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Apigenin, a natural flavonoid, promotes autophagy and ferroptosis in human endometrial carcinoma Ishikawa cells *in vitro* and *in vivo*

Yancui Liang[a], Qian Zhong[a,b], Runhui Ma[a,b], Zhijing Ni[a,b], Kiran Thakur[a,b], Jianguo Zhang[a,b], Zhaojun Wei[a,b,*]

[a] *School of Food and Biological Engineering, Hefei University of Technology, Hefei 230009, China*
[b] *School of Biological Science and Engineering, Collaborative Innovation Center for Food Production and Safety, North Minzu University, Yinchuan 750021, China*

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ABSTRACT

Apigenin, a natural flavonoid has been reported against a variety of cancer types. However, it is unclear whether apigenin can promote autophagy and ferroptosis in Ishikawa cells. There are few reports on the mechanism of apigenin on autophagy and ferroptosis of endometrial cancer Ishikawa cells. We found that iron accumulation, lipid peroxidation, glutathione consumption, p62, HMOX1, and ferritin were increased, while, solute carrier family 7 member 11 and glutathione peroxidase 4 were decreased. Ferrostatin-1, an iron-death inhibitor could reverse the effects of apigenin in Ishikawa cells. On the other hand, apigenin could promote autophagy via up-regulating *Beclin 1*, *ULK1*, *ATG5*, *ATG13*, and *LC3B* and down-regulating *AMPK*, *mTOR*, *P70S6K*, and *ATG4*. Furthermore, apigenin could inhibit tumor tissue proliferation and restrict tumor growth via ferroptosis *in vivo*.

1. Introduction

According to the latest report, 19.3 million patients were diagnosed with cancer and 10 million cancer deaths were reported worldwide in 2020 [1]. So far, endometrial carcinoma (EC) has been recognized as the most common malignant epithelial uterine tumor [2]. Traditional cancer treatments, including surgical excision, chemotherapy, and radiation are known to damage the normal cells to varying degrees. By 1960s, organometallic anticancer drugs were developed and among them cisplatin showed desirable results in clinical application. However, these drugs are reported with serious nephrotoxic effects and drug resistance challenge. Therefore, finding alternative drugs with targeted approach accompanied by less toxicity and minimal side effects has attracted the widespread focus of researchers [3].

Among the various strategies, programmed cell death (induction of apoptosis) is deemed to be one of the major methods for oncotherapy because regulation of apoptosis is tightly in connection with the appearance and inhibition of tumors. Many new forms of non-apoptotic cell death have been reported in recent years, including ferroptosis and autophagy, revealing molecular mechanisms beyond apoptosis. Recent studies have offered possible targets and new methods for the development of oncotherapy [4]. Autophagy plays a key role in cell death and it is recognized as a self-degrading mechanism which is related to the modification of autophagy-related proteins ATGs, Beclin 1, mTOR, and p53 [5]. Ferroptosis is non-apoptotic cell death based on lipid peroxidation activated by ROS mediated via the disintegration of the lipid membrane [6]. Ferroptosis (iron-dependent process) is regulated by ROS generation, GPX4-catalyzed glutathione (GSH) reaction with ROS, and lipid

* Corresponding author at: School of Food and Biological Engineering, Hefei University of Technology, Hefei 230009, China
E-mail address: zjwei@hfut.edu.cn (Z.J. Wei)
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peroxidation. Therefore, studies have reported that factors that regulation of iron levels, iron reserve, and transport may influence the occurrence of ferroptosis [7]. The primary regulation and control process of ferroptosis involves the neutralization of excess ROS by GSH catalyzed by glutathione peroxidase 4 (GPX4) [8]. One recent study reported that β-elemene inhibited the colorectal cancer cells growth, and displayed the double membrane structure of autophagic vesicles, accompanied by up-regulation of LC3B and SQSTM1 and induced autophagy. Further, β-elemene inhibited tumor growth and induced autophagy in nude mice [9]. Another study reported that autophagy was involved in the anti-tumor effect of *Ficus carica* fruit extract in pancreatic cancer [10]. Sinapine, an alkaloidal amine could induce ferroptosis in human NSCLC cell lines, accompanied by up-regulation of transferrin and transferrin receptor and down-regulation of SLC7A11 [11]. Zhang et al. reported that bufotalin promoted ferroptosis in NSCLC cells through ubiquitination and degradation of GPX4 [12]. According to the above-mentioned data, natural compounds inducing autophagy and ferroptosis is a novel research area for cancer treatment.

Among the natural compounds, flavonoids are vital secondary metabolites found in most fruits and vegetables [13]. Widely known for their *in vitro* and *in vivo* anticancer properties [14]. Apigenin has attracted a great interest in food industry because of the wide range of its biological activities including antioxidant and anti-inflammatory[15]. In recent years, apigenin, a flavonoid has been recognized for its anti-cancer properties involving JAK/STAT and NF-κB. Previous studies have reported its effect on cell cycle arrest, counteracting oxidative stress, apoptosis induction, and immunestimulating effects [16]. Li et al. reported that apigenin could regulate the CyclinD1/CDK4 expression and inhibit the growth of HCC cells via the P38 MAPK-P21 pathway [17]. In another study, apigenin targeting hyaluronic acid-lipid nanoparticles could induce Nrf2-dependent apoptosis in lung cancer cells [18]. To date, no data is available on whether apigenin can induce autophagy and ferroptosis in Ishikawa cells. Therefore, we explored the effect of apigenin promoting EC Ishikawa cell death through autophagy and iron death, as well as related molecular mechanism. Our results demonstrated that apigenin can promote Ishikawa cell death through autophagy and ferroptosis, providing a basis for further development and utilization of apigenin as a promising anti-cancer agent.

2. Materials and methods

2.1 Chemicals

Apigenin (purity ≥ 98%) (Muenster, Chengdu, China); Dulbecco's modification of Eagle's media and serum (Hyclone, Logan, UT); primary and secondary antibodies (CST, Danvers, MA; Abcam, Shanghai, China); RT-qPCR kit (Novoprotein, Beijing, China); Iron analysis kit (Abcam, Shanghai, China); lipid peroxidation malondialdehyde (MDA) assay kit (Beyotime, Shanghai, China); GSH and glutathiol (GSSG) assay kit (Beyotime, Shanghai, China); bicinchoninic acid (BCA) assay kit (Servicebio, Wuhan, China).

2.2 Cell culture and treatment

Human endometrial carcinoma (EC) Ishikawa cells were cultured in a high sugar medium including 5% serum and 5% PS at 37 °C and 5% CO_2. When the density of Ishikawa cells in the Petri dishes was above 70%, digestion was performed with 5% trypsin for 3 min to shed most of the cells, and digestion was terminated with the right amount of medium. After centrifugation, the mixture of trypsin and culture medium was discarded, and the cells were suspended in a fresh medium [19]. Ishikawa cells (1×10^5 cells/well) were inoculated in petri dishes and inoculated for 12 h to allow the cells adhere to the wall. Then, the old culture medium in the petri dish was discarded and the fresh culture medium containing different concentrations of apigenin (0, 30, 50, and 70 μmol/L) was added. After 48 h of treatment, the cells were collected for follow-up experiments.

2.3 3-(4,5)-Dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay and lactate dehydrogenase (LDH) assay

According to previously reported method with slightly modifications, Ishikawa cells (8×10^4 cells/well) were uniformly laid in the 96-well plate and inoculated for 12 h. The cells were allowed to adhere to the wall, and then the old medium in the 96-well plate was discarded. Further, the fresh medium containing concentration-gradient apigenin and caspase inhibitor Z-VAD-FMK was added for 48 h followed by the addition of fresh medium containing MTT (5 mg/mL). Then, dimethyl sulfoxide (150 μL) was added after 4 h and absorbance values were measured at 490 and 630 nm [20].

The release of LDH was detected using LDH cytotoxicity detection kit [21], and the cells were divided into four groups: background blank control well, sample control well, sample maximum enzyme activity control well and drug treatment sample well. The concentration gradient of apigenin was added into the well of the treated cells for 48 h, and the LDH releasing reagent was added into the control well of the maximum enzyme activity of the sample 1 hour before the detection point. Finally, 120 μL of supernatant from each well and 60 μL of LDH working fluid were added to another 96-well plate, and the absorbance value was measured at 490 nm.

2.4 Cell colony formation assay

Ishikawa cells (1 000 cells/well) were inoculated in 6-well plates for 12 h to allow the cells adhere to the cell wall. Then, the old medium was discarded and fresh medium containing the autophagy inhibitor chloroquine (CQ) or the ferroptosis inhibitor ferrostatin (Fer)-1, medium containing apigenin and medium containing CQ or Fer-1 and apigenin were added respectively. After 48 h, the old medium was replaced every two days for five consecutive times. Finally, the culture holes were cleaned with phosphate buffer saline (PBS), and then the cells were fixed with methanol for 30 min and stained with 0.1% crystal violet solution for 10 min and the cells were cleaned with PBS, and the colony count was performed with Image J (BIO-RAD) [22].

2.5 Iron assay

According to recent study and following the Iron assay kit

instructions [23], cells were exposed to concentration-gradient apigenin. The cells number in each group was maintained and 1 mL of FerroOrange working solution (1 μmol/L) was added to each group and cultured for 30 min at 37 °C with 5% CO_2. Finally, a fluorescence spectrophotometer (F97Pro, shanghai, China) was used to measure the fluorescence intensity of each cell group.

2.6 MDA assay

According to the previous study and following the MDA test kit instructions [24], the collected cells were lysed with Western and IP cell lysates, centrifuged, and supernatant was collected. BCA kit was used to determine the protein content in the supernatant, and the MDA kit was used to determine MDA content in the supernatant. The MDA content in the original sample was expressed by protein content per unit weight, such as μmol/mg protein.

2.7 GSH assay

GSH content in Ishikawa cells was determined according to previous study and following the GSH and GSSG test kit instructions [25]. The GSH content in the original sample was expressed by GSH content /mg of protein. After the cells were collected by centrifugation, protein removal reagent M solution was added and cells were vigorously vortexed. The samples were then freeze-thawed twice using liquid nitrogen and a water bath at 37 °C. The content of total GSH in the supernatant was determined after centrifugation. The protein content was determined by the BCA kit, and the GSH content in the original sample was expressed by protein content per unit weight, such as μmol/mg protein.

2.8 In vivo tumor xenograft models

All the *in vivo* experiments were approved by the Institutional Animal Care and Use Committee of the Hefei University of Technology. 1 mL of Ishikawa cell suspension (1×10^7 cells/mL) (1 mL) was inoculated subcutaneously in the right axilla of BALB/c nude mice to allow the tumor volume growth up to 100 mm^3. Then, the mice were separated into an untreated group and an apigenin treated group. The former group was intraperitoneally injected with normal saline (200 μL/20 g), and the apigenin treated group was intraperitoneally injected with 50 mg/kg and 200 μL/20 g for 21 days. After 21 days, mice sacrificed and tumor was obtained and weighed, and the heart, liver, spleen, lung and kidney of the mice were collected for further processing [26].

2.9 H&E staining assay

According to previous study and following the instructions of the hematoxylin-eosin dye kit [27], tissue was embedded in paraffin blocks and cut into slices with thickness of 4−5 μm Xylene was used for dewaxing, and ethanol and PBS were used for cleaning. The tissue sections were stained with hematoxylin for 3−5 min and washed with running water. And then the tissues were separated with 1% hydrochloric acid and ammonia, washed with running water, and stained with eosin solution for 2−3 min. Finally, the tissue sections were dehydrated with alcohol and xylene, observed under a microscope (BX43, Olympus, Japan).

2.10 RT-qPCR

According to the recent study and following the reverse transcription kit instructions [28], RNA extraction and reverse transcription were performed. Trizol was used for each group to detach cells and then collect them into EP tubes. After 5 min, chloroform was added to each EP tube and allowed to stand for another 5 min. After centrifugation again for 15 min, the uppermost layer of liquid was transferred into the new EP tubes, and isopropyl alcohol was used and centrifuged for another 10 min. Finally, it was cleaned with 75% ethanol, and the resulting white precipitate was obtained as RNA. Reverse transcription was arranged based on the requirements of the reverse transcription kit. Finally, the SYBR Green method was used for quantitative analysis of mRNA by PCR (Roche, Basel, Switzerland). Table 1 itemized the sequences of the relative PCR primers.

Table 1
Primers for quantitative real-time PCR.

Gene	Primer	Sequence (5′-3′)
AMPK	Forward	CGGCAAAGTGAAGGTTGGCAAAC
	Reverse	CCACATCAAGGCTCCGAATCTTCTG
mTOR	Forward	TTATGGGCAGCAACGGACAT
	Reverse	CTTCTCCCTGTAGTCCCGGA
P70S6K	Forward	ACTGGAAGCCTTGGAATGGG
	Reverse	CCTTGCCGACCACAGTATGT
ULK1	Forward	CCAAGGCTGACTTGTGGAGT
	Reverse	GTTCCTCAGATGGCTGGAAG
Beclin1	Forward	ACATCTGGCACAGTGGACAGTTTG
	Reverse	AGCATGGAGCAGCAACACAGTC
ATG4	Forward	TTGAGAGCCTACCCCTCTGA
	Reverse	CGCACCAGCTCTGTACCTTT
ATG5	Forward	GGACAGTTGACACTAGGAGATC
	Reverse	CTCAGATGTTCACTCAGCCACTGC
LC3B	Forward	GAACCGTAGGAGAAGACCT
	Reverse	CGGTCTTCTCCGACGGCAT
ATG13	Forward	GTACCCAGGCTGACCAGGAGAG
	Reverse	CCGTCCCTCACTGCTGTTTGATAC
P62	Forward	CACCTACTGTGAGGGGTGCT
	Reverse	CTGAGTCCCCTTGACTCTGG
HMOX1	Forward	CTCGGTTTCCCCATCTGTAA
	Reverse	GTGGGCAACATCAGGAACTT
FTL	Forward	GATCTTCATGCCCTGGGTTCTGC
	Reverse	TGGAGGTTGGTCAGGTGGTCAC
FHC	Forward	GCTACAAGTGCCAGTGTGAGGAAG
	Reverse	GTTGGTGAAGAGGTAGGCGATG
SLC7A11	Forward	GGCAGTGACCTTTTCTGAGC
	Reverse	TCATTGTCAAAGGGTGCAAA
GPX4	Forward	TCAGCAAGATCTGCGTGAAC
	Reverse	GGGGCAGGTCCTTCTCTATC
β-actin	Forward	TCTCCCAAGTCCACACAGG
	Reverse	GGCACGAAGGCTCATCA

2.11 Western blot

According to Liu's research [29], the collected cells were lysated with cells lysate containing 1% phenylmethylsulfonyl and placed on ice for 30 min. After centrifugation, the supernatant was collected into new EP tubes and concentration was measured using a BCA kit. Finally, the sample loading buffer was added to the supernatant and boiled at 98.3 °C

for 5 min. The prepared samples were placed in a refrigerator at −80 °C for further use. The proteins were separated by SDS-PAGE gel electrophoresis and transferred to the polyvinylidene fluoride (PVDF) membrane. Then the primary antibody was incubated overnight at 4 °C. The PVDF membranes were cleaned with PBS containing twain (PBST), and the secondary antibody was incubated in an 80 rotary shaker at 37 °C. Finally, the PVDF membrane was cleaned with PBST, exposed to a Tanon 4600 Exposure instrument (Thermo Scientific, USA) and photographed.

2.12 Statistical analysis

The mean values were shown as mean ± SD and the statistical analysis between different groups was processed by *t*-test, and the results were analyzed by SPSS 17.0 using Origin 2018.

3. Results

3.1 Apigenin induced Ishikawa cell death in vitro

In our previous study, we found that apigenin could inhibit Ishikawa cell activity and promote Ishikawa cell apoptosis with the IC_{50} of 45.55 μmol/L [30] (Fig. 1A). In recent years, novel cell death modes have been reported. To further explore whether apigenin could cause Ishikawa cell death through other signaling pathways than reported earlier, we added Z-VAD-FMK, a broad-spectrum caspases inhibitor. As shown in Fig. 1, apigenin could still cause Ishikawa cell death at IC_{50} value of 141.44 μmol/L (Fig. 1A). We also reported the effect of apigenin on Ishikawa cell viability by detecting the release amount of LDH at IC_{50} of 86.45 μmol/L (Fig. 1B).

3.2 Apigenin inhibits tumor growth

To explore whether apigenin can affect Ishikawa cells *in vivo*, we conducted a tumor xenotransplantation experiment, and the results are shown in Fig. 2. Apigenin significantly inhibited tumor growth *in vivo*. As shown in Fig. 2A and Fig. 2B, the frontal volume and weight of the tumor significantly decreased. Contrary to the control group, the tumor volume decreased by 50.49% (Fig. 2C) and weight decreased by 50.63% (Fig. 2D). Apigenin had no distinct influence on the body weight of mice (Fig. 2E). As was shown in Fig. 2F, the results of H&E staining indicated that the tumor tissue in the control group was compact and no new vessels were observed in the interstitium. There was no central necrosis in the tumor tissue, but focal necrosis area was reported, which accounted for 1/4 of the total tumor area. Inflammatory cells were infiltrated in the necrotic area. In the apigenin treated group, the texture of tumor tissue was loose, apoptotic cells could be seen, and there were no new vessels

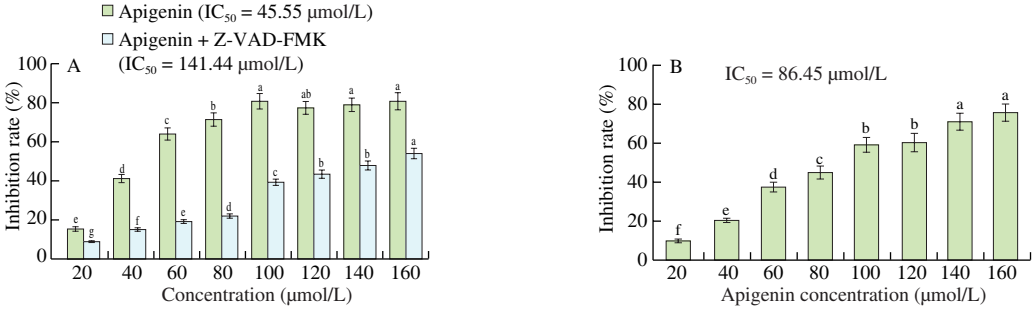

Fig. 1 Inhibitory action of apigenin on Ishikawa cells. (A) Inhibition rate of apigenin and apigenin + Z-VAD-FMK on the proliferation of Ishikawa cells. (B) LDH cytotoxicity. The data were presented as means ± SD of triplicates experiments.

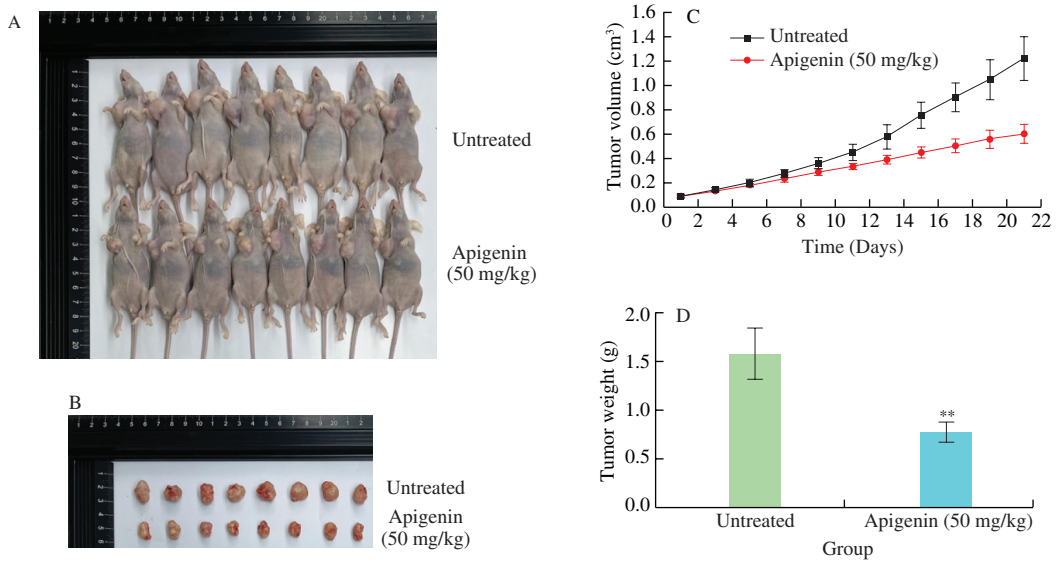

Fig. 2 Tumor growth inhibition of apigenin (50 mg/kg) on Ishikawa xenograft. (A, B) Images of Ishikawa xenograft mice and excised tumors at the end of treatment (n = 8). (C) Tumor volume of Ishikawa xenograft mice. (D) Tumor weight of Ishikawa xenograft. (E) Body weight at the end of treatment. (F) Representative results of H&E staining in tumors. Values were means ± SD of 3 replicates, and $^{*}P < 0.05$ and $^{**}P < 0.01$ were relative to the control group.

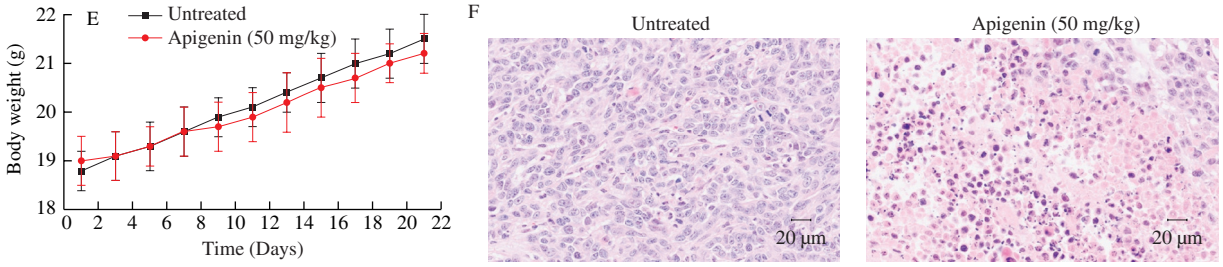

Fig. 2 (*Continued*)

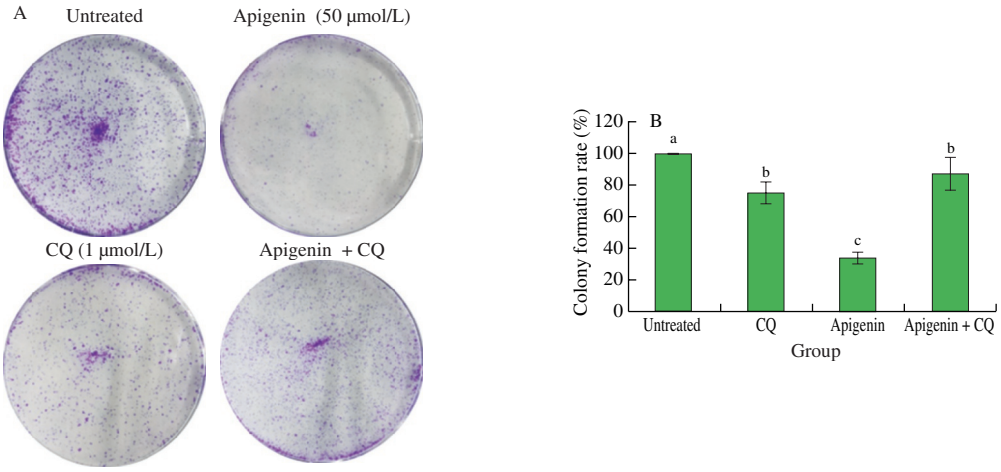

Fig. 3 Apigenin inhibits colony formation. (A) The colony formation after apigenin, CQ and apigenin + CQ treatment. (B) The quantitative analysis of data is mentioned in (A). Values were means ± SD of three replicates. Different letters meant significant differences between the two groups ($P < 0.05$).

in the interstitium. The necrotic area of tumor tissue increased, and the central necrotic area was more than 1/2 of the total tumor area. Inflammatory cells were infiltrated in the necrotic area.

3.3 *Apigenin promoted autophagy in Ishikawa cells*

To investigate whether apigenin promotes autophagy, we added autophagy inhibitor CQ for the colony experiment. Our results (Fig. 3A–B) showed that the colony formation rate in the CQ and apigenin groups increased compared with the apigenin group alone. We further verified whether apigenin could promote Ishikawa autophagy at the gene and protein levels. For *in vitro*, mRNA and protein expression trends were the same, both of which showed down-regulation of *AMPK*, *mTOR*, *P70S6K*, and *ATG4*, up-regulation of *ULK1*, *Beclin 1*, *LC3B*, *ATG5*, and *ATG13* (Fig. 4A–C). After the addition of autophagy inhibitor CQ, the regulatory effect of apigenin on Ishikawa cell proteins was weakened. Compared with the apigenin group, the protein levels were up-regulated for AMPK, mTOR, P70S6K, and ATG4 in the apigenin and CQ group, while the ULK1, Beclin 1, ATG5 and LC3B showed down-regulation (Fig. 4D–E). The similar changes were observed in genes and proteins under *in vivo* conditions. (Fig. 4F–H).

3.4 *Apigenin induced ferroptosis in Ishikawa cells*

To explore whether apigenin can cause ferroptosis in Ishikawa cells, we added Fer-1, an inhibitor of ferroptosis, to conduct the colony experiment, and the results showed that the apigenin treated group significantly inhibited the colony formation rate compared to untreated group (Fig. 5A). The colony formation rate of the apigenin and Fer-1 group was more than that of the apigenin group alone. These results manifested that Fer-1 reversed the inhibitory effect of apigenin on Ishikawa cells. To further verify the impact of apigenin on ferroptosis in Ishikawa cells, we measured the contents of iron ion, GSH, and MDA in Ishikawa cells. The results demonstrated that apigenin increased the iron ion (Fig. 5B), decreased the amount of GSH (Fig. 5C), and increased the content of MDA (Fig. 5D). These results suggested that apigenin could promote ferroptosis in Ishikawa cells.

We further verified that apigenin can promote ferroptosis in Ishikawa cells at the gene and protein levels (Fig. 6). RT-qPCR results indicated that the *p62*, *HMOX1*, *FTL*, and *FHC* were up-regulated, while the *SLC7A11* and *GPX4* were down-regulated *in vivo* and *in vitro* (Fig. 6A and F). The protein expression trend (Fig. 6B, C) was the same as that of genes, which showed up-regulation of P62, HMOX1, and ferritin, while down-regulation of SLC7A11 and GPX4. Compared with the apigenin group, HMOX1 and ferritin proteins were decreased and GPX4 proteins were increased after the addition of Fer-1 (Fig. 6D, E). HMOX1 and ferritin protein expression was increased, while GPX4 protein expression was decreased under *in vivo* conditions (Fig. 6G, H). Our results suggested that apigenin promoted ferroptosis in Ishikawa cells both under *in vivo* and *in vitro* conditions.

3.5 *Effect on other organs*

To explore the toxicity of apigenin, a pathological analysis in mice model was performed. As shown in Fig. 7, all the analyses were in contrast with the untreated group. Apigenin has no obvious effect on the heart, liver, spleen, lungs, and kidney. For the liver, the lobule structure was normal, there was no degeneration of liver cells,

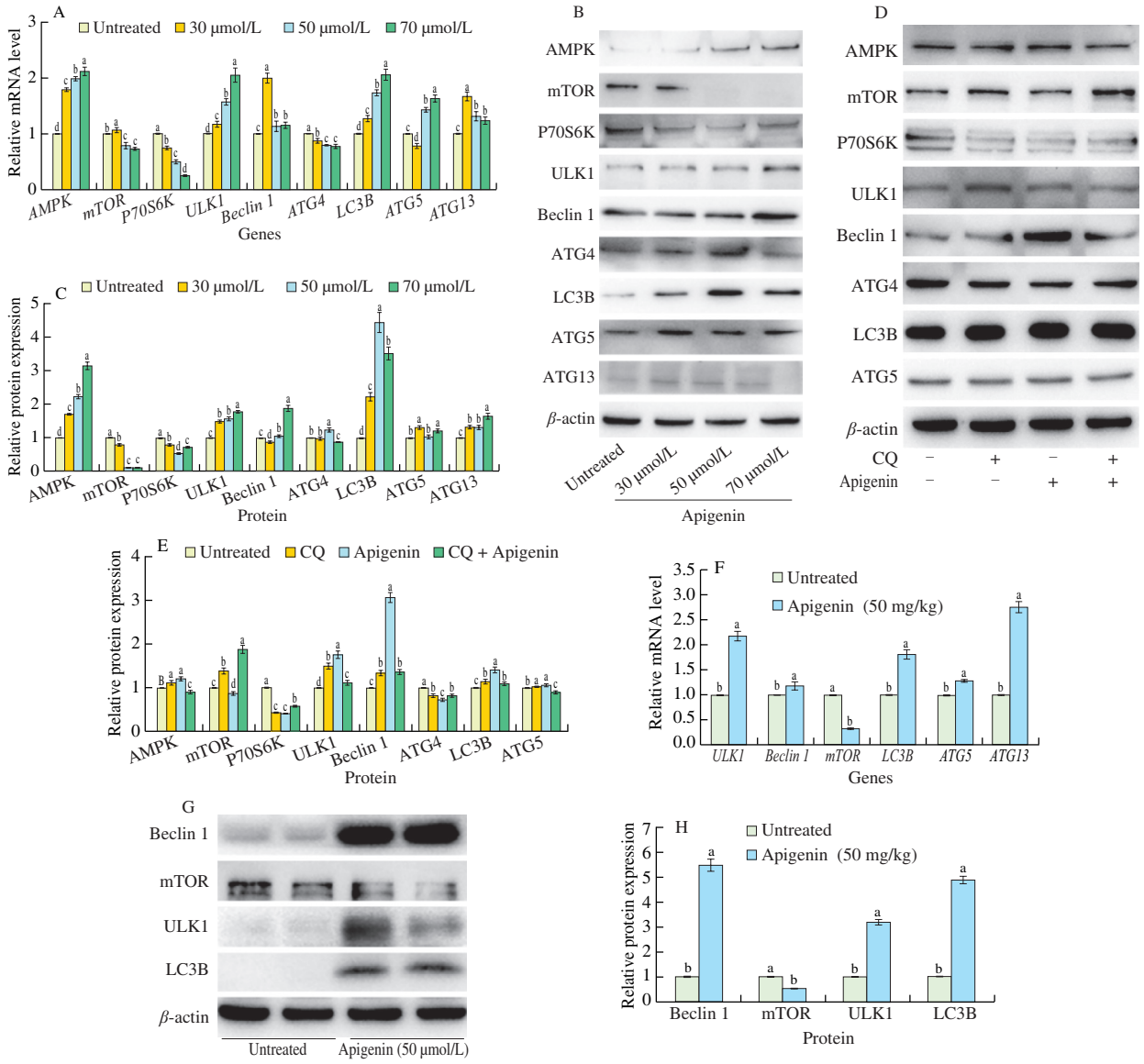

Fig. 4 Apigenin inhibits the autophagy of Ishikawa cells and the autophagy-related gene and protein. (A) Relative mRNA level. (B) Relative protein bands by Western blotting analyses. (C) The quantitative analysis of protein expression data is mentioned in (B). (D) Effect of autophagy inhibitor (CQ) on apigenin-induced autophagy of Ishikawa cells. (E) The quantitative analysis of protein expression data is mentioned in (D). (F) Relative mRNA level in the tumor. (G) Relative protein bands in tumors. (H) The quantitative analysis of protein expression data is mentioned in (G). Values were means ± SD of three replicates. Different letters meant significant differences between the two groups ($P < 0.05$).

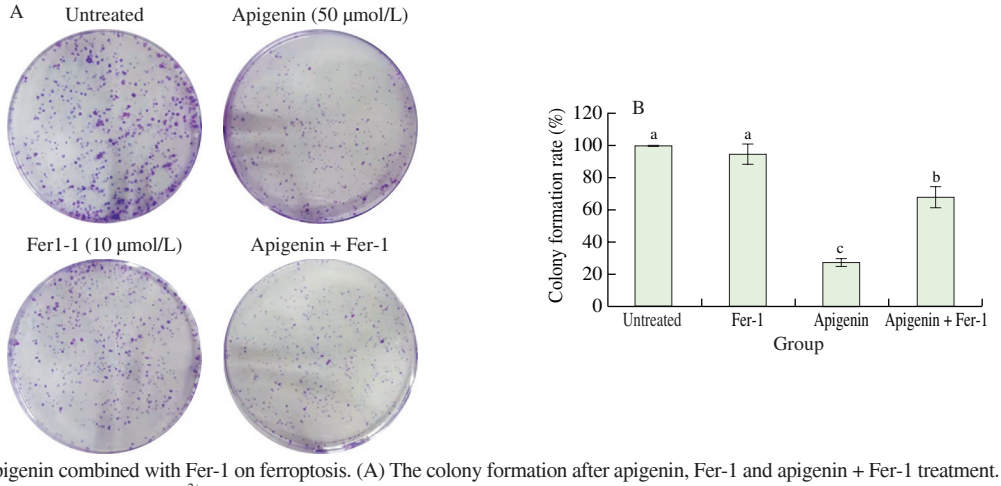

Fig. 5 Effects of apigenin combined with Fer-1 on ferroptosis. (A) The colony formation after apigenin, Fer-1 and apigenin + Fer-1 treatment. (B) The quantitative analysis of data mentioned in (A). (C) Fe^{2+} content. (D) GSH. (E) MDA. The different superscripts (a, b, c, and d) represent significant differences at $P < 0.05$.

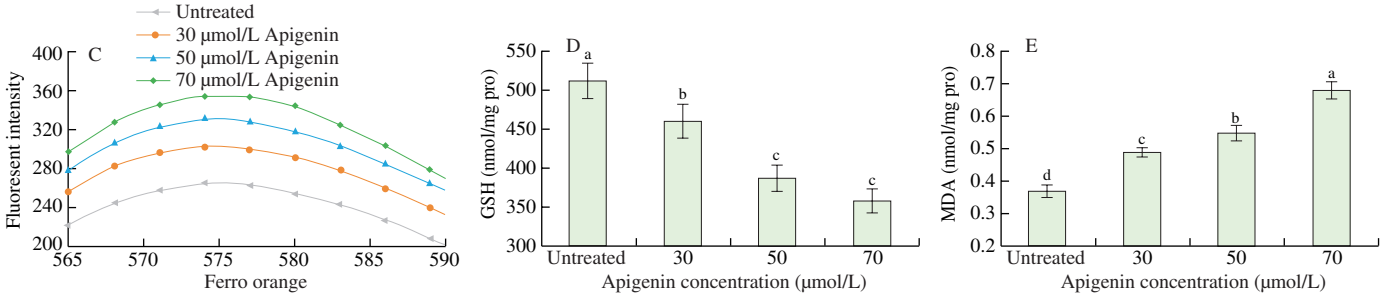

Fig. 5 (*Continued*)

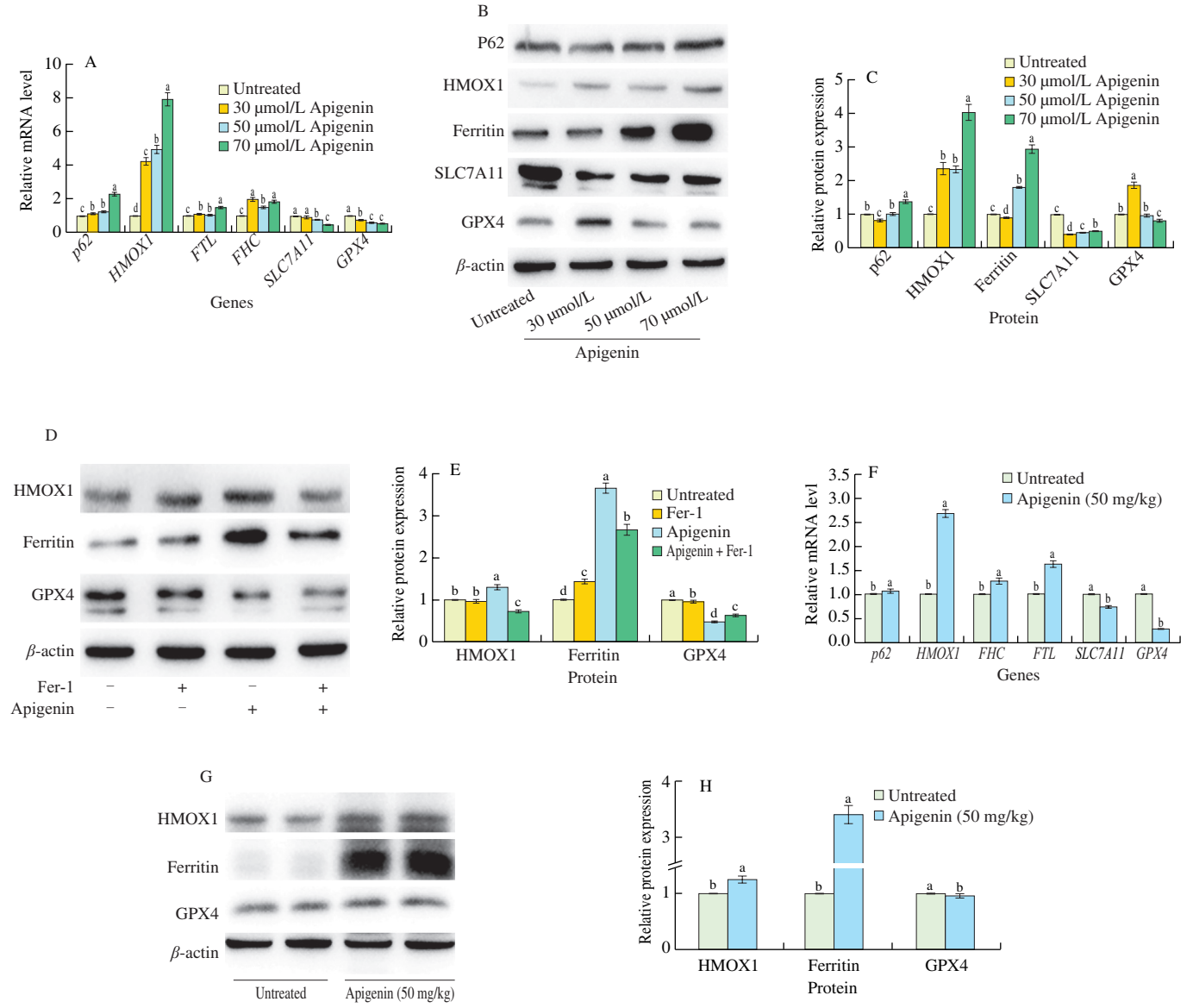

Fig. 6 Apigenin inhibits the ferroptosis of Ishikawa cells and the ferroptosis-related gene and protein. (A) Relative mRNA level. (B) Relative protein bands by Western blotting analyses. (C) The quantitative analysis of protein expression data is mentioned in (B). (D) Effect of ferroptosis inhibitor (Fer-1) on apigenin-induced ferroptosis of Ishikawa cells. (E) The quantitative analysis of protein expression data is mentioned in (D). (F)Relative mRNA level in the tumor. (G) Relative protein bands in the tumor. (H) The quantitative analysis of protein expression data is mentioned in (G). Values were means ± SD of three replicates. Different letters meant significant differences between the two groups ($P < 0.05$).

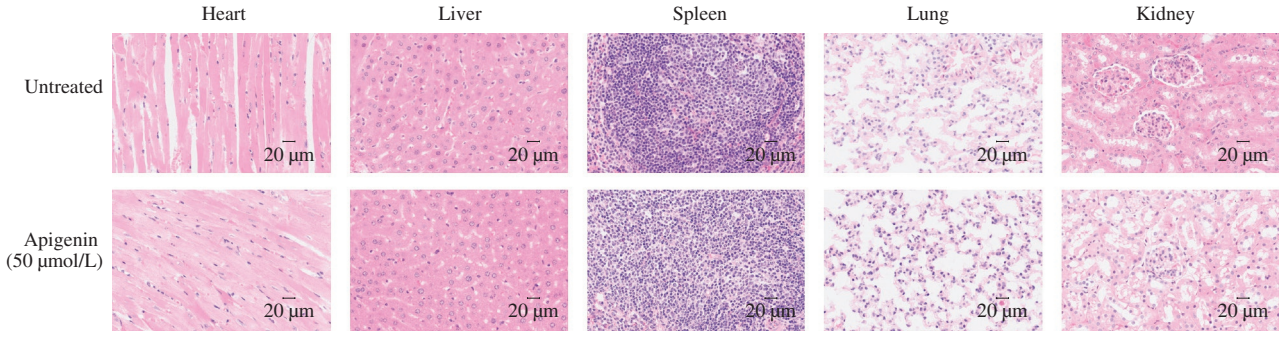

Fig. 7 Representative results of H&E staining in heart, liver, spleen, lungs, and kidney.

no necrosis, and no congestion of hepatic sinuses. For the heart, the cardiomyocyte structure was normal, the boundary was not clear, and the myocardium was slightly deformed. There was no hyperemia and edema in the interstitium of the heart tissue, with occasional inflammatory cell infiltration. For the kidney, the glomerulus was well structured and normal in size, the tubules were not swollen, the interstitium was not edematous and there was no local hyperemia. As for the lungs, the alveolar wall structure and bronchial structure of mouse lung tissue were intact, without exfoliation of bronchial epithelial cells, no secretions in the lumen, and no thickening of the alveolar wall. For the spleen, the capsule was with complete and smooth structure. The trabecular artery, central artery, and peripheral lymphatic sheath were normal.

The possible mechanism of apigenin caused Ishikawa cells ferroptosis and autophagy as shown in Fig. 8.

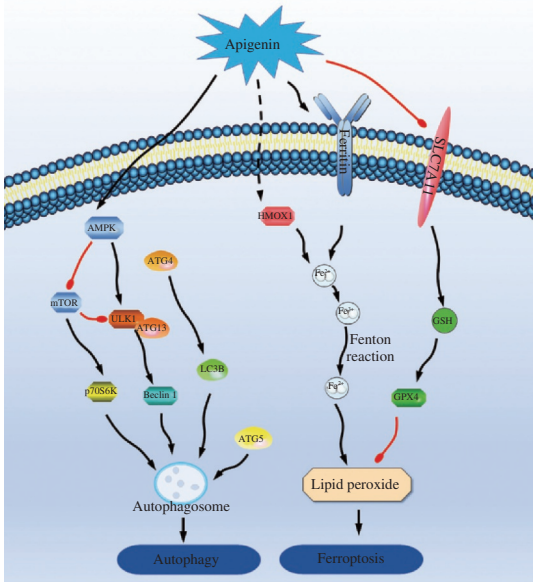

Fig. 8 Possible mechanism of apigenin caused Ishikawa cells ferroptosis and autophagy.

4. Discussion

Cancer is a widespread health problem that affects a large population and is the leading cause of death worldwide [31]. The number of cancer patients is predicted to increase by 47% by 2040, indicating the urgency of finding novel anti-cancer drugs [32]. The American Cancer Society estimated that 63 230 new cases of EC were diagnosed in the US in 2018, and about 11 350 women die from it each year [33]. EC remained the most usual type of cancer in women after breast, lung, bronchial, and colon and rectal cancers, with an incidence of 2.83% in China.

Among the natural flavonoids, apigenin has been widely studied in recent years because of its anticancer and low toxicity [34]. Apigenin has been shown to inhibit a variety of human malignancies *in vitro* and intravenously through a variety of organic actions, including inducing cell death and autophagy, preventing cell cycle progression, and inhibiting cell motility and invasion [35]. In our previous study, we confirmed that apigenin can block Ishikawa cells in the G2/M phase, promote apoptosis and inhibit cell migration and invasion [30]. Here in, we further demonstrated that apigenin can promote cell death through autophagy and ferroptosis.

Autophagy is a complex process and plays a vital role in the oncogenesis and progression of sickness [36]. Many factors, such as hunger and metabolic stress, can lead to autophagy activation through overexpression of AMPK and subsequent induction of ULK1 [37]. The ULK1 complex induces Beclin 1 expression, leading to the formation of the class III PI3K VPS34 complex. This process is necessary for phagocytic mass formation to begin [38]. Furthermore, AMPK has been claimed to inhibit the mTOR pathway and induce autophagy. Autophagosome formation is mediated by lipidation of LC3-I into LC3-II, and ATG5 and ATG16L1 are directly involved in the modification of LC3-I and subsequent autophagosome mechanisms [39]. It has been reported that the occurrence and development of cancer could be inhibited by promoting autophagy [40]. Apigenin treated Ishikawa cells showed lowered number of cells, up-regulated proteins ULK1, Beclin 1, LC3B, ATG5, and ATG13, accounting for Ishikawa cell death via autophagy. *In vivo*, apigenin also inhibited tumor growth and promoted autophagy in Ishikawa cells.

Recent studies have indicated that ferroptosis played a vital role in the development of tumors [41]. Inducing ferroptosis has become a potentially effective strategy for clinical cancer prevention and treatment. Although there are many pathways involved in ferroptosis, lipid metabolism and iron metabolism are the most important pathways in ferroptosis [42]. In general, cells maintain a balance between iron absorption, output, utilization and storage. When the intracellular iron content is extremely high, free Fe^{2+} with high oxidation ability is easy to react with peroxide lipids in the Fenton process, producing hydroxyl radicals, causing strong oxidative stress reaction, promoting ROS production and inducing ferroptosis [43].

Abnormal iron metabolism can increase the intracellular iron content and induce ferroptosis [44]. Excess iron in cells is stored in ferritin composed of FHC, which is responsible for rapid oxidation of Fe^{2+}, and FLC, which is responsible for Fe^{3+} storage. Previous research has shown that the expression of FLC and FHC was upregulated in the presence of iron overload when free iron was too high [45]. In our study, apigenin increased iron content and up-regulated ferritin protein expression in Ishikawa cells, providing preliminary evidence for the indication that apigenin promoted ferroptosis. It has been shown that ferroptosis was triggered by the high expression background of HMOX1 in osteosarcoma cells [46]. In our study, apigenin upregulated HMOX1 expression in Ishikawa cells, similar to previous studies. SLC7A11 is a cystine/glutamate reverse transporter with important functions including the transport of extracellular cysteine for glutathione biosynthesis and ROS detoxification. GPX4 is a lipid repair enzyme that can inhibit ferroptosis by lowering lipid ROS [47]. Inhibition of SLC7A11 and GPX4 interrupts intracellular GSH metabolism and promotes lipid peroxidation and subsequent ferroptosis [48]. Fer-1 is an iron death inhibitor that inhibits cell death due to ferroptosis. The proteins SLC7A11 and GPX4 were down-regulated, GSH down-regulated and MDA up-regulated in apigenin treated Ishikawa cells.

5. Conclusion

Overall, apigenin could promote autophagy via up-regulating the protein expressions of ULK1, Beclin1, LC3B, ATG5, and ATG13, and down-regulating the mTOR, P70S6K and ATG4. In addition, apigenin treatment increased the iron concentration, decreased the GSH content of, and increased MDA content. Apigenin also increased the protein expressions of P62, HMOX1, and ferritin, decreased the protein expressions of SLC7A11 and GPX4, and induced ferroptosis in Ishikawa cells. For *in vivo*, apigenin inhibited tumor growth through autophagy and ferroptosis. The normal physiological functions of the heart, liver, spleen, lung, and kidney and bodyweight of mice were not affected. Our data illustrate that apigenin can be highly effective anti-cancer agent with low toxicity.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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