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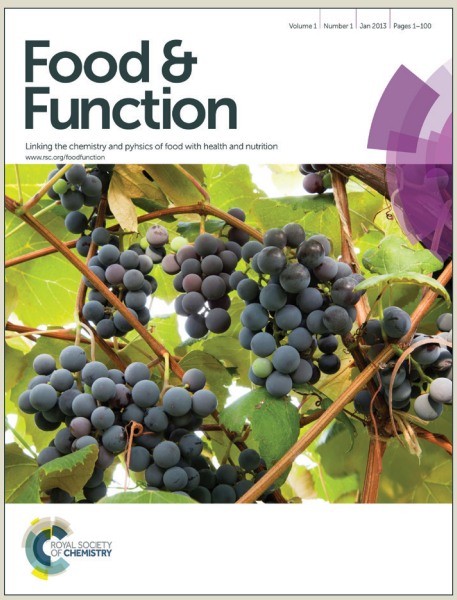

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Nutrient values and bioactivities of the extracts from three fern species in China: a comparative assessment

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Abstract

Pteridium aquilinum, *Osmunda cinnamomea* Linn, and *Athyrium multidentatum* (Doll.) Ching are three fern species widely consumed as potherbs and traditional medicinal herbs in China. Nevertheless, no detailed comparative assessments of their nutrient values and bioactivities have been reported. In this paper, we examined the nutrient content of these ferns and the bioactivities of their extracts with comparative method. The results indicated that they were nutrient dense for protein, carbohydrates, fat and minerals. Compared with *Pteridium aquilinum* and *Osmunda cinnamomea* Linn, extracts from *Athyrium multidentatum* (Doll.) Ching was found to possess the strongest antioxidant activity, protective effect on biomolecules, cellular antioxidant activity, and antiproliferative effects owing to its highest total phenolic (476.52±11.26 mg GAE/g extract) and total flavonoid (924.81±4.25 mg RNE/g extract) contents. Further, *Athyrium multidentatum* (Doll.) Ching can lead to caspase-3 activation, poly ADP-ribose polymerase cleavage, mitochondrial membrane potential (MMP) reduction and inhibition of wound-healing in a dose-dependent manner in HepG2 cells. These results demonstrate the remarkable potential of *Pteridium aquilinum*, *Osmunda cinnamomea* Linn, and *Athyrium multidentatum* (Doll.) Ching as valuable sources of nutrients and natural antioxidants, and among which, *Athyrium multidentatum* (Doll.) Ching has the potential of anticancer property.

Keywords

Fern; Nutrient values; Antioxidant activity; Cellular antioxidant activity; Apoptosis; Comparative method

1. Introduction

Ferns are a group of sporous plants with a diverse germplasm and metabolites. Approximately 12,000 species are extensively distributed across the world, from remote mountain regions to bodies of water, dry desert zones, and open fields. In China, approximate 2600 species of ferns are found,[1] many of them with possession of alkaloids, flavonoids, and terpenoids as major bioactive compounds,[2-4] used as traditional medicinal herbs in hepatitis and skin tumefaction treatment, as well as antipyretics.[5]

Pteridium aquilinum (PAQ), *Osmunda cinnamomea* Linn (OSC), and *Athyrium multidentatum* (Doll.) Ching (AMC) are three high-yield fern species widely distributed in China. PAQ is one of the most common ferns in China, which possess anti-inflammatory, anti-cancer, anti-viral, and anti-bacterial bioactivities.[6,7] A water-soluble polysaccharide purified from PAQ revealed strong reductive power.[8] OSC serves as an edible and medicinal material, and also a famous ornamental house plant,[9] mainly distributes in the northeast and Qin Ba mountain area of China. Yang Ji et al. found that flavonoids extracted from OSC exhibit a strong antioxidant activity and broad antibacterial spectrum.[10] AMC is a well-known nutritious potherb and traditional Chinese medicine native to northeast China, especially in the Changbai Mountain area. AMC has been widely used as diuretic and tranquilizer. And also, it can lower the blood pressure. Moreover, Sheng, Jiwen et al. reported that polysaccharides extracted from AMC possess high antioxidant activity.[11]

Imbalances in reactive oxygen species (ROS) production and ROS clearance can lead to oxidative stress in an organism.[12] Excessive production of ROS is universally considered to be a contributing factor to the development of many pathological processes, such as cardiovascular disease, cancer, brain and immune system diseases.[13] To delay, prevent or stop this process, antioxidants obtained from the diet may act as ideal reducing agents, free radical scavengers or singlet oxygen quenchers.

Liver cancer is one of the most prevalent malignant diseases and the second leading cause of cancer-related death worldwide.[14] The induction of apoptosis is an effective approach to preventing cancer and treating cancer cells.[15] Reductions in the mitochondrial membrane potential (MMP), activation of caspase-3, and the cleavage of poly ADP-ribose-polymerase (PARP) are the primary markers of the mitochondrial-mediated apoptotic pathway.[16] Natural phytochemicals are considered as good sources of potential cancer chemopreventive and chemotherapeutic agents. Thus, great attention has been paid to the highly active phytochemical antioxidants and agents.[17]

The objectives of this study were to assess the nutrient values and bioactivities of the extracts from three fern species in China with comparative method. First, the total phenolic content (TPC) and total flavonoid content (TFC) of each extracts was determined using colorimetric method. Further, the antioxidant activity of each extracts was evaluated by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Hydroxyl radical (•OH), and Ferric reducing antioxidant power (FRAP) assays. Then, the protective

effects of them on biomolecules were assessed by protein oxidation damage, protein carbonyl, and lipid peroxidation models. To further confirm the antioxidant activity of the extracts, a cellular antioxidant activity (CAA) assay was used, either. Finally, we investigated antiproliferation, inhibition of wound-healing and pro-apoptotic effect of AMC on HepG2 cells.

2. Materials and methods

2.1. Chemicals and reagents

For these experiments, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2,4,6-tris (2-pyridyl)-s-triazine (TPTZ), 6-hydroxy-2, 5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), 20,70-dichlorofluorescein diacetate (DCFH-DA), vitamin C, and linoleic acid (LA) were purchased from Sigma (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 cell culture media were purchased from Thermo Fisher (Shanghai, China). JC-1, DAPI, and AO/EB were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Poly (ADP-ribose) polymerase (PARP) (9542) and cleaved caspase-3 (9662) antibodies were provided by Cell Signaling Technology (Shanghai, China). All other chemicals were of analytical grade and were produced in China.

2.2. Preparation of plant extracts

PAQ and OSC were collected in Hanzhong, Shaanxi province (China) from April 23th -28th, 2014, and AMC was collected in Ji'an, Jilin province (China) from May 10th-16th, 2014. The samples were the dried at 65-70˚C for 2-3 days. Portions of

the dried plants were used to determine nutrient values at the Northwest A&F University TC (Test Center). The remainder of the samples was powdered with a grinder and filtered through a 40-mesh screen. Desiccated samples (200 g) were mixed with 2 L ethanol (60% (v/v)) and subjected to ultrasound for 2 h at 40˚C. After filtration, the residues were re-extracted twice under the same conditions. A rotary evaporator was used to concentrate the extracts at 40˚C. After degreasing with petroleum ether, the concentrated samples were purified using an AB-8 macroporous resin. Finally, the purified extracts were vacuum freeze-dried and stored at -20˚C until analysis.[3, 18]

2.3. Determination of total phenolic content (TPC)

The total phenolic content was determined using the Folin-Ciocalteu colorimetric method.[19] The results were reported as mg gallic acid equivalents (GAE) per gram of extract.

2.4. Determination of total flavonoid content (TFC)

The total flavonoid content was determined using a modified colorimetric method.[20] The results were reported as mg rutin equivalents (RNE) per gram of extract.

2.5. Antioxidant assay

The EC_{50} value (μg extract per ml) was obtained by interpolation from linear regression analysis and represents the effective concentration at which radicals were scavenged by 50%.

2.5.1. DPPH free radical scavenging activity

The DPPH scavenging activity of the extracts was measured by assessing the absorbance of DPPH at 517 nm.[18, 21]

2.5.2. ABTS radical scavenging activity

The determination of ABTS radical scavenging was carried out as reported by Dorman and Hiltunen.[22]

2.5.3. Hydroxyl radical (•OH) scavenging activity

The scavenging of hydroxyl radicals was measured by the method of Chandini.[23]

2.5.4. Ferric reducing antioxidant power (FRAP)

To determine the antioxidant capacity, the FRAP method was used as previously described.[18, 24]

2.6. Protective effects of PAQ, OSC, and AMC on biomolecules

2.6.1. Protection of protein oxidation damage

AAPH-induced oxidation of albumin from bovine serum (BSA) was carried out following previously described methods.[25] To determine the amount of protein damage, band intensity was estimated using Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA). The optical density of each band was estimated and standardized with respect to the control group.

2.6.2. Protection of protein carbonylation

The determination of protein carbonylation was carried out by previously described methods.[26] The carbonyl contents are determined by the absorbance at 366 nm using a molar absorption coefficient of 22.0 μM/ (L·cm). The protein carbonyl content was calculated using the following formula:

$$\text{Carbonyl content } (\mu M/mg) = \frac{A}{22.0 \mu M/(L \cdot cm)} \times 1000 \quad (1)$$

where A refers to the absorbance of the sample at 366 nm.

2.6.3. Protection of lipid peroxidation

The TBARS assay was carried out using the method previously described.[27] The amount of TBARS was estimated using a molar absorption coefficient of 1.56×10^5/ (M·cm). The inhibition of TBARS formation was calculated using the following formula:

$$\text{Inhibition } (\%) = (A_1\text{-}A_2)/(A_1\text{-}A_0)\times100 \quad (2)$$

where A_0 refers to the absorbance of the control group (blank, without Fe^{2+}/vitamin C and extracts), A_1 refers to the absorbance of the Fe^{2+}/vitamin C-treated group (without extracts), and A_2 refers to the absorbance of the Fe^{2+}/ vitamin C+ extracts group.

2.7. Cellular antioxidant activity (CAA)

To further confirm the antioxidant activities of PAQ, OSC, and AMC in cells, the intracellular antioxidant activity of the extracts was measured using the CAA assay reported by Wolfe and Liu,[28] with some modifications.

2.7.1. Cell Culture

HepG2 cells were grown in growth medium (RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin) and were incubated at 37˚C and 5% CO_2.

2.7.2. Cytotoxicity

The cytotoxicity of extracts toward HepG2 cells was measured, as described previously.[28] The median cytotoxic concentration (CC_{50}) was calculated for each extract.

2.7.3. CAA of three extracts

HepG2 cells were seeded in 100 μL of growth medium at a density of 6×10^4/ mL in 96-well plates. The seeded HepG2 cells were cultured for 24 h in an incubator at 37 ˚C and 5% CO_2. The growth medium was removed and the wells were washed with 100 μL phosphate buffer saline (PBS). Then, the HepG2 cells were treated for 1 h with 100 μL of extracts at different concentrations plus 25 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA) dissolved in antioxidant treatment medium. When a PBS wash was utilized, the wells were washed with 100 μL of PBS. Then, 600 μM AAPH was applied to the cells in 100 μL of hanks balanced salt solution (HBSS), and the 96-well microplate was placed into a Multimode plate reader (Tecan, Switzerland). The fluorescence reading was measured every 5 min for 1 h at 538 and 485 nm excitation and emission, respectively.

After subtracting the blank value and the initial fluorescence values, the area under the curve for fluorescence versus time was integrated to calculate the CAA value for each concentration of extracts as:

$$\text{CAA unit} = 100-(\int SA / \int CA)\times 100 \quad (3)$$

where ∫SA was the integrated area under the sample fluorescence versus time curve and ∫CA was the integrated area from the control curve. The median effective dose (EC_{50}) was determined for extracts from the median effect plot of log (fa/fu)

versus log (dose), where fa was the affected fraction (CAA unit) and fu was the unaffected fraction (100 - CAA). The EC_{50} value was stated as the mean±SD for triplets of data obtained from the same experiments. The EC_{50} value was converted to the CAA value expressed as micromoles of quercetin equivalents (QE) per g extract.

2.8. Antiproliferative effects

The antiproliferative activity of the three extracts against human HepG2 liver cancer cells was determined using a method reported previously.[29] Briefly, HepG2 cells in growth media were placed in each well of a 96-well plate at a density of 2.5×10^4 cells/well. After 4 h of incubation at 37 ˚C with 5% CO_2, the growth medium was replaced by medium containing different concentrations of extracts (0-800 μg/mL). After 72 h of incubation, the MTT test (microculture tetrazolium test) was used to measure the cell proliferation rate. Cell proliferation (percent) was determined from the absorbance at 570 nm for each concentration compared to the control. A minimum of three replications for each sample was used to determine the antiproliferative activity.

2.9. AO-EB and DAPI staining assay

To explore whether the antiproliferative activity of HepG2 cells was mainly due to apoptosis or necrosis after AMC treatment for 24 h, the nuclear morphology was analyzed by acridine orange/ethidium bromide (AO-EB) and 4',6-diamidino-2-phenylindole (DAPI) staining. Cells were fixed with paraformaldehyde (4%) for 10 min and then permeated with Triton X-100 (0.1%) for 15 min at room temperature and stained with DAPI (5 μg/mL) for 30 min in the dark after washing twice with

PBS. The cells were then observed using a fluorescence microscope (Olympus Optical, Tokyo, Japan).

For the AO-EB staining assay, 5 μL of AO-EB (100 μg/mL) was added to HepG2 cells in the dark at room temperature, followed by observation under a fluorescence microscope after washing twice with PBS.[30]

2.10. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was assessed using the mitochondria-specific lipophilic cationic fluorescence dye JC-1 to detect the disruption of mitochondrial membrane potential (MMP). The cells were seeded in 96-well plates at a density of 1 $\times 10^5$ cells/mL and treated with different concentrations of AMC (50, 100, 200 μg/mL) for 24 h, followed by incubation with JC-1 (5 μg/mL) for 1 h at 37 ˚C in the dark. Relative fluorescence intensities were monitored by a Multimode plate reader (Tecan, Switzerland) with settings of FL1 (red) at 585 nm and FL2 (green) at 538 nm and were qualitatively analyzed by fluorescence microscopy (Olympus Optical, Tokyo, Japan).

2.11. Western blot analysis

After treatment with different concentrations of AMC (50, 100, 200 μg/mL), the cells were lysed with 150 μL of lysis buffer containing 20 mM NaF and 1% phenylmethanesulfonyl fluoride (PMSF) on ice for 10 min. After centrifugation at 15,000 rpm/min for 10 min at 4 ˚C, the supernatant was collected and quantified using the bicinchoninic acid (BCA) protein assay. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and

electrophoretically transferred onto a PVDF membrane. Blocking was performed with 5 % defatted milk powder in tris buffered saline + Tween (TBST) at 25 ˚C for 2 h. Then, the membranes were washed three times with TBST and incubated with the primary antibodies at 4 ˚C overnight. Being added with secondary antibodies, the samples were incubated at 25 ˚C for 2 h and washed three times with TBST. The bands were revealed by enhanced chemiluminescence using a commercial ECL kit (Bio-RAD ChemiDoc XRS).

2.12. Scratch assay

The spreading and migratory capabilities of HepG2 cancer cells were assessed using the scratch wound assay of Fronza,[31] with minor modifications. The cells were seeded into 24-well cell culture plates at a concentration of 3×10^5 cells/mL for 24 h at 37 ˚C and cultured in medium containing 10% FBS to produce a 90% confluent cell monolayer. Then, a linear wound was generated in the monolayer with a sterile 100 μL plastic pipette tip. Any remaining cellular debris was removed by washing the wells with PBS. RPMI-1640 medium (1 mL) was used as the control; vascular endothelial growth factor (VEGF) (8 ng/mL) was used as the positive control and VEGF (8 ng/mL) plus different concentrations of AMC extracts (50, 100, and 200 μg/mL) were the test group. The width of the wound was measured again after 24 h of incubation.

2.13. Statistical analysis

The data was representative of three independent experiments. The results were expressed as the mean±standard deviation (SD). Significant differences between

measurements for the control and treated samples were analyzed using one-way factorial analysis of variance (ANOVA), followed by Tukey's test (SPSS 16.0). A $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Nutrient content of PAQ, OSC, and AMC

Knowledge of the nutrient values of foods is fundamental to virtually all nutrition-related projects, including public health studies and educational policies, as well as to inform consumers.[32] The nutrient compositions of PAQ, OSC, and AMC were given in Table 1. The protein content of the three fern varieties was the highest in AMC (24.65±1.34 g/100 g, DW) and the lowest in OSC (15.45±1.07 g/100 g). AMC had the highest fat content (2.09±0.02 g/100 g, DW), higher than that of *Cucurbita maxima* (0.13±0.03 g/100 g, FW) and *Vigna unguiculata* (0.11±0.03 g/100 g, FW).[33] The highest carbohydrate content was found in OSC (24.62±0.63 g/100 g) and the lowest in AMC (17.82±0.42 g/100 g, DW). No significant difference was found in the crude fiber and calorie content of the three ferns. PAQ contained the highest concentration of Calcium (1943±26.47 mg/kg, DW), Iron (94.43±3.35 mg/kg, DW), Zinc (56.60±2.06 mg/kg, DW) and Potassium (12196.67 ±168.62 mg/kg, DW), while OSC contained the highest Sodium (294.63±9.29 mg/kg, DW) and Selenium (2920±26.46 mg/kg, DW) levels. The Zinc content of the ferns was higher than that of Chinese cabbage (0.30±0.06 mg/100 g, FW),[34] and the Iron level was higher than in Indian lettuce (0.52±0.04 mg/100 g, FW) and Spanish joint fir (0.40±0.05 mg/100 g, FW).[35] These results suggest that PAQ, OSC, and

AMC are ideal potherb resources, full of protein, carbohydrates, fat, and minerals (calcium, iron, zinc, potassium).

3.2. Total phenolic and flavonoid contents of PAQ, OSC, and AMC

Phenolic compounds are considered to be the major contributor to antioxidant activity.[36] The weights of the PAQ, OSC, and AMC extracts were 3.95 g, 3.83 g and 4.12 g, respectively. The TPC of AMC (476.52±11.26 mg GAE/g extract) was statistically higher ($p < 0.01$) than that of PAQ (246.51±3.31 mg GAE/g extract) and OSC (208.63±5.26 mg GAE/g extract).

A similar trend was also observed for total flavonoid content, with values of 924.81±4.25 mg RNE/g extract for AMC, 547.46±2.89 mg RNE/g extract for PAQ, and 436.24±5.84 mg RNE/g extract for OSC. Previous studies reported that the total flavonoid content of 19 species of ferns ranged from 8.6 to 306.4 mg/g (w/w) in China.[3] Compared to those data, the TFC values for PAQ, OSC, and AMC were not high enough. This may be partly due to partial loss of the flavonoids during purification across the AB-8 macroporous resin.

3.3. *In vitro* antioxidant assay

To evaluate the antioxidant activities of PAQ, OSC, and AMC, four different radical scavenging assay systems were used, the DPPH, ABTS, •OH, and FRAP assays. Fig 1 shows the results of the antioxidant activity of the three extracts.

DPPH scavenging is a well-known parameter in the identification of extracts with potential free radical scavenging activity.[37] PAQ, OSC, and AMC all demonstrated a dose-dependent DPPH radical scavenging activity (Fig. 1A), with

EC_{50} values of 24.17 μg/mL, 46.71 μg/mL and 15.01 μg/mL. In the ABTS assay (Fig. 1B), AMC showed a significantly ($p < 0.05$) higher scavenging activity (EC_{50} of 4.63 μg/mL) than PAQ (EC_{50} of 7.90 μg/mL) and OSC (EC_{50} of 15.17 μg/mL). The results of the •OH assay show that the scavenging activity of AMC was significantly ($p < 0.05$) higher (EC_{50} of 48.01 μg/mL) than that of PAQ (EC_{50} of 92.08 μg/mL) and OSC (EC_{50} of 113.25 μg/mL) (Fig. 1C). As shown in Fig 1D, the FRAP value of AMC (EC_{50} of 13.20 μg/mL) is significantly ($p < 0.05$) higher than that of PAQ (EC_{50} of 27.85 μg/mL) and OSC (EC_{50} of 46.40 μg/mL). The antioxidant capacity of AMC, PAQ and OSC assayed in terms of the four methods was obviously attenuated, among which AMC possess the strongest antioxidant ability.

3.4. Protection against Protein oxidation

Free radical damage to biomolecules is associated with a variety at chronic disease processes. Such damage is caused by ROS and is characterized by the formation of carbonyl groups and protein fragmentation.[38] Fig. 2A shows the effects of PAQ, OCS, and AMC in preventing AAPH-induced BSA oxidant damage. A rapid increase in protein (BSA) oxidant damage was observed after treatment with AAPH in the absence of extracts. However, pre-incubation of PAQ, OCS, and AMC (5-100 μg/mL) restored protein oxidation levels in a dose-dependent manner. Used at the highest concentrations (100 μg/mL), each extract can protect almost completely against protein oxidant damage. AMC revealed the strongest protective effect for protein oxidation damage, followed by PAQ and OCS, but the difference was not significant.

3.5. Protection against Protein carbonylation

Besides protein fragmentation, the oxidation of arginine, threonine, lysine and proline may also generate carbonyl derivatives. The appearance of carbonyl groups has therefore been considered to be a marker of ROS-mediated protein oxidation.[39] As shown in Fig. 2B, PAQ, OCS, and AMC in the range of 5 to 100 μg/mL efficiently inhibited BSA carbonyl in a dose-dependent manner. Similar to the protein oxidation results, the protective effect of the protein carbonyl of AMC was significantly ($p < 0.05$) higher than that of PAQ and OSC, and that of PAQ was slightly higher than OSC, the difference was not significant.

3.6. Protection against lipid peroxidation

Free radical formation may lead to oxidative damage of cellular components, including lipid membranes, which are considered to be associated with the pathology of many diseases, such as Alzheimer's disease, asthma, and endotoxin liver injury.[40] Therefore, the inhibition of lipid peroxidation is an important index of antioxidant potential. The inhibitory effects on TBARS formation of various concentrations of the extracts are shown in Fig. 2C. PAQ, OCS, and AMC all have tremendous potential inhibiting lipid peroxidation in a dose-dependent manner. The lipid peroxidation inhibitory effects of AMC were slightly higher than OSC, while PAQ had the lowest protective effect. Taken together, these results suggest that PAQ, OSC, and AMC had powerful protective effect on biological macromolecules including proteins and lipids, indicating their potential use in the chemoprevention of diseases related to ROS.

3.7. Cellular antioxidant activity of PAQ, OSC, and AMC

Typical chemical antioxidant activity assays, such as DPPH and ABTS, have many limitations. Cellular antioxidant activity provides an approach that is cost-effective and relatively fast. It addresses some issues of uptake, distribution, and metabolism of the antioxidant.

Two protocols (PBS wash and no PBS wash) were used to measure the cellular antioxidant activities of the three extracts. The concentrations of extracts in this experiment were lower than the level that is cytotoxic to HepG2 cells. The CAA values of PAQ, OSC, and AMC are shown in Table 2. According to the results, AMC had the highest CAA value (115.85±11.87 μM QE/g extract), followed by PAQ (79.17±5.28 μM QE/g extract), whereas OSC had the lowest CAA value (77.12±3.86 μM QE/g extract) in the no PBS wash protocol. Similar to the results of the no PBS wash protocol, in the PBS wash protocol, AMC had the greatest cellular antioxidant activity, with CAA values of 88.14±5.32 μM QE/g extract, followed by PAQ (81.84±1.26 μM QE/g extract) and OSC (48.14±3.59 μM QE/g extract). The CAA value of AMC (81.84±1.26 μM QE/g extract, i.e., 168.59±2.60 μM QE/100 g sample, dry weight) was significantly higher than those of common vegetables (range from 0.80 to 5.61 μM QE/100 g sample, FW) [41] and adlays (range from 0.53 to 1.37 μM QE/100 g sample, DW) [42] in the PBS wash protocol, indicating that AMC is an excellent natural antioxidant and may play a significant antioxidant role against peroxyl radicals formed in cells.

3.8. Antiproliferative effects of the extracts

The antiproliferative activity against human HepG2 liver cancer cells for the

three extracts is shown in Fig. 4C. AMC significantly inhibited cell proliferation in a dose-dependent manner, and PAQ expressed little antiproliferative activity, while OSC revealed no inhibitory effect.

3.9. AMC induces apoptosis in HepG2 liver cancer cells

Because AMC exhibited a significant inhibition on HepG2 liver cancer cells (62% lower than that of control cells at 200 μg/mL), the possible underlying cellular mechanism of the inhibitory effect was investigated. As shown in Fig. 3A, after treatment with AMC, the cells shrank. And they were smaller and rounder compared to the control. To better determine whether AMC induced cell death by apoptosis, the cells were observed by fluorescence microscopy under AO/EB double staining and DAPI staining after AMC treatment for 24 h. EB was used to stain dead or apoptotic cells emitting a red-orange fluorescence while AO produced a yellow-green fluorescence. Apoptotic cells display condensed chromatin and fragmented nuclei and non-apoptotic cells maintain uniformity in DAPI staining assays. The red fluorescence cells after EB staining increased in a dose-dependent manner (Fig. 3B) and the cells stained with DAPI (Fig. 3C) showed condensed nuclei and apoptotic bodies after AMC treatment.

A change in mitochondrial membrane potential (MMP) is one of the early events leading to mitochondrial functional alteration. Fluorescence microscopy showed that the red fluorescence was significantly reduced, while the green fluorescence was increased, after treating cells with 50-200 μg/mL AMC for 24 h (Fig. 3D). AMC was associated with a significant loss of MMP (76.3% lower than that of control cells at

200 μg/mL) in a dose-dependent manner (Fig. 3F).

Caspases are necessary initiators and executors of apoptosis. The cleavage of PARP is an important index of cell apoptosis and caspase-3 activation. As shown in Fig. 4B and 4C, cleaved caspase-3 and the cleavage of PARP were observed in a dose-dependent and time-dependent manner after treatment with AMC.

3.10. The effect of AMC on HepG2 cell wound healing

Nowadays, chemotherapy and surgery are the primary treatments for liver cancer. However, recrudescence and metastasis are common, owing to cell migration and invasion.[43] As shown in Fig. 4A, VEGF enhances the wound healing of HepG2 cells compared with the control ($P< 0.05$), while AMC (100 and 200 μg/mL) can significantly inhibit the wound healing stimulated by VEGF (Fig. 4B). These results suggest that AMC can inhibit cell migration and wound healing.

4. Discussion

Dietary factors account for approximately one third of cancer deaths, which are similar to the impact of smoking.[44] It is advisable to reduce this risk by adjusting the diet properly. Thus, much interest has focused on the vital role of many plants that are thought to prevent chronic diseases, especially plants that are rich in natural antioxidants. It is well known that PAQ, OSC, and AMC are wild and pollution-free fern plants in China.[6] We found that PAQ, OSC, and AMC were nutrient dense for protein, carbohydrates, fat and minerals that human requires every day (Table 1). Thus, the use of their rhizomes and fronds as food appears to be widespread. Young croziers are consumed fresh, canned, dried, or pickled, and they are regarded as a

delicacy in many countries.[8] Extracts of AMC possessed the highest total phenolic contents and total flavonoid contents, followed by PAQ and OSC. These differences could be explained by different families and environmental factors, such as: growing conditions (soil, climate, rainfall, altitude), harvesting, among other factors that can directly interfere with the levels of phenolic compounds.[4, 45] The antioxidant capacity values assayed in terms of the four methods evidenced a similar tendency. Overall, Extract of AMC revealed the best antioxidant properties (Fig. 1). In previous research, a significant negative linear correlation between the phenolic content and the EC_{50} antioxidant activity value was observed. The results of antioxidant assays were in agreement with the theory that phenolic compounds were the major contributor to anti-oxidant activity.[46, 47] Besides the level of antioxidants, the interactions among constituents in plants are also key factors in antioxidant activity of ferns. However, it is still not clear that which factor dominates.

At long-term exposure and higher concentrations, ROS can cause incredible damage and disrupt the fundamental physiological functions of biomolecules, such as lipids and proteins, leading to protein oxidative damage, protein carbonylation, and lipid peroxidation.[48] When attacked by ROS, protein fragmentation can occur as a result of peptide bond cleavage. ROS also can modify amino acid residues of proteins, resulting in the hydroxylation, nitration, nitrosylation, sulfoxidation, chlorination of different amino acids. Protein carbonyl groups are generated by direct oxidation of amino acid residues, particularly lysine, arginine, threonine residues, with various reaction mechanisms.[49] In the present investigation, PAQ, OSC, and AMC all have

excellent performance in protecting proteins and lipids from free radical-mediated oxidative damage (Fig. 2). Lipids and proteins are compounds often found in the environment surrounding phenolic compounds. Growing evidence suggests that polyphenols have many potential bioactivities in the human body which are affected by interactions of polyphenols with other macromolecules.[50] So the total phenolic content, phenolic species, and the interaction between polyphenols and molecules (hydrogen bonds, covalent bonds) may explain the difference protective capacity of three extracts on biomolecules.[51]

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and about 600,000 patients suffer from HCC annually.[52] Numerous studies have shown that the activation of the apoptotic pathway in tumor cells is a major protective mechanism against the development and progression of cancer.[53] Apoptosis is a programmed cell death mechanism that can be driven by two major apoptotic pathways, the cell death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway.[30] Reduction of mitochondrial membrane potential, caspase-3 activation and cleavage of PARP are important index of mitochondrial-mediated apoptotic pathway.[54] In this study, we evaluate the anticancer effect of extracts of three fern species in HepG2 cells. It was observed that AMC exhibited significantly higher antiproliferative activity than PAQ and OSC (Fig. 4C). This may partly be due to the high level of TFC and TPC in AMC extracts. There may also be some specific compounds in AMC that account for the high antiprolifereative activity. For example, Xia, Xian et al. discovered *Selaginella frondosa* exhibited the

highest inhibition against A549 cells, with a very low total flavonoid concentration (13.4 mg/g).[3] To explore the mechanism underlying AMC-induced apoptosis in HepG2 cells, mitochondrial membrane potential, activated caspase-3 and cleavage of PARP were examined. We noted that AMC significantly promoted the expression of activated caspase-3 and cleavage of PARP of HepG2 cells in a time-and dose-dependent manner (Fig. 3F). Reduction of mitochondrial membrane potential in HepG2 cells was also observed after treatment with different concentration of AMC (Fig. 3D and Fig. 3E). Taken together, these results indicate that AMC-induced cell death may due to apoptosis via a mitochondria-mediated pathway. Further studies are needed to investigate the specific molecular mechanism of apoptosis caused by AMC. Cell migration and proliferation plays a vital role in the wound healing process. Also for the course of cancer, cell migration plays a central role as it is the key mechanism underlying metastasis, cell scattering and tissue invasion.[55] We observed that AMC significantly inhibited wound healing of HepG2 in a dose-dependent manner (Fig. 4A and Fig. 4B). These date suggested that AMC may have good potential as a therapeutic and chemopreventive agent for liver cancer.

5. Conclusion

Pteridium aquilinum (PAQ), *Osmunda cinnamomea* Linn (OSC), and *Athyrium multidentatum* (Doll.) Ching (AMC) are three fern species that full of nutrient content (protein, carbohydrates, fat, and minerals) in China. The results in this study demonstrated that flavonoids and phenolic rich extracts from PAQ, OSC, and AMC demonstrated remarkable *in vitro* antioxidant activity and cellular antioxidant activity,

as well as the protection effects of biomolecules. And that the bioactivities of the extracts from AMC, PAQ, and OSC were obviously attenuated. Further, extracts of AMC possessed excellent antiprolifereative and inhibition of wound healing capacity in HepG2 cells. In addition, AMC-induced cell death of cancer cells is mainly due to apoptosis via a mitochondria-mediated pathway. These results demonstrate the remarkable potential of PAQ, OSC, and AMC as valuable sources of nutrition and natural antioxidants to be used in functional foods and health products. Notwithstanding, further investigation should be performed to isolate and identify the antioxidant compounds present in three extracts.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1- Nutrient content of PAQ, OSC, and AMC

Notes: The data are presented as the mean±standard deviation (SD) on a dry basis.

Table 2- Cellular Antioxidant Activities of PAQ, OSC, and AMC extracts expressed as EC_{50} and CAA Values (Mean ± SD, n = 3)

Fig. 1- *In vitro* antioxidant activities of PAQ, OSC, and AMC extracts. The data are shown as the mean ± SD (n = 3). (A) DPPH free radical scavenging activity. (B) ABTS radical scavenging activity. (C) Hydroxyl radical (•OH) scavenging activity. (D) Ferric reducing antioxidant power assay.

Fig. 2- Protective effects of PAQ, OSC, and AMC on biomolecules. The data are shown as the mean ± SD (n = 3). (A) Effects of PAQ, OSC and AMC on protein oxidation damage. BSA was incubated with AAPH in the presence or absence of the extracts, as described in the Materials and Methods section. BSA samples were separated by SDS-PAGE and stained with Coomassie brilliant blue R-250. The band intensity was determined. (B) Effects of PAQ, OSC, and AMC on protein carbonylation. BSA was incubated with AAPH in the presence or absence of extracts, as described in the Materials and Methods section. Carbonyl content was determined using a colorimetric method. (C) Effects of PAQ, OSC, and AMC on lipid

peroxidation. Linoleic acid was incubated with Fe^{2+}/vitamin C in the absence or presence of extracts, as described in the Materials and Methods section. The amount of TBARS formed was determined. * P<0.05, ** P<0.01 versus the control group. # P<0.05, ## P<0.01 versus the treatment group (Treatment, BSA with AAPH or linoleic acid with Fe^{2+}/vitamin C in absence of extracts).

Fig. 3- AMC induced morphological changes, mitochondrial membrane potential reduction and apoptosis in HepG2 liver cancer cells. Cells were treated with or without AMC (50, 100 and 200 μg/mL) for 24 h and photographed (A) or observed after staining with AO/EB (B), DAPI (C) or JC-1(D) using a fluorescence microscope (×100). The ratio of fluorescence intensity (E) was obtained using Multimode plate readers. Cleaved caspase-3 and PARP cleavage were assessed by western blot analysis; β-actin was used as an internal control (F). ** P<0.01 versus the control group.

Fig. 4- Antiproliferation of three extracts and the effect of AMC on HepG2 cell wound healing. The cells were seeded into 24-well cell culture plates for 24 h at 37 ˚C and cultured in medium containing 10% FBS to produce a 90% confluent cell monolayer. Then, a linear wound was generated in the monolayer with a sterile 100 μL plastic pipette tip and the width of the wound measured. Any remaining cellular debris was removed by washing the wells with PBS. RPMI-1640 medium (1 mL) was used as a control; vascular endothelial growth factor (VEGF) (8 ng/mL) was used as a

positive control and VEGF (8 ng/mL) plus different concentrations of AMC extracts (50, 100 and 200 μg/mL) were the test group. The width of the wound was measured again after 24 h of incubation. A qualitative analysis (A) and a quantitative analysis (B) of wound- healing were undertaken. (C) Inhibition of proliferation of HepG2 human liver cancer cells by the three extracts. The absolute value obtained for each sample concentration is expressed as the percent relative to the absolute value obtained for the untreated HepG2 cells and set at 100%.** P<0.01 versus the control group.

Table 1:

Nutrient	*Pteridium aquilinum*	*Osmunda cinnamomea* Linn	*Athyrium multidentatum*(Doll.) Ching
Protein (g/100g)	20.45 ± 0.79	15.45 ± 1.07	24.65 ± 1.34
Fat (g/100g)	1.83 ± 0.06	1.48 ± 0.04	2.09 ± 0.02
Carbohydrate (g/100g)	20.33 ± 0.7	24.62 ± 0.63	17.82 ± 0.42
Crude fiber (g/100g)	21.45 ± 0.05	18.41 ± 0.29	21.94 ± 0.26
Calories (KJ/100g)	179.63 ± 2.58	173.60 ± 6.83	188.75 ± 4.6
Vitamin B_2 (mg/100g)	0.415 ± 0.01	0.46 ± 0.02	0.32 ± 0.01
Sodium (mg/kg)	238.46 ± 3.61	294.63 ± 9.29	214.84 ± 5.44
Calcium (mg/kg)	1943 ± 26.47	715.96 ± 10	1456.8 ± 54.27
Iron (mg/kg)	94.43 ± 3.35	91.19 ± 2.65	80.86 ± 2.62
Zinc (mg/kg)	56.60 ± 2.06	38.24 ± 1.08	49.52 ± 0.87
Selenium (mg/kg)	0.0039 ± 0.0002	0.174 ± 0.005	0.0221 ± 0.0004
Potassium (mg/kg)	12196.67 ± 168.62	2920 ± 26.46	6253 ± 31.5

Table 2:

Sample	no PBS wash		PBS wash		cytotoxicity
	EC_{50}(μg /mL)	CAA (μM QE/g extracts)	EC_{50}(μg /mL)	CAA (μM QE/g extracts)	CC_{50}(μg /mL)
Pteridium aquilinum	186.17±10.54	79.17±5.28	200.87±3.62	81.84±1.26	>800
Osmunda cinnamomea Linn	191.14±15.89	77.12±3.86	341.53±21.06	48.14±3.59	>800
Athyrium multidentatum(Doll.) Ching	127.23±11.86	115.85±11.87	186.52±0.75	88.14±5.32	>100

Fig. 1:

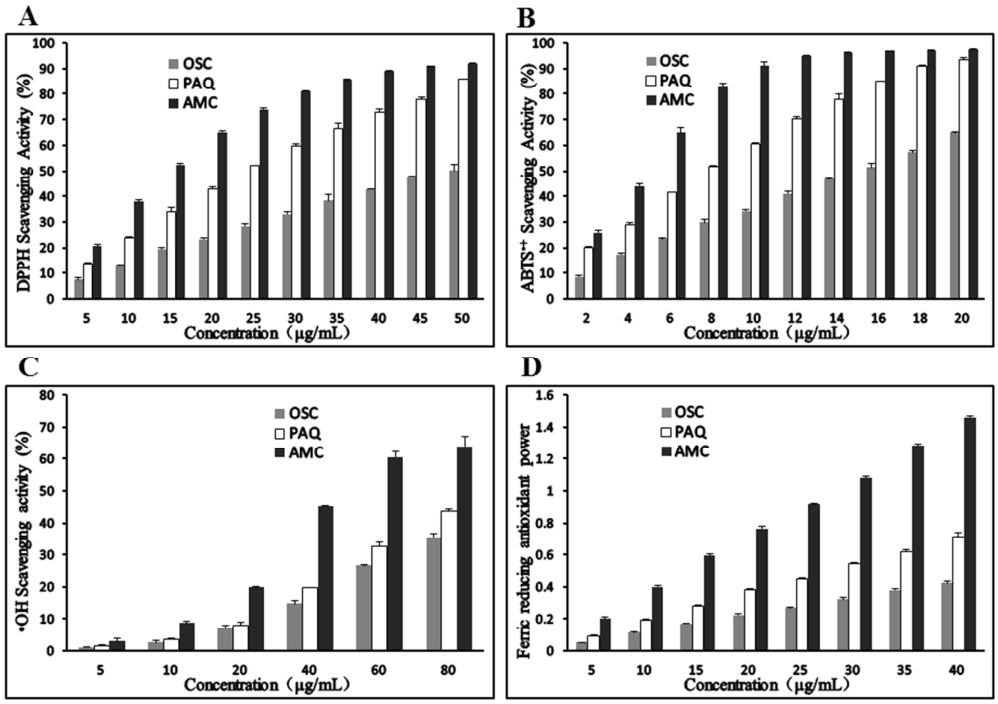
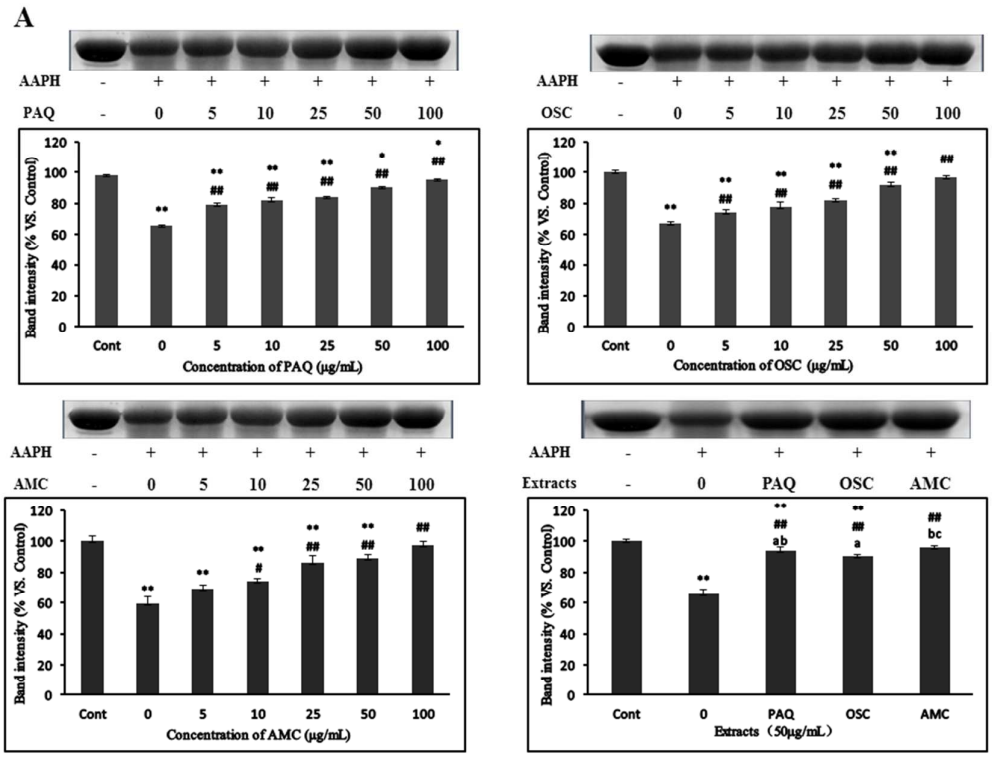
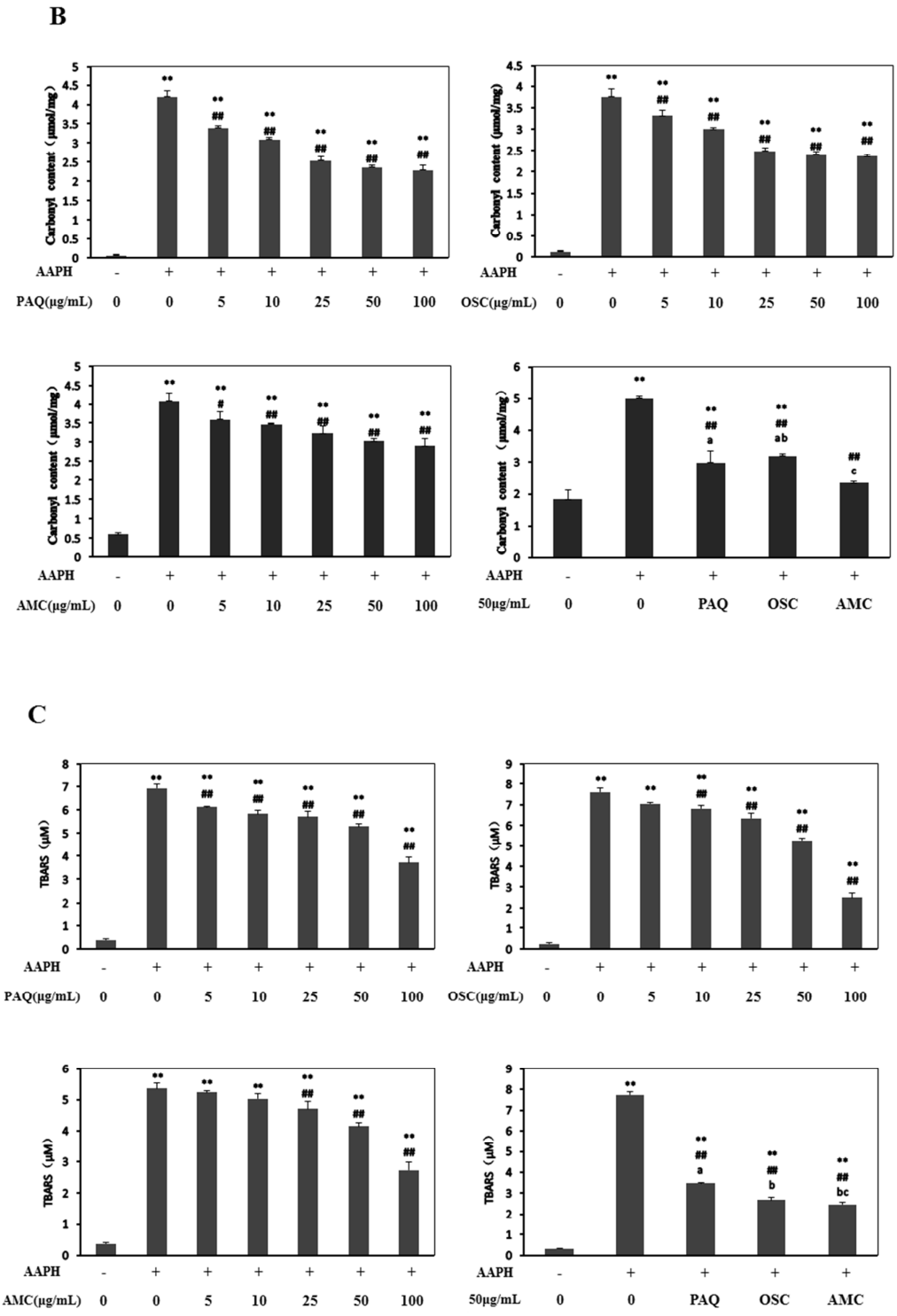


Fig. 2:





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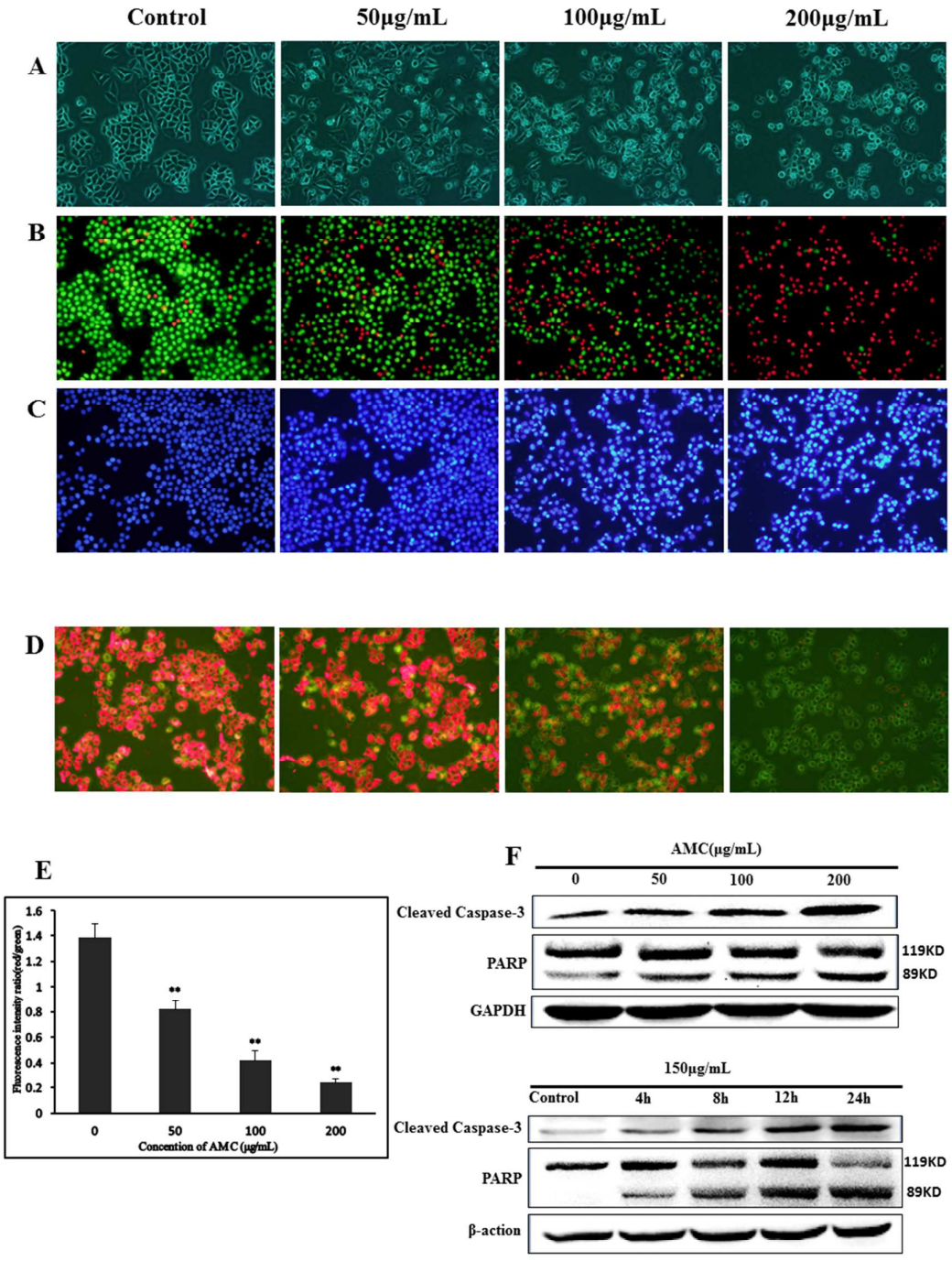

Fig. 4: