study in Hokkaido University.

References

Alam, B., Majumder, R., Akter, S., & Lee, S. H. (2015). Piper betle extracts exhibit antitumor activity by augmenting antioxidant potential. *Oncology Letters*, *9*(2), 863-868.

Bangham, A. D., Standish, M. M., & Watkins, J. C. (1965). Diffusion of univalent ions across the lamellae of swollen phospholipids. *Journal of Molecular Biology*, *13*(1), 238-252.

Bartsch, H., & Nair, J. (2006). Chronic inflammation and oxidative stress in the genesis and perpetuation of cancer: role of lipid peroxidation, DNA damage, and repair. *Langenbeck's Archives of Surgery / Deutsche Gesellschaft Für Chirurgie*, *391*(5), 499-510.

Bozzuto, G., & Molinari, A. (2015). Liposomes as nanomedical devices. *International Journal of Nanomedicine*, *10*, 975-999.

Calviello, G., Serini, S., Piccioni, E., & Pessina, G. (2009). Antineoplastic effects of n-3 polyunsaturated fatty acids in combination with drugs and radiotherapy: preventive and therapeutic strategies. *Nutrition and Cancer*, *61*(3), 287-301.

Colomer, R., Moreno-Nogueira, J. M., García-Luna, P. P., García-Peris, P., García-de-Lorenzo, A., Zarazaga, A., Casimiro, C. (2007). N-3 fatty acids, cancer and cachexia: a systematic review of the literature. *The British Journal of Nutrition*, *97*(5), 823-831.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*(4), 495-516.

Finocchiaro, C., Segre, O., Fadda, M., Monge, T., Scigliano, M., Schena, M., Canuto, R. A. (2012). Effect of n-3 fatty acids on patients with advanced lung cancer: a double-blind, placebo-controlled study. *The British Journal of Nutrition*, *108*(2), 327-333.

Goustard-Langelier, B., Guesnet, P., Durand, G., Antoine, J. M., & Alessandri, J. M. (1999). n-3 and n-6 fatty acid enrichment by dietary fish oil and phospholipid sources in brain cortical areas and nonneural tissues of formula-fed piglets. *Lipids*, *34*(1), 5-16.

Gullberg, E., Leonard, M., Karlsson, J., Hopkins, A. M., Brayden, D., Baird, A. W., & Artursson, P. (2000). Expression of specific markers and particle transport in a new human intestinal M-cell model. *Biochemical and Biophysical Research Communications*, *279*(3), 808-813.

Gupta, M., Mazumder, U. K., Kumar, R. S., & Sivakumar, T. (2004). Antitumor activity and antioxidant status of *Caesalpinia bonducella* against Ehrlich ascites carcinoma in Swiss albino mice, *184*, 177-184.

Hossain, Z., Kurihara, H., Hosokawa, M., & Takahashi, K. (2006). Docosahexaenoic acid and eicosapentaenoic acid-enriched phosphatidylcholine liposomes enhance the permeability, transportation and uptake of phospholipids in Caco-2 cells. *Molecular and Cellular Biochemistry*, *285*(1-2), 155-163.

Klaunig, J. E., Kamendulis, L. M., & Hocevar, B. A. (2010). Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic Pathology*, *38*(1), 96-109.

Kucharzik, T., Lügering, N., Rautenberg, K., Lügering, A., Schmidt, M. A., Stoll, R., & Domschke, W. (2000). Role of M cells in intestinal barrier function. *Annals of the New York Academy of Sciences*, *915*, 171-183.

Larsson, S. C., Kumlin, M., Ingelman-Sundberg, M., & Wolk, A. (2004). Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am J Clin Nutr*, *79*(6), 935-945.

Liu, X., Cui, J., Li, Z., Xu, J., Wang, J., Xue, C., & Wang, Y. (2014). Comparative study of DHA-enriched phospholipids and EPA-enriched phospholipids on metabolic disorders in diet-induced-obese C57BL/6J mice. *European Journal of Lipid Science and Technology*, *116*(3), 255-265.

Lowe, S. W. (2000). Apoptosis in cancer. *Carcinogenesis*, *21*(3), 485-495.

Lu, F. S. H., Nielsen, N. S., Timm-Heinrich, M., & Jacobsen, C. (2011). Oxidative stability of marine phospholipids in the liposomal form and their applications. *Lipids*, *46*(1), 3-23.

Mizoguchi, K., Ishiguro, H., Kimura, M., Takahashi, H., Sakamoto, N., Tanaka, T., & Takeyama, H. (2014). Induction of apoptosis by eicosapentaenoic acid in esophageal squamous cell carcinoma. *Anticancer Research*, *34*(12), 7145-7149.

Murakawa, K., Fukunaga, K., Tanouchi, M., Hosokawa, M., Hossain, Z., & Takahashi, K. (2007). Therapy of myeloma in vivo using marine phospholipid in combination with Agaricus blazei Murill as an immune respond activator. *Journal of Oleo Science*, *56*(4), 179-188.

Nag, O. K., & Awasthi, V. (2013). Surface engineering of liposomes for stealth behavior. *Pharmaceutics*, *5*(4), 542-569.

Perin, N., Jarocka-Cyrta, E., Keelan, M., Clandinin, T., & Thomson, A. (1999). Dietary lipid composition modifies intestinal morphology and nutrient transport in young rats. *Journal of Pediatric Gastroenterology and Nutrition*, *28*(1), 46-53.

Prevot, A. F., & Mordred, F. X. (1976). Utilisation des colonnes capillaries de verre pour l'analyse des corp gras par chromatographie en phase gazeuse. *Rev. Fse. Corps. Gras.*, *23*, 409-423.

Rahman, A., Sultan, T., Islam, R., & Biomol, I. J. (2012). Apoptosis and cancer : insights molecular mechanisms and treatments. *Int. J. Biomol. & Biomed.*, *2*(1), 1-16.

Ray, S., Murmu, N., Adhikari, J., Bhattacharyya, S., Adhikari, S., & Banerjee, S. (2014). Inhibition of HepG2 hepatic cancer cell growth and CCl_4 induced liver cytotoxicity in Swiss albino mice by Mahua extract. *Journal of Environmental Pathology, Toxicology and Oncology : Official Organ of the International Society for Environmental Toxicology and Cancer*, *33*(4), 295-314.

Rein, D. T., & Kurbacher, C. M. (2001). The role of chemotherapy in invasive cancer of the cervix uteri: current standards and future prospects. *Anti-Cancer Drugs*, *12*(10), 787-795.

Reuter, S., Gupta, S. C., Chaturvedi, M. M., & Aggarwal, B. B. (2010). Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology & Medicine*, *49*(11), 1603-1616.

Stillwell, W., Ehringer, W., & Jenski, L. J. (1993). Docosahexaenoic acid increases permeability of lipid vesicles and tumor cells. *Lipids*, *28*(2), 103-108.

Takahashi, M., Tsuboyama, N., Nakatani, T., Ishii, M., Tsutsumi, S., Aburatani, H., & Ezaki, O. (2002). Fish oil feeding alters liver gene expressions to defend against PPARalpha activation and ROS production. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *282*(2), G338-348.

Tu, H., Sun, H., Lin, Y., Ding, J., Nan, K., Li, Z., Wei, Y. (2014). Oxidative stress upregulates PDCD4 expression in patients with gastric cancer via miR-21. *Current Pharmaceutical Design*, *20*(11), 1917-1923.

Van Breemen, R. B., & Li, Y. (2005). Caco-2 cell permeability assays to measure drug absorption. *Expert Opinion on Drug Metabolism & Toxicology*, *1*(2), 175-185.

Visconti, R., & Grieco, D. (2009). New insights on oxidative stress in cancer. *Current Opinion in Drug Discovery & Development*, *12*(2), 240-245.

Wang, W., Guo, Q. L., You, Q. D., Zhang, K., Yang, Y., Yu, J., Wang, X. T. (2006). The anticancer activities of wogonin in murine sarcoma S180 both *in vitro* and *in vivo*. *Biological & Pharmaceutical Bulletin*, *29*(6), 1132-1137.

Wijendran, V., Huang, M. C., Diau, G. Y., Boehm, G., Nathanielsz, P. W., & Brenna, J. T. (2002). Efficacy of dietary arachidonic acid provided as triglyceride or phospholipid as substrates for brain arachidonic acid accretion in baboon neonates. *Pediatric Research*, *51*(3), 265-272.

Zhang, Y., Han, L., Qi, W., Cheng, D., Ma, X., Hou, L., Wang, C. (2015). Eicosapentaenoic acid (EPA) induced apoptosis in HepG2 cells through ROS-Ca($^{2+}$)-JNK mitochondrial pathways. *Biochemical and Biophysical Research Communications*, *456*(4), 926-932.

Zhang, Y., Li, Q., Shu, Y., Wang, H., Zheng, Z., Wang, J., & Wang, K. (2015). Induction of apoptosis in S180 tumour bearing mice by polysaccharide from Lentinus edodes via mitochondria apoptotic pathway. *Journal of Functional Foods*, *15*, 151-159.

Table 1 Sequences of the primers used in this study.

Table 2 Fatty acid composition of the phospholipids used in this study.

Table 3 The mean survival time of S180 ascitic tumour-bearing mice. S180 cells (10^6 cells/mouse) were injected intraperitoneally into mice. After 24 hours, the mice in each group (n=8) were administered intragastrically with normal saline (NS), SFP or SCP liposomes (100 mg/kg BW) for totally 14 consecutive days. After the final drug administration, the mice were allowed free access to water and food until death and then recorded the survival time. Values are expressed as mean ± SEM (n = 8), $^{*}P < 0.05$, $^{**}P < 0.01$ versus model control mice.

Table 1 Sequences of the primers used in this study

Gene name	Forward primer	Reverse primer
Bax	GGTTGCCCTCTTCTACTTTGC	CCACCATTCCCACCCCTC
Bcl-2	GCTACCGTCGTGACTTCGC	ACCCAGCCTCCGTTATCC
caspase 3	TGACTGGAAAGCCGAAACT	CTGGATGAACCACGACCC
caspase 9	ACAATAAATCTTCGGCAATAGG	TGGGGACCAGGCTCACTT
β-actin	CAGGCATTGCTGACAGGATG	TGCTGATCCACATCTGCTGG

Table 2 Fatty acid composition of the phospholipids used in this study

Fatty acid composition (%)	SFP	SCP	Soy-PL
C14:0	0.71 ± 0.01	0.28 ± 0.01	0.07 ± 0.01
C16:0	3.40 ± 0.23	5.51 ± 0.23	18.9 ± 0.32
C16:1	3.19 ± 0.18	4.24 ± 0.03	0.20 ± 0.04
C18:0	8.52 ± 0.32	11.5 ± 0.52	2.37 ± 0.18
C18:1	6.51 ± 0.31	6.13 ± 0.31	9.68 ± 0.61
C18:2	-	0.36 ± 0.06	62.7 ± 1.09
C18:3 n-3	-	0.64 ± 0.04	3.65 ± 0.39
C20:1	5.57 ± 0.15	7.78 ± 0.15	0.07 ± 0.01
C20:2	0.51 ± 0.33	-	-
C20:3	0.09 ± 0.06	-	-
C20:4 n-6 (AA)	8.85 ± 0.07	4.70 ± 0.07	-
C20:5 n-3 (EPA)	42.0 ± 0.83	47.9 ± 0.83	-
C22:6 n-3 (DHA)	6.84 ± 0.41	2.08 ± 0.41	-
Others	13.8	8.88	2.36
∑SFA	12.6	17.3	19.3
∑MUFA	15.3	18.2	7.95
∑PUFA	58.3	55.7	70.4
∑n-3 PUFA	48.8	50.6	3.65

Table 3 The mean survival time of S180 ascitic tumour-bearing mice

Groups	Dose (mg/kg BW)	Survival days (day)
Model	NS	14.8 ± 0.88
SFP	100	19.9 ± 1.33**
SCP	100	20.1 ± 0.74**

Fig. 1 Effects of SFP and SCP liposomes on cell proliferation of several kinds of cancer cells. S180, P388, HepG2 and HeLa cells were treated with various concentrations of SFP and SCP liposomes for 24, 48 and 72 h. Each value is expressed as mean ± SEM of three independent experiments.

Fig. 2 Effects of phospholipids liposomes on transport and uptake in Caco-2 cell monolayer (Caco-2 cell model) and M cell monolayer (M cell model). One mM calcein encapsulated liposomes composed of SFP, SCP or soy-PL was added to the apical chamber. The transported liposomes in the basolateral chamber or the liposomes which were absorbed into Caco-2 or M cells were determined using fluorescence spectrophotometer after 2 h incubation at 37 °C. The fluorescent intensity was measured at excitation and emission wavelengths of 490 and 520 nm, respectively. Values are expressed as mean ± SEM (n = 4), different letters indicate significant difference at $P < 0.5$ among each value determined by Tukey's post hoc test.

Fig. 3 Survival curve of SFP and SCP liposomes in S180 ascitic tumour bearing-mice. S180 cells (10^6 cells/mouse) were injected intraperitoneally into mice. After 24 hours, the mice in each group (n=8) were administered intragastrically with normal saline, SFP or SCP liposomes (100 mg/kg BW) for totally 14 consecutive days. After the final drug administration, the mice were allowed free access to water and food until death. The survival rate was followed up to 24 days after inoculation.

Fig. 4 Antitumour effects of SFP and SCP liposomes in S180 ascitic tumour bearing-mice. The S180 ascitic tumour bearing-mice in each group (n=8) were administered intragastrically with normal saline, SFP or SCP liposomes (100 mg/kg BW) for 14 days. The ascitic fluid

weight and ascitic tumour cells viability in mice were measured and calculated at day 15. Values are expressed as mean ± SEM (n = 8), $^{*}P < 0.05$, $^{**}P < 0.01$ versus model control mice.

Fig. 5 Effects of SFP and SCP liposomes on hepatic (A) glutathione (GSH) content, (B) superoxide dismutase (SOD) and (C) catalase (CAT) activities in mice. The normal mice and S180 ascitic tumour bearing-mice in each group (n=8) were administered intragastrically with normal saline, SFP or SCP liposomes (100 mg/kg BW) for 14 days. Values are expressed as mean ± SEM (n = 8), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus normal control mice, $^{*}P < 0.05$, $^{**}P < 0.01$ versus model control mice.

Fig. 6 Effects of SFP and SCP liposomes on induction of ascitic tumour cells apoptosis. The changes in cell morphology were examined by AO and EB staining after administered intragastrically with normal saline, SFP or SCP liposomes (100 mg/kg BW) for 14 days in S180 ascitic tumour bearing-mice. The images manifested that the viable tumour cells with normal morphology stained bright green (arrow 1) were observed in model control group (A), while the later apoptotic tumour cells with condensed and fragmented chromatin as well as apoptotic bodies (arrow 2 and 3) stained bright orange were exhibited after administration of SFP (B) or SCP (C) liposomes.

Fig. 7 Effects of SFP and SCP liposomes on the mRNA expression of mitochondrial apoptotic pathways. The mRNA expression of Bax, Bcl-2, caspase 9, caspase 3 and β-actin in S180 ascitic tumour cells of tumour bearing-mice were measured by real-time PCR after treated with SFP and SCP liposomes (100 mg/kg BW) for 14 days. Data normalization was

accomplished using the endogenous reference β-actin. Value are expressed as mean ± SEM (n = 8), $^{*}P < 0.05$, $^{**}P < 0.01$ versus model control group.

Fig. 8 Effects of SFP and SCP liposomes on the protein expression of mitochondrial apoptotic pathways. The protein expressions of Bax, Bcl-2, cytochrome c (mitochondria), cytochrome c (cytosol), cleaved-caspase 9, cleaved-caspase 3 and β-actin in S180 ascitic tumour cells of tumour bearing-mice were determined by western blotting after treated with SFP and SCP liposomes (100 mg/kg BW) for 14 days. Normalization of protein expression was carried out using β-actin as control. Value are expressed as mean ± SEM (n = 8), $^{*}P < 0.05$, $^{**}P < 0.01$ versus model control group.

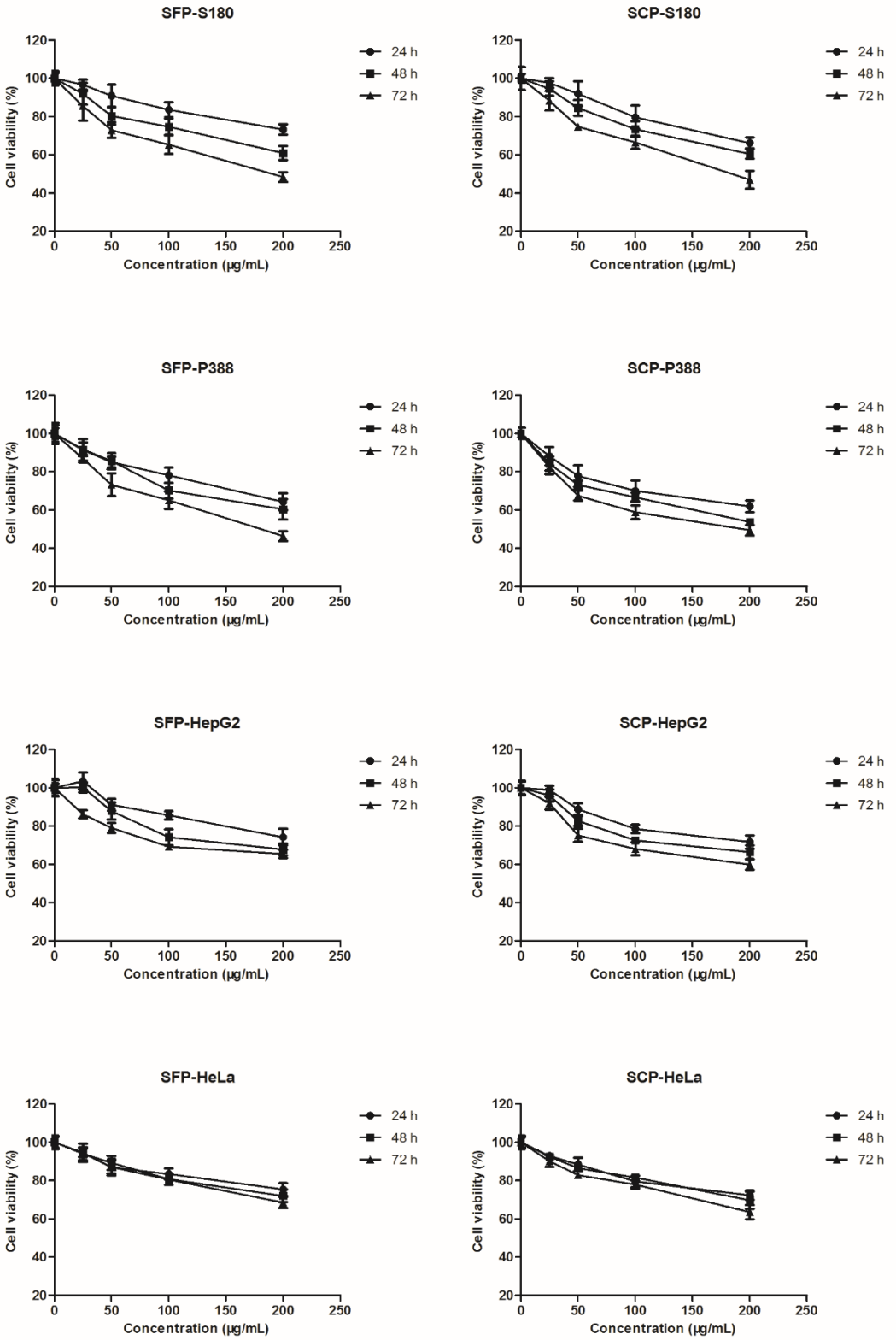

Fig. 1 Effects of SFP and SCP liposomes on cell proliferation of several kinds of cancer cells.

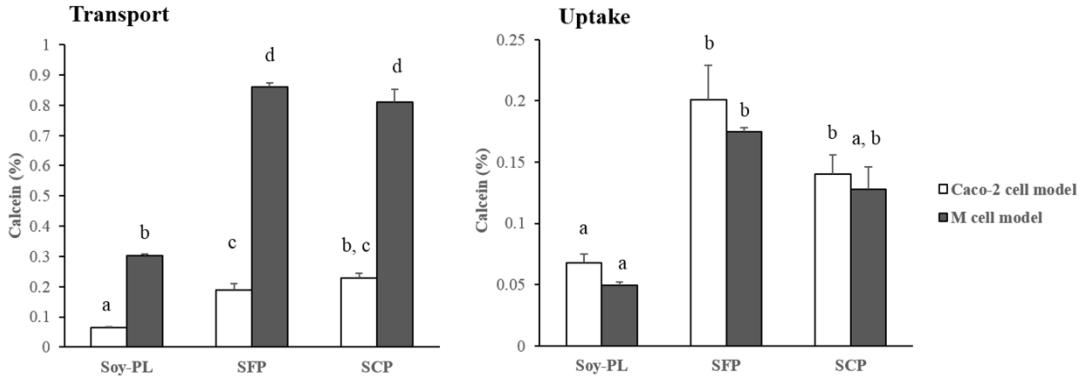

Fig. 2 Effects of phospholipids liposomes on transport and uptake in Caco-2 cell monolayer (Caco-2 cell model) and M cell monolayer (M cell model).

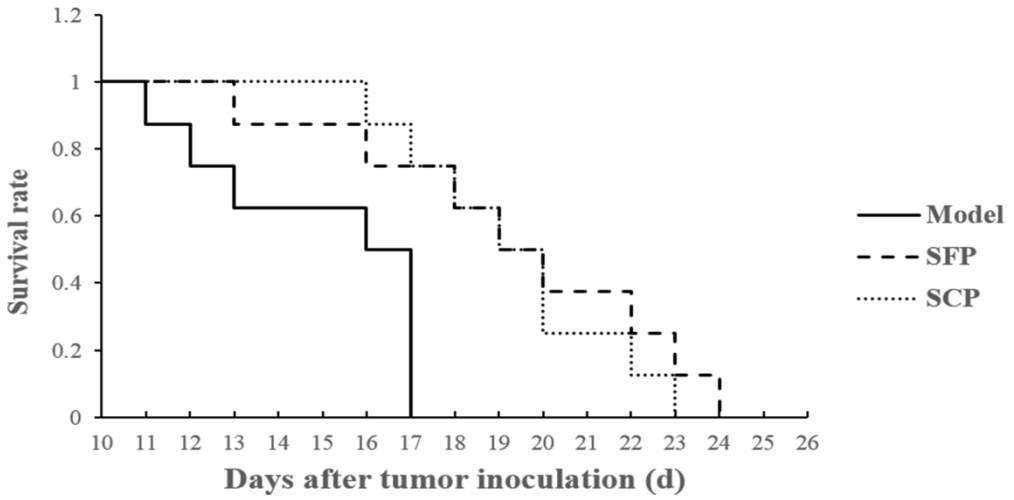

Fig. 3 Survival curve of SFP and SCP liposomes in S180 ascitic tumour-bearing mice.

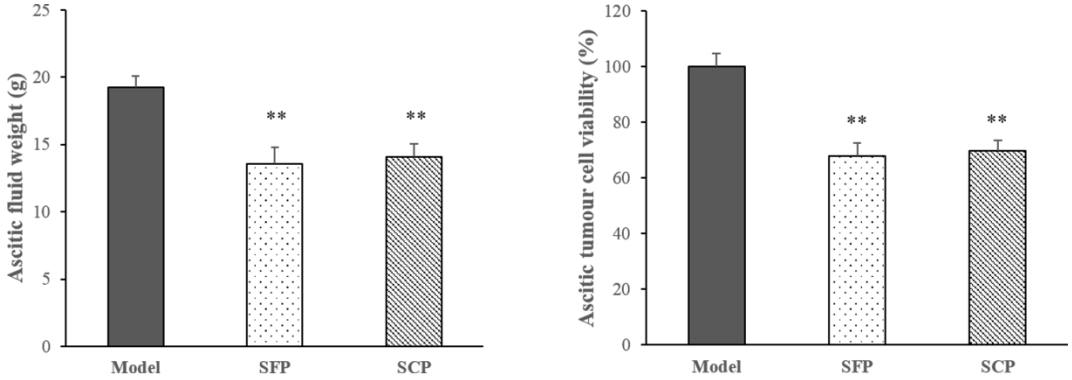

Fig. 4 Antitumour effects of SFP and SCP liposomes in S180 ascitic tumour-bearing mice.

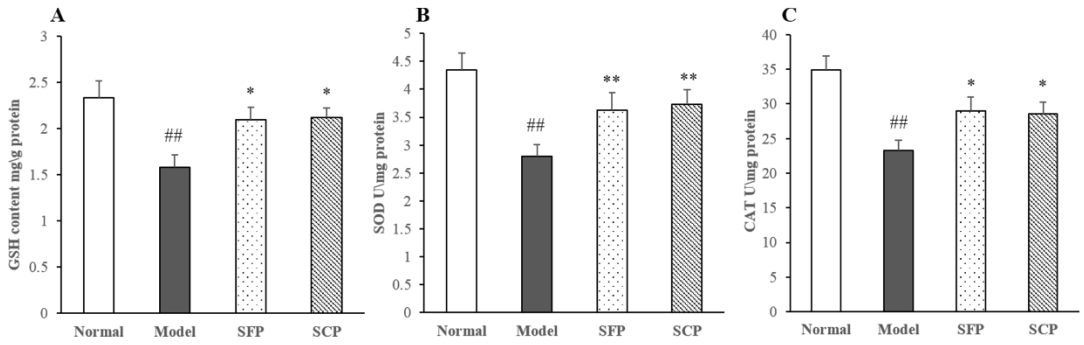

Fig. 5 Effects of SFP and SCP liposomes on hepatic (A) GSH content, (B) SOD and (C) CAT activities in mice.

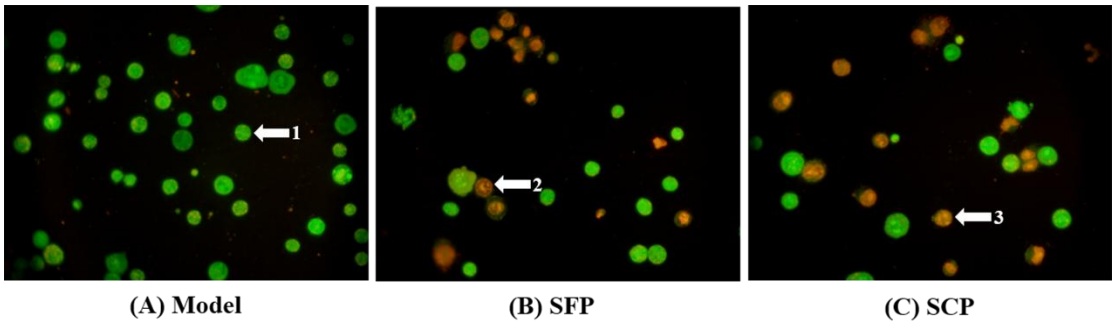

Fig. 6 Effects of SFP and SCP liposomes on induction of ascitic tumour cells apoptosis.

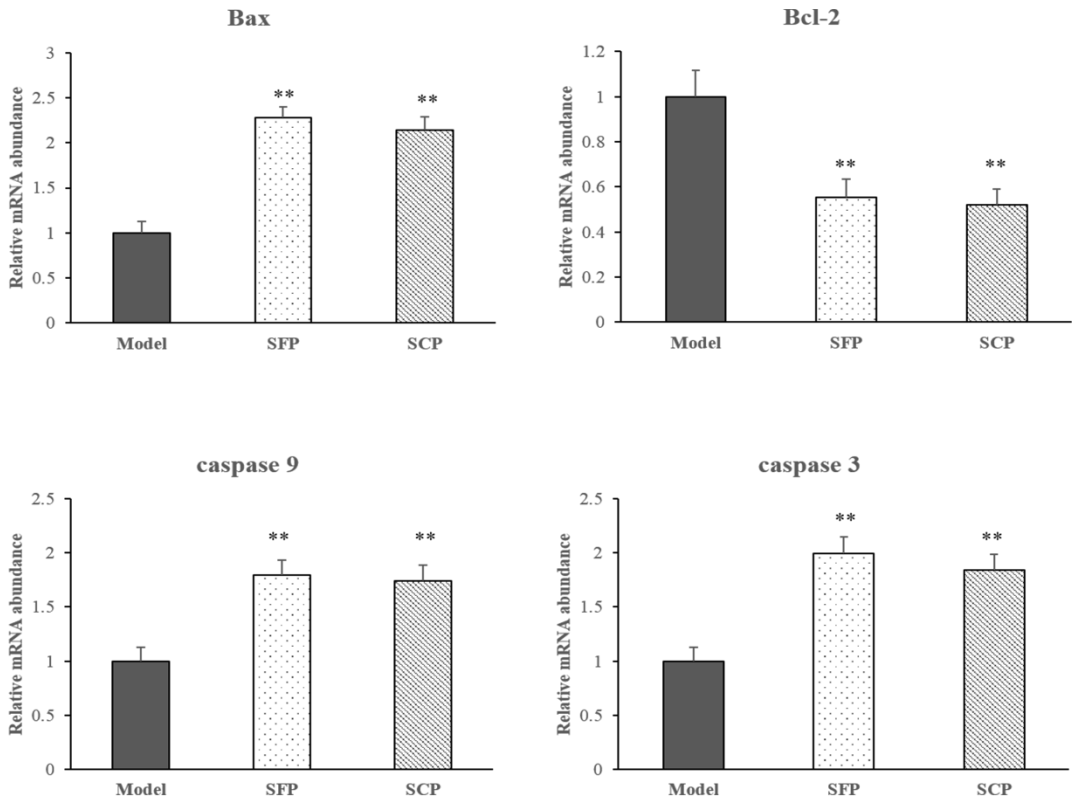

Fig. 7 Effects of SFP and SCP liposomes on the mRNA expression of mitochondrial apoptotic pathways.

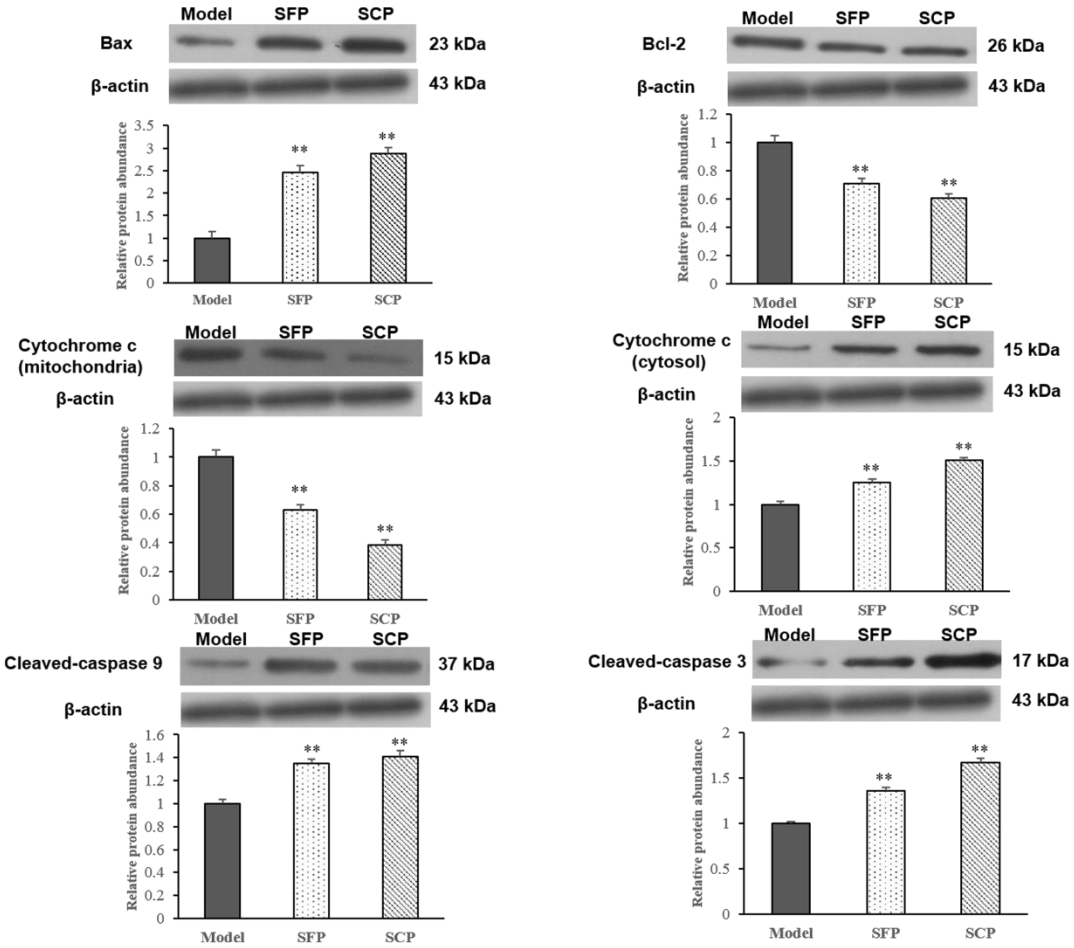

Fig. 8 Effects of SFP and SCP liposomes on the protein expression of mitochondrial apoptotic pathways.