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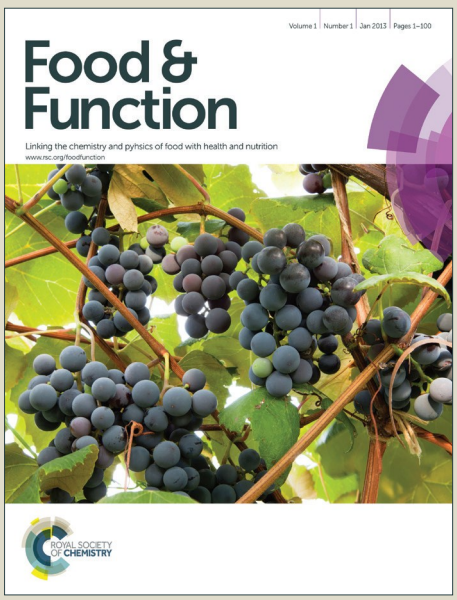

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Evaluation of antioxidative and antitumor activities of extracted flavonoids from Pink Lady Apple in human colon and breast cancer cell lines.

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Abstract

The antioxidative and anticancer effect of extracted flavonoids from Pink Lady Apple on human colon cancer LoVo cells and breast cancer MCF-7 cells were evaluated. It was found the antioxidative property of peel-flavonoids (Peel-F) was more effective than flesh-flavonoids (Flesh-F). Meanwhile, both Peel-F and Flesh-F can inhibit the cancer cell growth in a dose-dependent manner, with the IC50 values of 110.33 ± 2.52 mg/mL and 378.14 ± 1.64 mg/mL for LoVo cells and 58.42 ± 1.39 mg/mL and 296.06 ± 3.71 mg/mL for MCF-7 cells, which led to the conclusion that Peel-F was more effective against cancer cells than Flesh-F and that scavenging ROS effects was significantly higher in Peel-F *in vitro*. Moreover, we also found the generation of ROS is a critical mediator in apple flavonoids-induced cell apoptosis and the induction effect of Peel-F was significantly higher than that of Flesh-F.

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1. Introduction

Advances in dietary modification and clinical studies in past years have unified as well as resolved the anticancer researches in a preventive manner. Many researches reveal that good dietary patterns and dietary constituents are effective in prevention and cure against several types of cancers.[1,2] The high absorption of fruits and vegetables is beneficial in the decrease of cancer and cardiovascular disease.[3,4] Phytochemicals, widely distributed in fruits and vegetables, are regarded as the major anticancer components for their extensive bioactivities in reducing the expression of cancer genes.[5] Among the natural phytochemicals found in herbal remedies, flavonoids are promising in candidate anticancer agents.[6]

Flavonoids have a remarkable scope of healthy benefits with a variety of high bioactivities in antioxidation and antiproliferation. For example, flavonoids are effective in scavenging reactive oxygen species (ROS) and activating an enzymatic antioxdant system in the field of antioxidation and are also regarded as the most utilizable bioactive substances in antiproliferation due to their structures. Such compounds commonly have the basic skeleton of a phenylbenzopyrone deruvative (C_6-C_3-C_6) and the 2 aromatic rings (A and B) are linked by 3 carbons with a central pyran oxygenated ring. Up to now, the molecular structures of more than 9,000 kinds of dietary flavonoids have been identified, and some new structures are still being discovered .[7-9]

Dietary flavonoids are widely distributed in various natural plants-based food and are closely related to people's daily diet. According to statistics, human beings take a

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large number of flavonoids everyday, especially quercetin and rutin. In New Zealand, the average daily intake of quercetin is about 23mg/d.[10] Since the properties of dietary flavonoids contain antitumor, antiviral and anti-aging functions and thus are beneficial for the treatment of cardiovascular and cerebrovascular diseases as well as for the senile osteoporosis. Fine diet and lifestyle play significant roles in prevention of malignant tumor. Some researchers have studied food-induced cancers and have made general recommendations[11] as large-scale epidemiological studies show that the occurrence of malignant tumor is directly linked to people's daily dietary structure and life habits. A large number of researches have actively contributed to the field of relationship between reasonable diet and cancers in the hope that malignant tumors can be prevented by improving and optimizing food collation. And only recently researchers have confirmed the antitumor activity of fruits and vegetables and proposed that flavonoids in plant-based food are major bioactive ingredients for antitumor.[12]

Relevant studies have shown that, vegetables and fruits are able to prevent cancer among which flavonoids are main bioactive substances and have potential medicinal value. However, the relationship between their molecular structure and activity as well as their complex antitumor mechanism still remains to be clarified. As a consequence the antitumor activity of widespread flavonoids like quercetin, rutin, baicalein and resveratrol needs to be determined. In this paper, Pink Lady Apple was selected as the matter for investigation, because of their high content of flavonoids in extracts from peel and flesh, respectively. Then, flavonoids are extracted from peel

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and flesh, respectively, and their contents are also determined. Furthermore, multiple evaluation systems are adopted to conduct a comparative study of in-vitro antioxidant and antitumor activity of flavonoids in peel and flesh.

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2. Material and methods

2.1 Extraction of Apple Flavonoids

Pink Lady Apple, which was purchased from a store in China Xi'an City, was used to extract the flavonoids. 1 mm fresh peel of Pink Lady Apple was cut, the core was removed and then 10 g peel and flesh were weighed and mashed respectively. The two sample were disposed in 80 % ethyl alcohol, respectively, to extract apple flavonoids for 2 h under the condition of room temperature out of sunlight with ultrasonic extractor, and then conduct vacuum filtration, the filtrate was then packaged and put into refrigerator with -20 °C .

The flavonoids were isolated as previously described, then the crude products of peel and flesh flavonoids will be obtained after drying, the dried products were stored into refrigerator with 4 °C.[13]

2.2 Determination of Apple Flavonoids content

10 g of crude products (peel and flesh flavonoids, respectively) was dissolved with methyl alcohol to 5 mL. The absorbance will be measured based on determination method of standard curve, calculate the flavonoids contents of peel and flesh respectively according to the regression equation of Rutin standard curve.

2.3 DPPH• scavenging activity

Based on a method described in the literature and making relevant change.[14] Briefly, 3 mL DPPH• solution with 0.1 Mmol/L methyl alcohol was mixed with 1 mL of sample solution (0.05, 0.1, 0.2, 0.4, 0.8 mg/mL) of peel and flesh flavonoids, and

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then absorbance value (*Ae*) was determined at 517 nm after 30 min standing with room temperature out of sunlight. The absorbance *Ao* was determined via liquids mixed by 1 mL distilled water and 3 mL DPPH• solution. The calculation formula of DPPH scavenging activity is: scavenging rate % = (1－*Ae*/*Ao*)×100.

2.4 Determination for the scavenging of superoxide anion $O_2•^-$

Concentrations of superoxide was determined by the reduction method of NBT with some modifications.[15] The reaction mixture consist of 1 mL 0.078 mol/L NBT solution,1 mL 0.468 mol/L NADH solution and 1 mL of sample solutions (0.05, 0.1, 0.2, 0.4, 0.8 mg/mL) of peel and flesh flavonoids with different concentration respectively and finally 400 μL 0.06 mol/L PMS solution was added to the reaction. The tubes were incubated for 5 min with room temperature and the absorbance value *Ae* were detected at 560 nm. The absorbance *Ao* was determined via liquids mixed with 1 mL distilled water and 1 mL NBT, 1 mL NADH solution and 400 μL distilled water. The calculation formula of $O_2•^-$ is that scavenging rate % = (1-*Ae*/*Ao*)×100.

2.5 Determination of total reducing power

1 mL of sample solutions (0.05, 0.1, 0.2, 0.4, 0.8 mg/mL) of peel and flesh flavonoids were transferred to 1 % $K_3Fe(CN)_6$ solution and 0.2 mol/L phosphate buffer (pH is 7.4). The mixture was put into water at 50 °C for incubation with 20 min, and then 10 % TCA was added to terminate the reaction. Centrifugation was performed to obtain suspension at 3000 r/min for 10 min. Ethyl alcohol and 0.1% $FeCl_3$ solution were added into suspension and then absorbance value was measured

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at the wavelength of 700 nm after being well mixed.[16]

2.6 Recovery and Cultivation of LoVo and MCF17 cellls

Human colon carcinoma LoVo cells and breast cancer MCF-7 cells were obtained from college of medicine, Xi'an Jiaotong University. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were matained in a humidified incubator, contained 5% CO_2, at 37 °C. All experiments were done independently in triplicate per experimental point.

2.7 Determining of tumor cell inhibition

MTT assay described to be available to cell vialibility. [17,18] LoVo cells or MCF-7 cells (2×10^5 cells/well) were seeded into 96-welll microplates and incubated for 24 h in 5 % CO_2 incubator at 37 °C for continuous cultivation. After 24 h (the cells were grown to 70% confluence), the cells were treated with serial concentrations of Peel-F or Flesh-F (0, 25, 50 and 100 mg/mL) for various periods (24 h and 48 h) and or 5-fluorouracil (5-Fu, 50 μM) was used as positive control. After the exposure time, 20 μL of MTT solution (5 mg/mL) in phosphate-buffered saline (PBS) was added to each well and then the plate was further incubated for 4 h. MTT-containing media were removed, and 100 μL of a solution containing 10% sodium dodecyl sulfate (SDS) plus 10 mM of HCl and 5% isobutyl alcohol was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After incubation overnight at 37 °C to ensure that all crystals were dissolved, the light absorption was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader (RT600, Guangdong,

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China).Viability was expressed as the percentage of absorbance in thetreated cells to that in the control cells: cell viability (%) = ODtest/ODcontrol ×100%.

2.8 Toxicity assessment of apple Flavonoids for tumor cell

The cell membrane injury was revealed by the leakage of LDH into the media, which could be detected with an assay kit (Jiancheng BioEngineering, Nanjing, China) by following the manufacturer instructions.[19] At the end of incubation, the culture supernatants with different treatments were supposed to be taken out at 20 μL for the activity analysis of extracellular LDH, and then reacted with 2,4-dinitrophenylhydrazine for the purpose of providing the brownish red in basic solution. The emission wavelength was set at 450 nm and the level of LDH activity could be stated as units per litre (U/L):

$$(U/L)=(OD_{test}-OD_{control})/(OD_{standard}-OD_{blank})\times C_{standard}\times N_{dilute\ times}\times 1000.$$

2.9 Cell cycle analysis by flow cytometry

Flow cytometry was used for analyzing the cell cycle after one day of culturing as previously described.[20,21] The density of 1×10^6 cells/mL of LoVo and MCF-7 cells in medium ,as the initial concentration, was needed in 6-well flat-bottomed plates and 80% confluence achieved by growing overnight, the medium was changed after 24 h. In the next period of 24 h, the cells were treated with vehicle alone (0.05% DMSO) or rutin (0, 25, 50 and 100 μM), respectively. After treatment, the detached cells in the medium were collected with PBS and combined with the remaining adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA, Sigma-Aldrich). After centrifugation, the acquisition of cell pellets was used for next steps by being

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resuspended and fixed with 1 mL of 70% ethanol at 4 °C overnight, washed in PBS, and resuspended in 1.0 mL of PBS containing 1.0 mg/mL RNase (Sigma-Aldrich). Finally, the samples was added in 0.5 mL of 50 μg/mL propidium iodine (PI, Sigma-Aldrich) stain solution, and incubated in the dark for 30 min at room temperature, and analyzed by the GUAVA® easyCyte™ 8HT flow cytometry (Millipore Corporation, Billerica, MA, USA).

2.10 Tumor cell apoptosis Detection

With the same treated described above, apoptotic cells of LoVo and MCF-7 were quantified by Annexin V-FITC/PI double staining assay.[22,23] Annexin V-FITC/PI Apoptosis Detection Kit (BestBio, Shanghai, China) was used for detecting changes in stage of preliminary and terminal, following the manufacturer's instruction. Briefly, cells (1×10^6) were treated with procedural steps of concentration, washing twice with PBS and suspension in 400 μL of binding buffer (adding 5 μL of annexin V-FITC and 10 μL of PI). The annexin V-FITC and PI staining were combined for the advantage of permit on simultaneous quantification of vital, apoptotic and necrotic cells. Thereafter, the samples were incubated in the dark for 10 min at 4 °C, and then analyzed on the flow cytometer. The number of annexin V-FITC-positive and PI-positive of cells in each field was determined by counting the cells directly. All experiments were done independently at least three times per experimental point.

2.11 Determination of reactive oxygen species (ROS) of tumor cell

DCFH-DA and DHE probes are specific dyes used for staining hydrogen

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peroxide (H_2O_2) and superoxide anion ($O_2\bullet^-$), respectively, which are produced by intact cells.[24] LoVo and MCF-7 cells (2×10^5 cells) were seeded in 6-well plates for 24 h, in the next period of 24 h the cells were exposed to rutin (0, 25, 50 and 100 μM) ,and then detached with trypsin-EDTA and washed once with PBS. The treated and control cells were immediately resuspended in 0.2 mL PBS containing DCFH-DA or DHE at the final concentration of 5 μM, reacting for 30 min at 37 °C. Finally, ROS production of cells was evaluated by flow cytometry.

2.12 Statistical analysis

Statistical analysis was carried out by using SPSS 18.0 software. All the data are mean ± SD. A one-way analysis of variance (ANOVA) was used for detecting the significant difference from the respective control for each experimental test condition and $p<0.05$ and $p<0.01$ were considered statistically significant.

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3. Results and Discussion

3.1 Flavonoids Contents in Apple Peel and Flesh

The flavonoids content in Pink Lady Apple was measured according to the relationship between concentration of rutin and absorbance. The values were presented in Table. 1. Flavonoids in peel was 2.2-fold to those in flesh with the value of 24.05 mg/g. The extraction yields of peel flavonoids (Peel-F) and flesh flavonoids (Flesh-F) were 2.4% and 1.1 %, respectively, which led the conclusion that flavonoids in peel was higher than those in flesh.

3.2 Antioxidant Activity of Flavonoids in Apple Peel and Flesh

To assess bioactivity of the compound, antioxidant activity analysis is a significant method to reveal the potential of a compound to inhibit free radical oxidation reaction in the way of direct effect on free radicals or indirect block of intermediate material that easily generates free radicals.

DPPH• is a nitrogen-centered organic synthetic radical, which is more stable in organic solvent, given its capacity to evaluate the antioxidation of matters. It has a single electron and can accept hydrogen ions or electrons. Its methanol solution has a obvious absorption peak at 517 nm. So when there is a free radical scavenger, it will be paired with DPPH• lone-pair electrons, with the absorbance value decreased. The scavenging rate is calculated according to changes in absorbance value. The higher scavenging rate indicates the stronger antioxidant capacity of the matter.

Fig. 1 reflected apple flavonoids' scavenging capacity on DPPH•. As shown in **Fig. 1**, apple flavonoids' scavenging effect on DPPH• was in a

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concentration-dependent manner. The scavenging effect enhanced with the increased treated concentration. Peel flavonoids' scavenging ability is higher than flesh flavonoids. When the sample concentration was 0.8 m /mL, the DPPH• scavenging rate of Peel-F and Flesh-F reached 64.38% and 17.41%, respectively, while quercetin at a concentration of 0.05 mg/mL had a scavenging rate of 87.84 percent, indicating that apple flavonoids' DPPH• scavenging capacity is lower than quercetin.

Superoxide anion radical is a weak oxidant exiting in body, which by disproportionation and a variety of other reaction pathways can produce hydrogen peroxide and hydroxyl radicals, thus is a source of free radicals in body.

Fig. 2 reflects apple flavonoids' O_2•− scavenging capacity related to its concentration. Peel flavonoids' scavenging rate is higher than flesh flavonoids. When the sample concentration was 0.8 mg/mL, the O_2•− scavenging rate of peel flavonoids (Peel-F) and flesh flavonoids (Flesh-F) reached 84.84% and 73.4%, respectively, while quercetin at a concentration of 0.05 mg/mL had a scavenging rate of 97.16%. This is consistent with DPPH• scavenging that apple flavonoids' O_2•− scavenging capacity is also lower than quercetin.

Antioxidants by sacrificing electrons can use their own reducing capacity, which result in a scavenging of free radicals. **Fig. 3** showed the total reducing power of apple flavonoids. The results demonstrate that apple flavonoids have a reduction potency, which is related to their increase in concentration. Peel flavonoids' reducing power was insignificant to flesh flavonoids, but was obviously lower than quercetin.

3.3 Effect of Apple Flavonoids on Inhibition of Tumor Cell Proliferation

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MTT assay was used to evaluate the effects of apple flavonoids on the growth of human cancer LoVo and MCF-7 cells.[25] **Fig. 4** showed the effect of flavonoids in different parts of Pink Lady Apple on viability of LoVo cells and MCF-7 cells for 24 h and 48 h. **Fig. 4A** showed that after 24 h of the treatment, the inhibition rate of 25 mg/mL, 50 mg/mL and 100 mg/mL peel flavonoids (Peel-F) on LoVo cell proliferation were 2.5%, 12.32% and 19.17%, respectively, while the inhibition rate of 100 mg/mL flesh flavonoids (Flesh-F) on LoVo cell proliferation was 6.83%. After 48 h of the treatment, the inhibition rate of 100 mg/mL Peel-F on LoVo cell proliferation reached 47.97%, while the inhibition rate of Flesh-F on LoVo cell proliferation increased to 20.1 %. **Fig. 4B** showed that after 48 h of treatment, the inhibition rate of 50 mg/mL Peel-F and Flesh-F on MCF-7 cell proliferation were 48.77% and 17.35%, which was in contrast to the control group ($P < 0.01$); where the concentration of Peel-F and Flesh-F flesh increased at 100 mg/mL, after 48 h of treatment their inhibition rate on MCF-7 cell reached 57.25% and 26.11%, and at this point the inhibition rate of Peel-F on cell proliferation rate was slightly higher than that after 24 h of treatment of the anticancer agent 5-Fu, which was 54.25%.

MTT assay results showed that the effect of flavonoids in different parts of Pink Lady Apple on anitumor-cell activity in a concentration- or time-dependent manner. Therefore, LoVo and MCF-7 carcinoma cells, when being exposed to higher concentration of apple flavonoids are exposed to a higher cytotoxic effect. After analysis, IC50 value produced by Peel-F and Flesh-F acting on LoVo cells were 110.33 ± 2.52 mg/mL and 378.14 ± 1.64 mg/mL, respectively, and IC50 value for

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MCF-7 cells were 58.42 ± 1.39 mg/mL and 296.06 ± 3.71 mg/mL, respectively, which showed that the inhibitory effect of Peel-F on cancer cells was significantly higher that of Flesh-F and Peel-F demonstrating a more pronounced antitumor effect on MCF-7 cells. This explains that apples are really good ingredients for anti-cancer.

3.4 Effect of Apple Peel Flavonoids on Changes in Tumor Cell Morphology

Adherent cells in the normal growth process will be promoted to attach to the wall by the existence of some special matters. In the process of cell culture, the pro-cell attachment factors first attach to the substrate, and then the spherical cells in suspension adhere to the substrate attached with pro-attachment factors, so that cells slowly attach to the wall and stretch into their normal form. When cells are subjected to external stimuli their adherence rate will be significantly lower. **Fig. 5** showed the cell morphology changes generated after 48 h of different concentrations of Peel-F acting on LoVo cells and MCF-7 cells.

As shown in **Fig. 5**, most LoVo cells in control group were in intact spindle, showing a normal adherent state. After the treatment by Peel-F with a concentration of 25 mg/mL, slight changes occurred compared to the cell morphology in the control group, showing a handful of spherical dead cells. When the drug concentration reached 50 mg/mL, the cell morphology showed great changes compared with that treated by 25 mg/mL Peel-F, when some cells were no longer as a spindle, but most gathered together, flocculent apoptosome formed. When the concentration was 100 mg/mL, the cell adherence rate decreased compared with the control group, showing a large number of spherical suspensoids, i.e. dead cells or apoptotic cells. For MCF-7

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cells, the cells in control group showed an intact regular polygon shape, and after treatment with the 25 mg/mL Peel-F, compared with the control group, only a few cells displayed changed shape into circular or spindle. When drug concentration reached 100 mg/mL, a lot of dead cells, apoptotic cells or cell debris appeared and the number of cells significantly reduced.

The observational experiment showed that the effect of different concentrations of Peel-F on cell morphology was also in a concentration-dependent manner, which means that the higher drug concentration exposed, the greater the destructive changes in cell morphology. This indicates that drug in low concentrations causes no significant damage to cells, while high doses lead to significant cell damage and significant reduction in the number of cells, which is consistent with the cell viability assay results. This reveals that Peel-F can reduce the attachment of cells, affect cell proliferation and survival, making apples one of the best foodborne medicines for anti-cancer.

3.5 The toxicity of apple peel flavonoids and flesh flavonoids to tumor cells

For the study of antitumor efficiency, the release of LDH was usually regarded as a index to assess the integrity of cell member.[26,27] In this research, LDH release was tested after the incubation for 24 h and 48 h at different treated concentration of drug. As shown in **Fig. 6A**, LDH activity produced by treating LoVo cells with 50 mg/mL peel flavonoids (Peel-F) and 50 mg/mL flesh flavonoids (Flesh-F) for 24 h were 466.34 U/L and 149.18 U/L, respectively. However, after 48 h of treatment LDH activity significantly increased to 653.07 U/L and 380.26 U/L, respectively; when the

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concentration increased to 100 mg/mL, the LDH activity produced by peel flavonoids and flesh flavonoids acting on cells for 48 h rose to 751.52 U/L and 478.79 U/L, respectively. As shown in **Fig. 6B**, the LDH activity of MCF-7cells treated with 50 mg/mL Peel-F and 50 mg/mL Flesh-F for 48 h were 678.57 U/L and 457.14 U/L, respectively. When the concentration increased to 100 mg/mL, LDH activity produced by Peel-F and Flesh-F acting on cells for 24 h significantly increased to 963.83 U/L and 789.50 U/L, respectively, after 48 h of treatment increased to 1118.76 U/L and 909.50 U/L. The results revealed that LDH activity with peel flavonoids was significantly higher than that with of the positive control group after 5-Fu treatment.

LDH activity assay results showed that the toxicity of Peel-F and Flesh-F to cancer cells in concentration- or time-dependent manner, suggesting that with increased concentration of flavonoids, LDH activity gradually enhanced and the toxicity to cells also exacerbated. The results on toxicity to two cell lines LoVo and MCF-7 and cell viability of flavonoids in different parts of apples were consistent. Peel flavonoids' effect was significantly better than flesh flavonoids and by comparison peel flavonoids were more sensitive to MCF -7 cells.

3.6 Effect of Apple Peel and Flesh Flavonoids on Tumor Cell Apoptosis

To assess the cell apoptosis, calculating the ratio of sum in period from initial to terminal stage, flow cytometry was performed by using annexin V-FITC and PI labeling at 48 h of exposure.[28] As shown in **Fig. 7A**, seldom apoptotic nuclei of LoVo cell were detected in the control cells, with the normal condition of 80.90 %. Significant amounts of apoptosis cells (21.24 % to Peel-F and 13.32 % to Flesh-F,

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p<0.01 vs control) were tested at 25 mg/mL and as the concentrations were increased to 100 mg/mL, 70.09 % and 31.88 % of the cells were induced by Peel-F and Flesh-F to enter early-stage apoptosis (FITC + /PI −), respectively. Histogram in **Fig. 7B** showed the cell apoptosis induced by different concentrations of Peel-F and Flesh-F. The amount of 100 mg/mL Peel-F could cause the apoptosis of 79.07 % cells ($P < 0.01$), which had a significant difference with the control group ($P < 0.01$) and 100 mg/mL flesh-F could induce the apoptosis of 34.76 % cells ($P < 0.05$). Peel-F caused greater apoptosis of LoVo cells than Flesh-F.

Fig. 8 showed the apoptosis of MCF-7 cells 48 h after being treated with different concentrations of apple flavonoids. As shown in **Fig. 8A,** 92.50% of the cells in control group were in normal state (FITC − /PI −). After being treated by 25 mg/mL Peel-F and Flesh-F, 28.00 % and 11.23 % of the cells were induced to enter early-stage apoptosis, respectively. When the concentration increased to 100 mg/mL, Peel-F and Flesh-F induced 66.43 % and 19.03 % of the cells into the late-stage apoptosis (FITC + /PI +). Histogram in **Fig. 8B** displayed cell apoptosis induced by different concentrations of Peel-F and Flesh-F. The amount of 100 mg/mL Peel-F and Flesh-F induced the apoptosis of 91.43 % and 45.26 % cells, respectively, which had a significant difference with the control group ($P < 0.01$).

The apoptosis detection results suggested that: Different parts of apple flavonoids can induce the apoptosis of tumor cells, which is directly correlated with dosage. With the increase of dosage, more cells will enter the apoptosis period, and Peel-F causes greater cell apoptosis than Flesh-F.

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3.7 Induction effect of Apple Peel and Flesh Flavonoids on Reactive Oxygen Species (ROS) of Tumor Cells

Further study was performed to explore whether apple flavonoids stimulate generation of ROS in both LoVo cells and MCF-7 cells. **Fig. 9** showed the generation of ROS detected by flow cytometer 12 h after the application of different parts of apple flavonoids to LoVo cells and MCF-7 cells. **Fig. 9A** indicated the contents of $O_2•^-$ and H_2O_2 in MCF-7 cells detected by DHE and DCFH-DA fluorescent probes after these cells were treated with Peel-F and Flesh-F. It could be seen from the figure that 25 mg/mL Peel-F and Flesh-F could induce 23.20 % and 12.13 % of the cells to present positive red fluorescence, and 37.47 % and 22.10 % of the cells to show positive green fluorescence, respectively. When the concentration of Peel-F and Flesh-F was increased to 100 mg/mL, 77.62 % and 33.87 % of the cells were induced to present red fluorescence, and 88.33 % and 42.5 % of the cells were induced to show green fluorescence. With the increase of the dosage, the fluorescence was significantly intensified, and the intensity of green fluorescence is significantly higher than that of red fluorescence. **Fig. 9B** indicated the changes in contents of $O_2•^-$ and H_2O_2 in LoVo cells after being treated with Peel-F and Flesh-F. It could be seen from the figure that 25 mg/mL Peel-F could induce 19.78 % and 28.45 % of the cells to present red and green fluorescence. When the concentration increased to 100 mg/mL, 61.84 % of the cells showed red fluorescence and 75.64 % presented green fluorescence. Meanwhile, the same concentration of Flesh-F could induce 26.93 % and 38.27 % of the cells to show red and green fluorescence. This detection results

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suggested that apple flavonoids could induce the generation of ROS within LoVo and MCF-7 cells. The induction effect of Peel-F was significantly higher than that of Flesh-F, leading to the generation of large amounts of ROS within cells. It was speculated that apple peel would effectively promote the ultimate death of cells.

Dietary flavonoids and their analogs have conventionally been used as a health food for the prevention and therapy of cancer.[29] In recent years, many papers have described the antiproliferative properties of flavonoids against various cancer cells.[30,31] The inhibitory effects against cancer cells by flavonoids have been suggested to be mediated by different mechanisms of action such as regulation of oncogene and tumor suppressor gene expression, inhibition of signal transduction pathways involving MAPK, NF-κB, Nrf and AP-1, and induction of cell-cycle arrest and apoptosis involving the p53, Bcl-2 and caspase families.[29,32-35] Therefore, this study was aimed at evaluating whether flavonoids in apple were effective on cancer cells (LoVo cells and MCF-7 cells) by controlled trails on the cell apoptosis and cell cycle progression. The results revealed that Peel-F and Flesh-F showed remarkable effects of antiproliferation and induced cell metabolic processes against LoVo cells and MCF-7 cells in a concentration- or time-dependent manner. Further researches of cytotoxicity were designed to demonstrate the effects of apple flavonoids in molecular and metabolic levels. Results from MTT assay and IC50 showed that Peel-F and Flesh-F effectively inhibited the growth of LoVo cells and MCF-7 cells, suggesting that apples are really good ingredients for anti-cancer. These results are in agreement with our previously research and other reports, describing that the antiproliferative

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activities of flavonoids including quercetin are associated with the induction of apoptosis.[30,36] Morphological changes of the two cells exposed in series of Peel-F and Flesh-F concentration further proved the inhibitory effect and revealed that MCF-7 cells was more sensitive to Peel-F and Flesh-F than LoVo cells, which was accorded with IC50.

The increasing evidence that inhibition of proliferation or action of apoptosis are the mainly reason chemotherapeutic agents induce tumor regression. The research of actions through which flavonoids performed chemopreventative effect in the cell life cycle were necessary for the precise understanding of the exposed cell biological process. In this study, the results from annexin V/PI assay clearly proved that the Peel-F and Flesh-F induced apoptosis and necrosis of LoVo cells and MCF-7 cells. Thus, Peel-F and Flesh-F displayed the abilities to interfere with the process of building up and expedite the tearing down of cancer cells.

Flavonoids has stronger antioxidant activity and could effectively scavenge free radicals *in vitro* which was in agreement with other reports and could significantly enhanced the activities of antioxidant enzymes *in vivo.*[37,38] Meanwhile, we found that apple flavonoids could induced the intracellular ROS generation in cancer cells which was in agreement with Qiao Zhang report [39], high levels of ROS could induce DNA damage-mediated protein expression, AKT, ERK inactivation and SAPKs activation. Furthermore, ROS production conspicuously blocked the migration of cancer cells, led to apoptotic cell-death and the growth inhibition of cancer cells. Mitochondria are a source of ROS during apoptosis and reduced mitochondria membrane potential,

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leading to increased generation of ROS and apoptosis.[40] ROS has been implicated as a second messenger in multiple signaling pathways and can also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell death pathway.[40,41] It was interesting that flavonoids played contrary roles out and in cancer cells. The antioxidant ability of flavonoids was due to their structure for more stability of electron migration and was expressed through progresses of aerobic respiration and lipid peroxidation to affect the production of free radicals in our body, while the ROS was induced via the mitochondrial pathway in cancer cells. The precise reason was unclear, so further studies should be done to understand this reason. The mechanism by which flavonoids inhibits cancer cells proliferation also should be further researched to elucidate the bioavailability of flavonoids via consumption of a flavonoids-rich dietary supplement in animal model.

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4. Conclusion

This study indicated that apple flavonoids have fine antioxidative ability in a concentration-dependent manner *in vitro*. High potential of antitumor activities of Peel-F and Flesh-F were shown in aspects of inhibitory effect on cell growth, decrease of cell attachment as well as induction of cytomembrane damage, cell apoptosis and intracellular ROS generation in LoVo human colon cancer cells and MCF-7 human breast cancer cells in a concentration-dependent manner and peel flavonoids displayed superior properties in not only antioxidation, but also antitumor aspect, resulting into apoptosis of tumor cells. The results suggested apple peel is a really good ingredient for anti-cancer as dietary food.

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Figure Captions

Fig. 1 The scavenging activity of apple flavonoids against DPPH• free radicals

Fig. 2 The scavenging activity of apple flavonoids for O2• ⁻ free radicals

Fig. 3 The total reducing activity of apple flavonoids

Fig. 4 Effects of flavonoids in different parts of apple on cell viability in LoVo (A) and MCF-7 (B) human cancer cells* $p<0.05$, ** $p<0.01$ compared with control group

Fig. 5 The morphological changes of peel flavonoids-exposed LoVo and MCF-7 human cancer cells were observed by Inverted Optic Microscope (Original magnification，× 100)

Fig. 6 Effects of flavonoids in different parts of apple on cell cytotoxicity in LoVo (A) and MCF-7 (B) human cancer cells* $p<0.05$, ** $p<0.01$ compared with control group

Fig. 7 Effects of flavonoids in different parts of apple on human colon cancer LoVo cell apoptosis. * $p<0.05$, ** $p<0.01$ compared with control group

Fig. 8 Effects of flavonoids in different parts of apple on human breast cancer MCF-7 cell apoptosis. * $p<0.05$, ** $p<0.01$ compared with control group

Fig. 9 Detection of ROS generation of MCF-7 (A) and LoVo (B) cells by flow cytometry* $p<0.05$, ** $p<0.01$ compared with control group

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Tables and Figures

Table 1 The flavonoids content in different parts of Pink Lady Apple

Position	Peel	Flesh
Content （mg/g）	24.048 ± 0.139	10.975 ± 0.246
Extraction yield（%）	2.40	1.10
Yield（%）	0.192	0.088
t	7.63	3.28
P	0.001	0.043

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Fig. 1

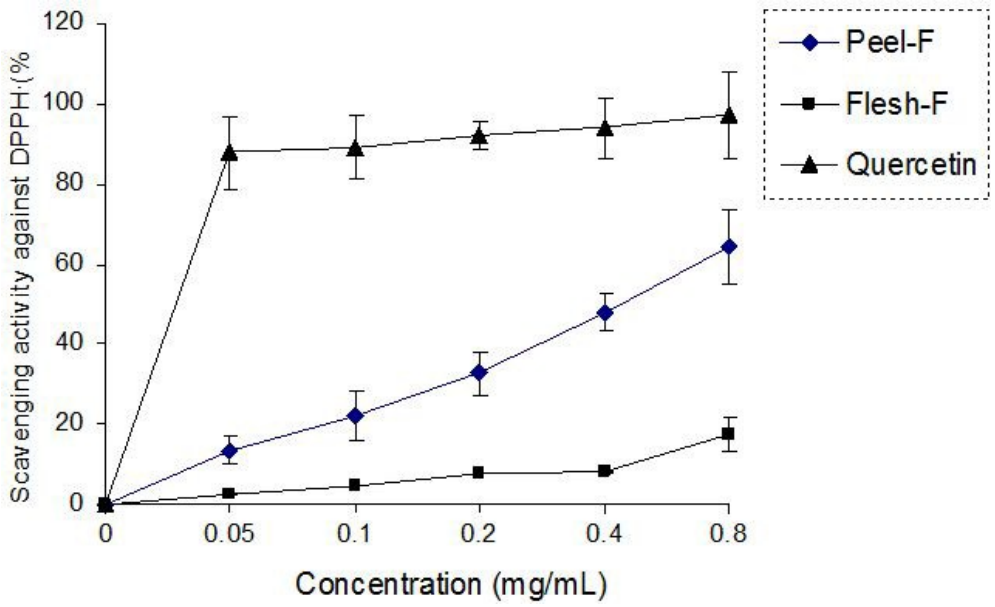

Fig. 1 The scavenging activity of apple flavonoids against DPPH• free radicals

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Fig. 2

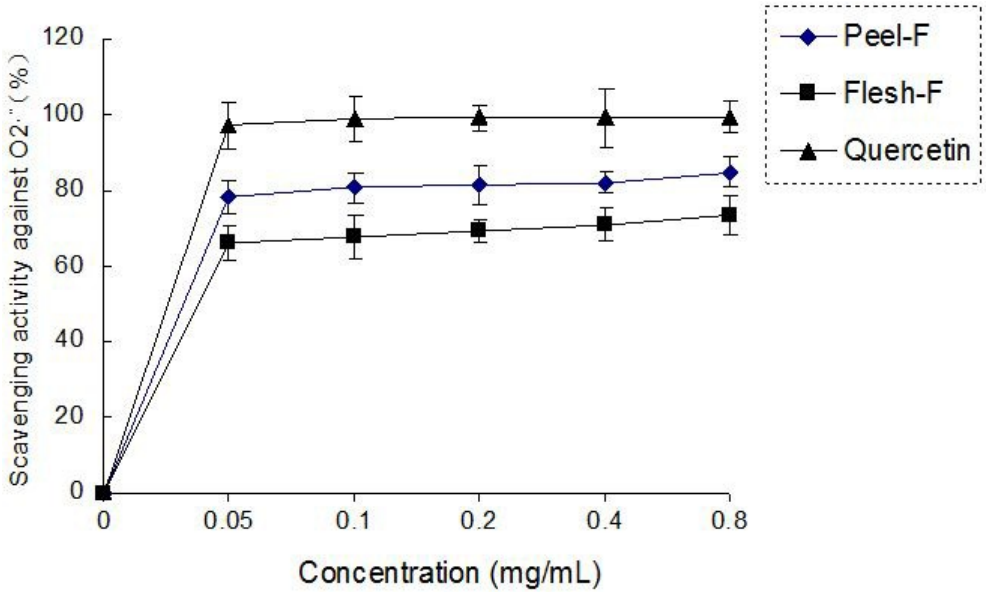

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Fig. 3

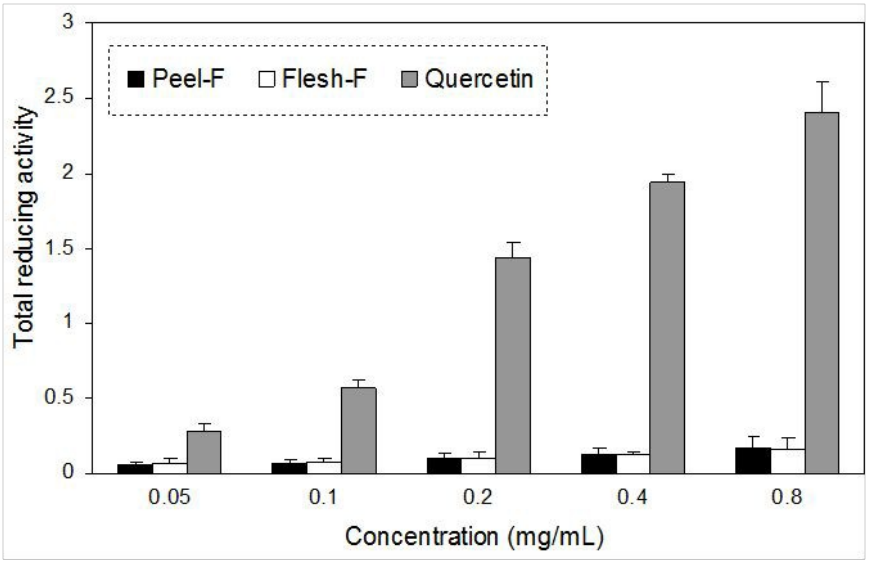

Fig. 3 The total reducing activity of apple flavonoids

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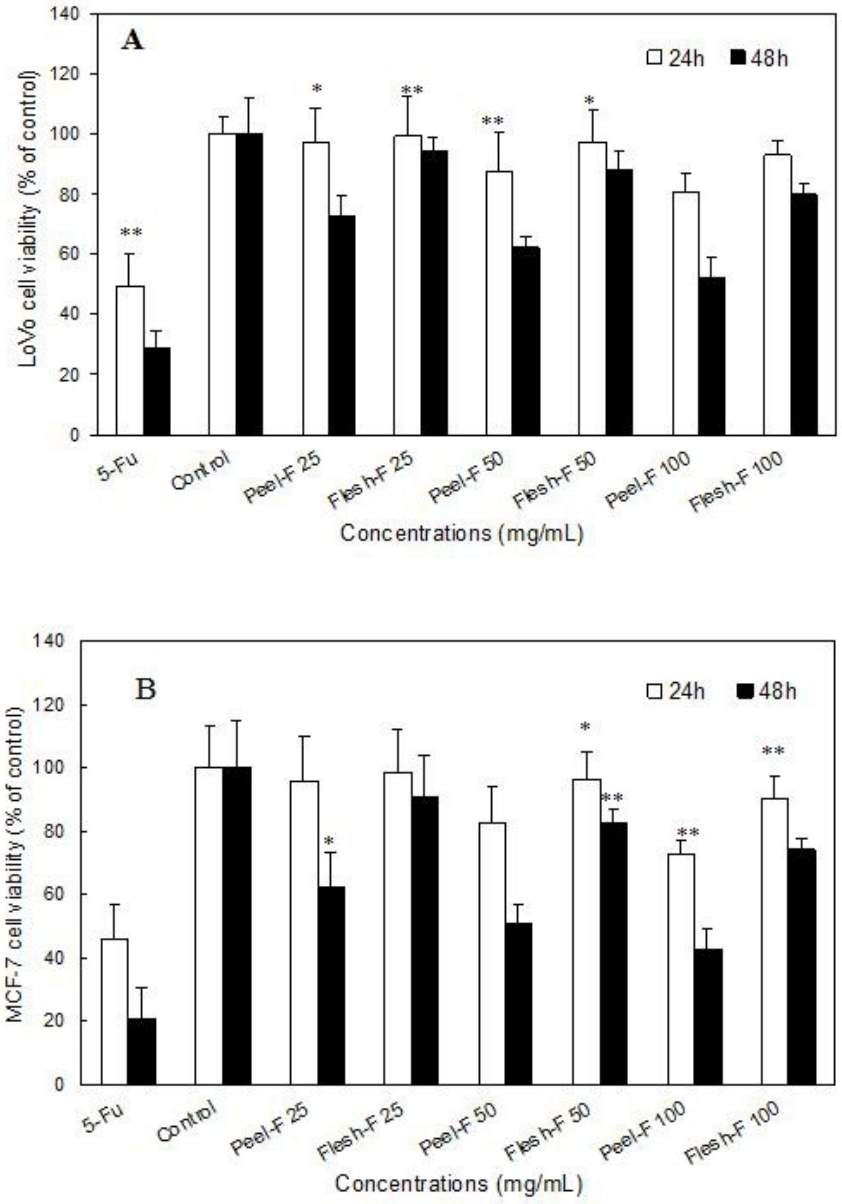
Fig. 4



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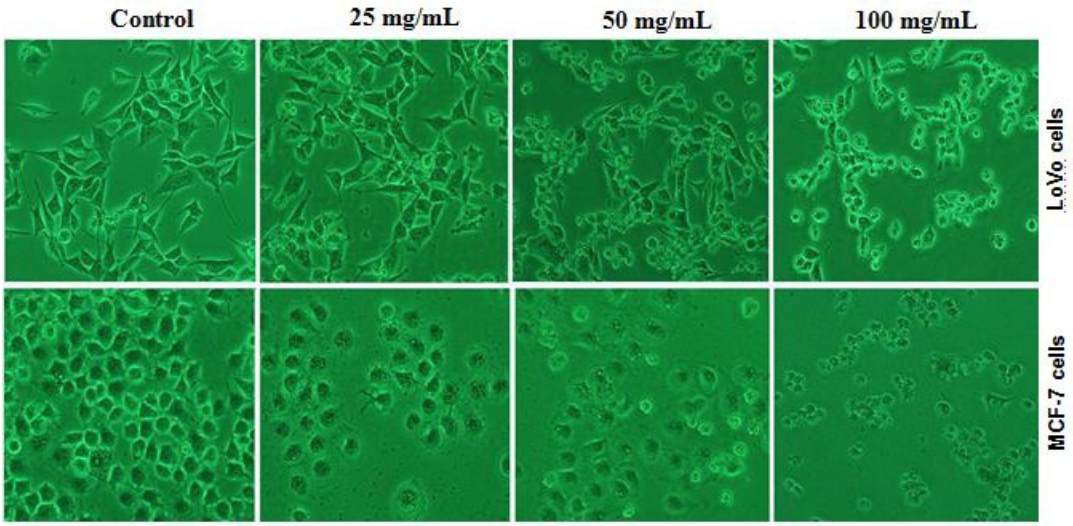

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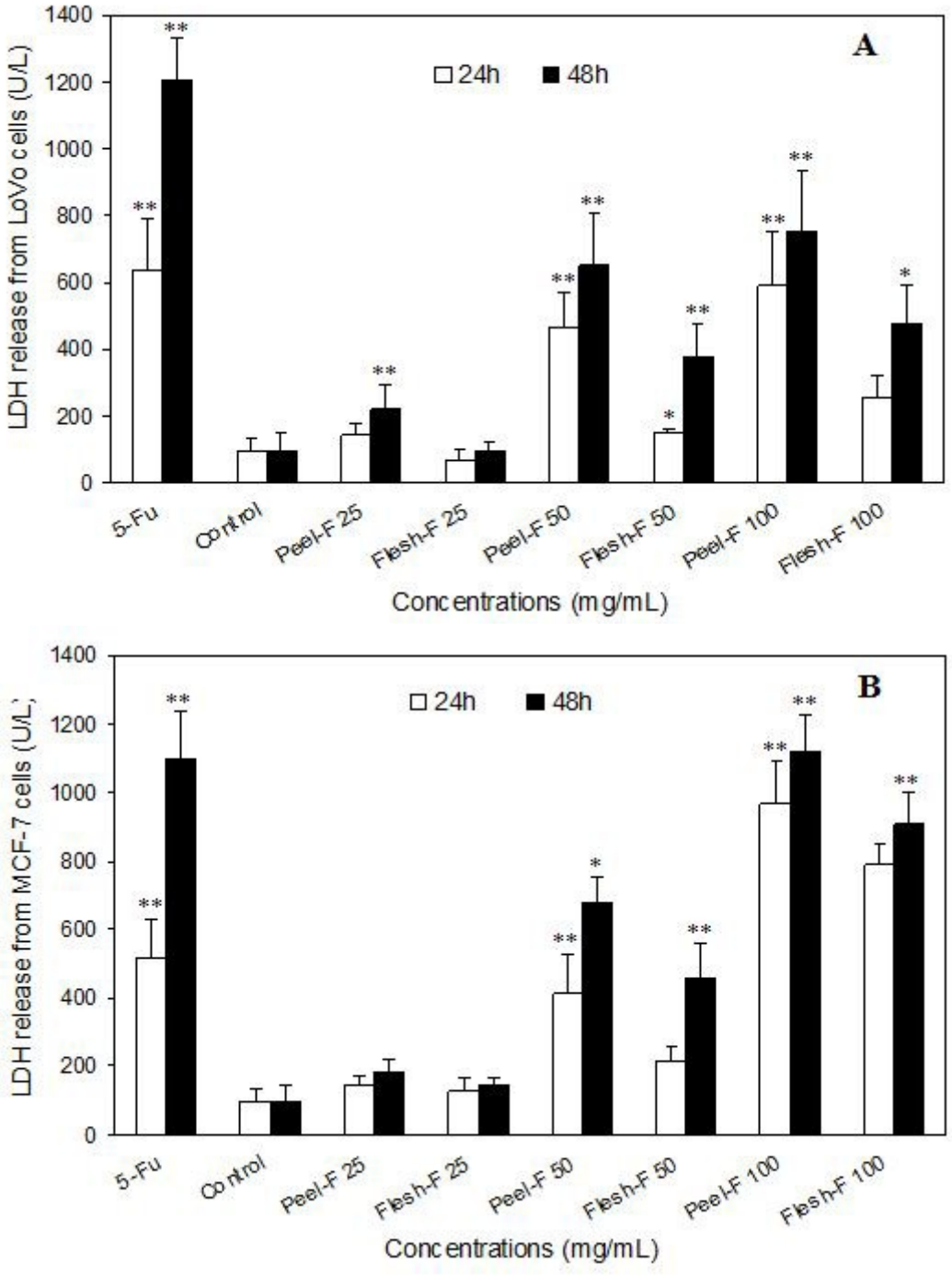

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Fig. 7

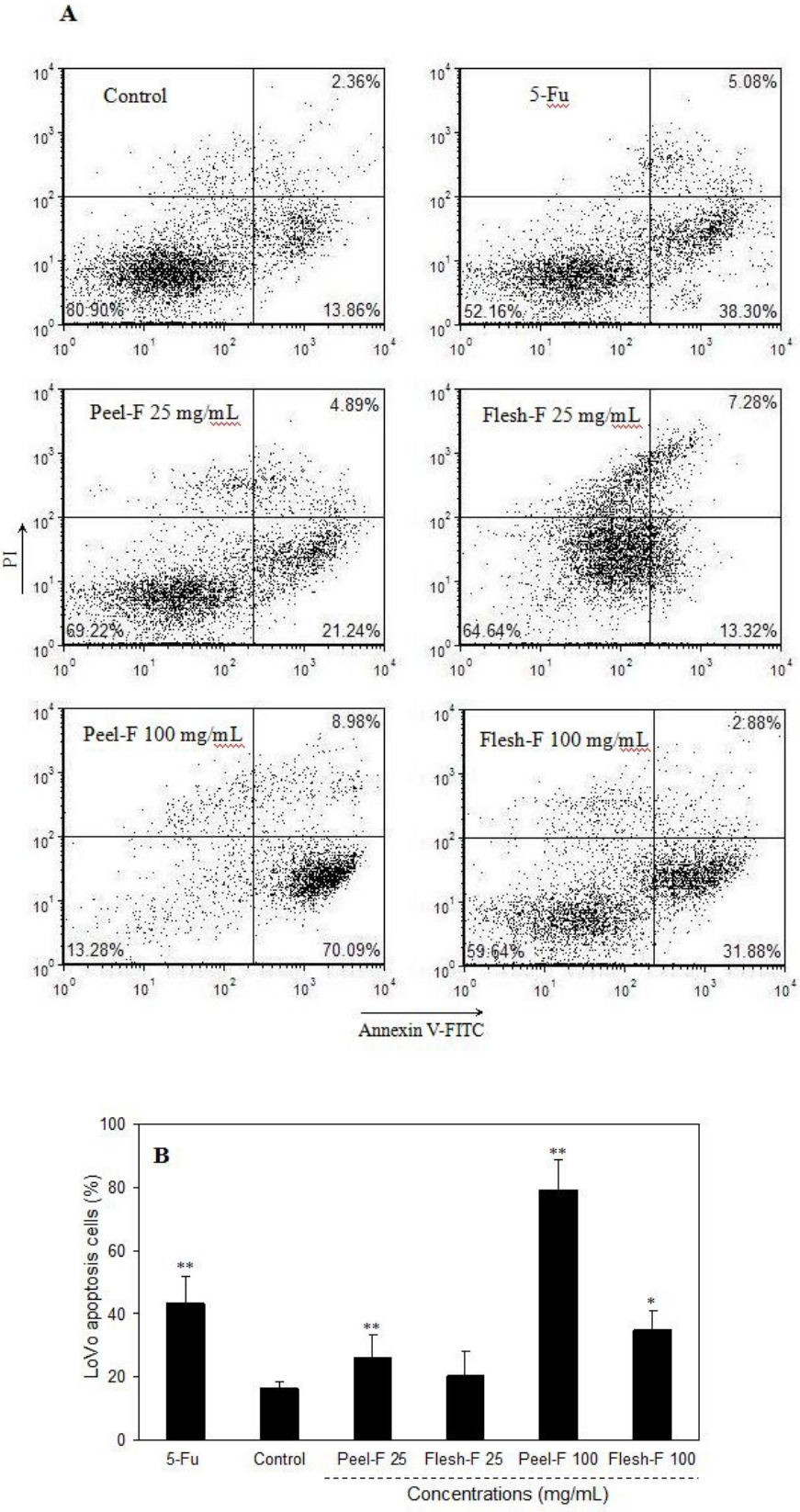

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Fig. 8

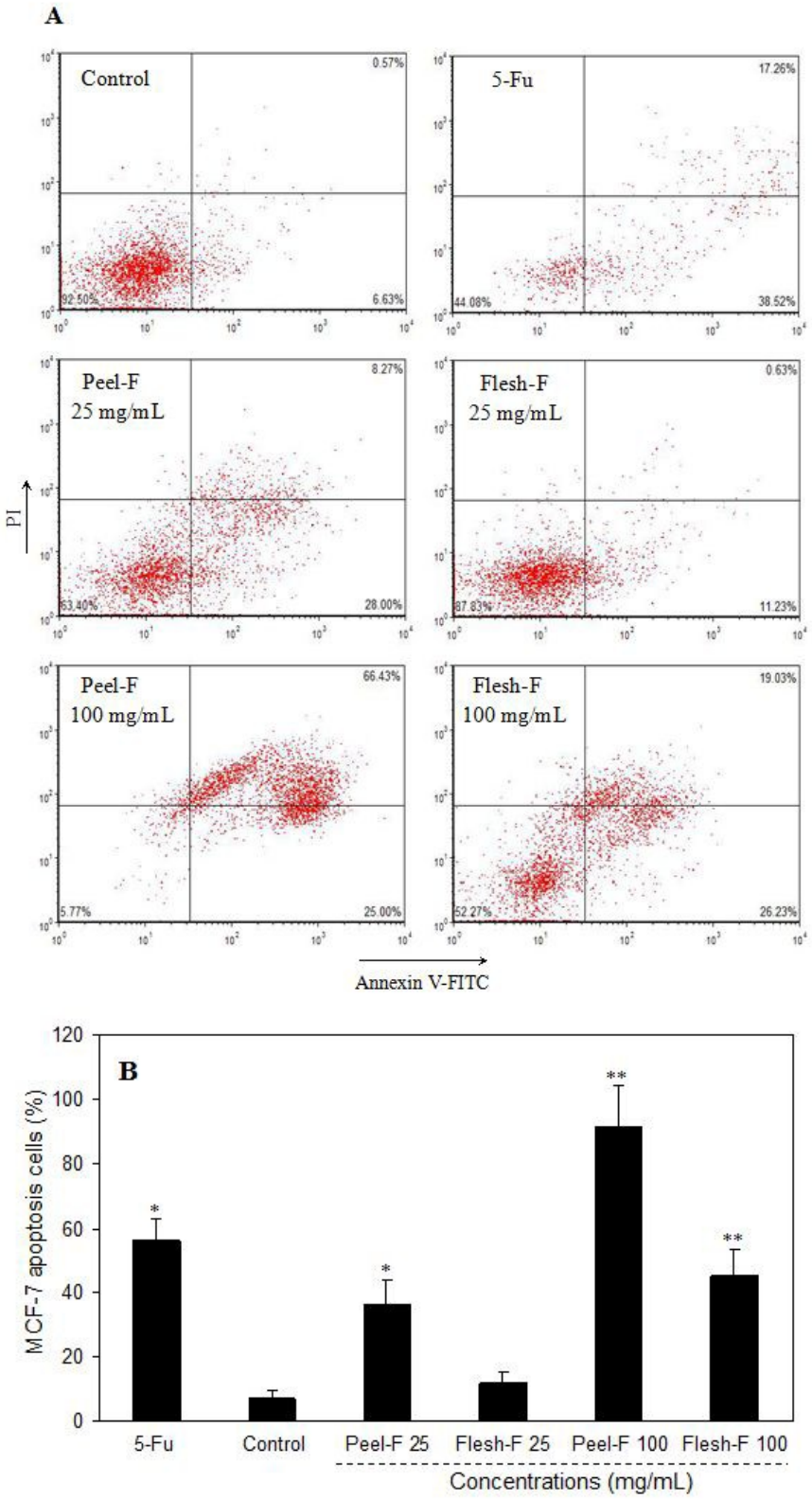

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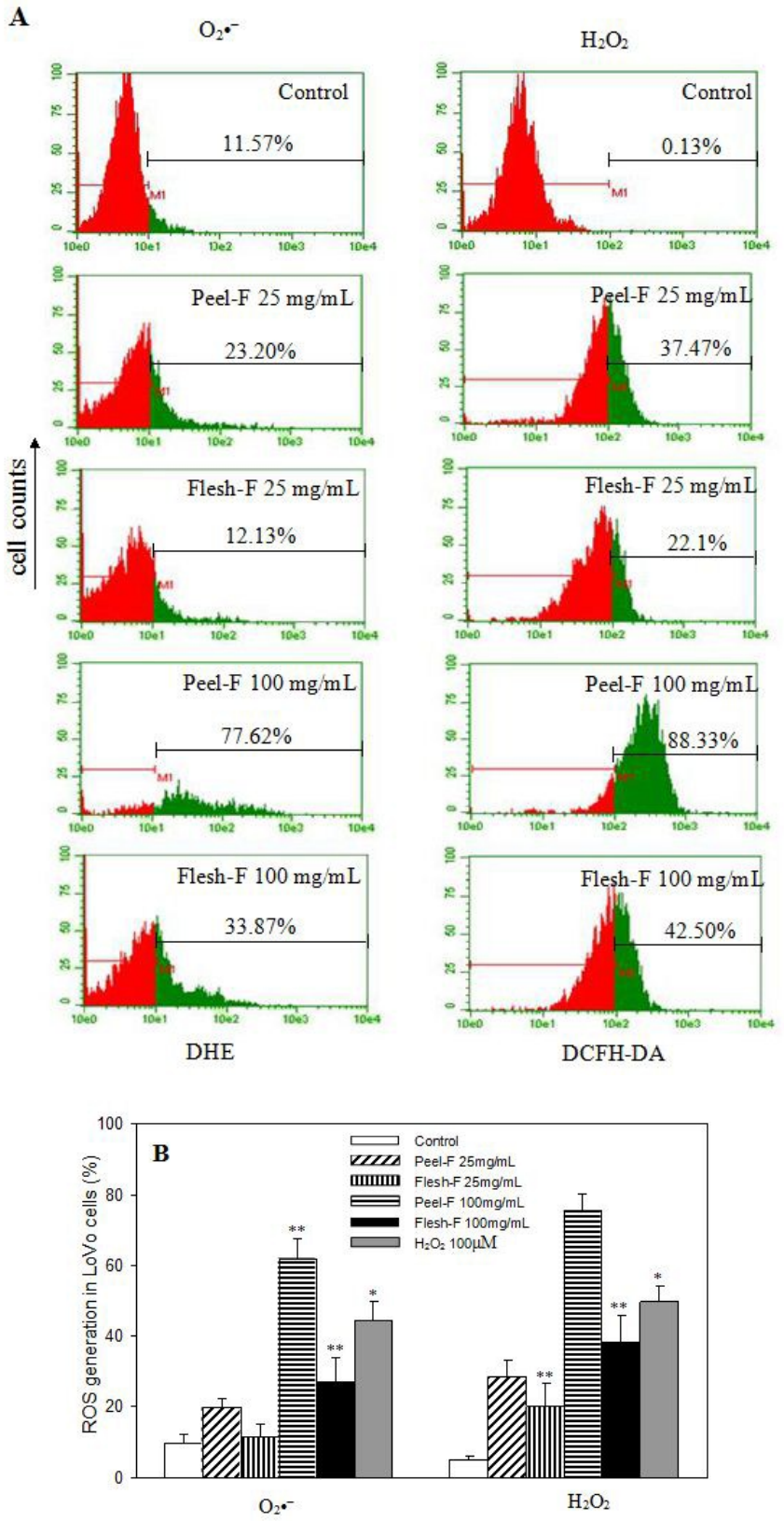
Fig. 9



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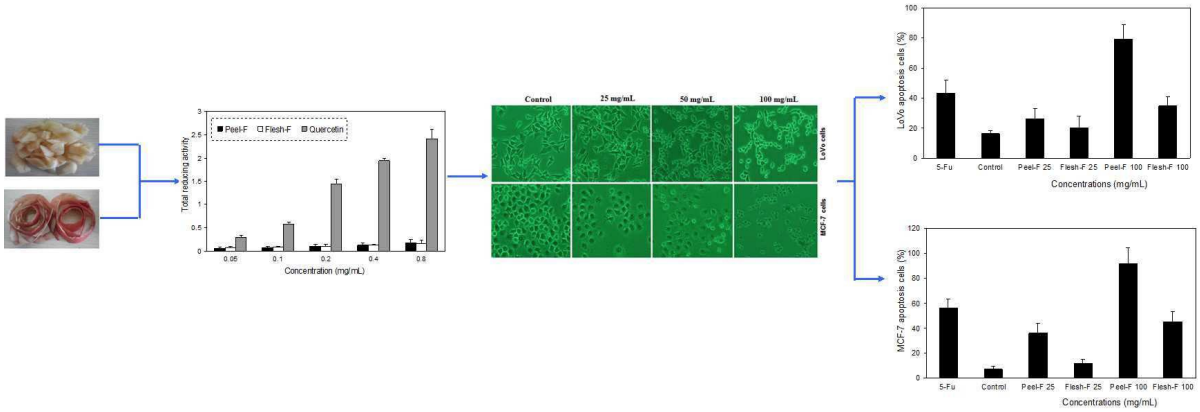
Peel-F and Flesh-F were proved to be immense foodborne medicines for anti-cancer for their property to induce tumor cells apoptosis.



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