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Anticancer effect and underlying mechanism of total flavonoids from Qinglongyi against colon cancer *in vitro* and *in vivo*

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ABSTRACT: Qinglongyi is the epicarp of the immature fruit of *Juglans regia* L. Qinglongyi flavones (QFs), the plant main active ingredients, have been shown to inhibit a variety of cancer cells. In this study, an MTT assay was used to evaluate the inhibitory effect of QFs on the proliferation of CT26 cells *in vitro*. Tumor-bearing BALB/c-nu mice were randomly divided into a model group, a 5-FU group, and high-, medium-, and low-dose QFs groups. Mice in the model group were given normal saline, those in the 5-FU group were given 5-FU, and those in the low-, medium-, and high-dose QFs groups were given a QFs solution by gavage once daily for 9 days. Subsequently, the tumor inhibition rate and pathological index were examined. The expression of RAS, phosphorylated Akt, and phosphorylated ERK1/2 proteins in the tumor tissue was detected. The *in vitro* experiment showed that QFs could significantly inhibit the proliferation of CT26 colon cancer cells and downregulate RAS gene expression in a dose-dependent manner. The in vivo experiments showed that QFs could significantly inhibit tumor growth in mice in a dose-dependent manner. The pathological examination showed that large areas of tumor tissue were necrotic in the different QFs-treated groups. Western blot analysis showed that QFs could downregulate the expression of RAS, phosphorylated Akt, and phosphorylated ERK1/2 proteins in the tumor tissue of mice. QFs have an anticancer effect against colon cancer both *in vivo* and *in vitro*, and their underlying mechanism may involve modulation of the RAS/AKT/ERK signaling pathway.

KEYWORDS: Qinglongyi, green walnut husks, total flavonoids, RAS/AKT/ERK signaling pathway, colon cancer

INTRODUCTION

Colon cancer is one of the most common malignant tumors in the digestive system, with a high incidence and a high mortality rate [1]. Colon cancer is mainly caused by the abnormal proliferation of intestinal epithelial cells, and these abnormal cells also have the ability to invade and spread, seriously threatening human health. According to WHO statistics, colon cancer is the third most common malignancy worldwide, and with changes in dietary habits and lifestyles, the incidence of colon cancer has been increasing year by year and colon cancer is one of the fastest growing malignant tumors in incidence [2, 3]. Although surgical resection combined with radiotherapy or chemotherapy is primarily used for the treatment of colon cancer and some therapeutic effects have been achieved, the adverse reactions induced by radiotherapy and chemotherapy can be substantial since they can kill tumor cells as well as normal cells at the same time, resulting in bone marrow hematopoietic suppression, organ function damage, and a decline in body immunity, seriously affecting the quality of life of patients [4, 5]. Therefore, it is of great significance to find new therapeutic drugs for the treatment of colon cancer.

In recent years, natural plant extracts have attracted widespread attention because of their high antitumor efficacy and low toxicity [6]. Natural plant extracts have shown unique advantages in many aspects, such as reducing toxicity and increasing the efficacy of radiotherapy and chemotherapy for malignant tumors [7], preventing postoperative recurrence and metastasis [8], and improving the immunity of patients [9]. Qinglongyi [green walnut husks (*Juglans regia* L.)] is the epicarp of the immature fruit of *Juglans regia* L., a plant of the family Juglandaceae, with the effects of clearing away heat and toxins, expelling wind, treating tinea, relieving pain and stopping dysentery [10]. Qinglongyi is often used in traditional Chinese medicine to treat stomachache, abdominal pain, dysentery, carbuncle, skin pruritus, etc. Only the immature exocarp, which is rich in flavonoids, can be used as traditional medicine [11]. Modern pharmacological studies have found that Qinglongyi has multiple effects, including anti-inflammatory effects [12, 13], analgesic effects [14], antibacterial effects [15, 16], antioxidant effects [17, 18], and antitumor effects [19], especially its therapeutic effects against digestive system tumors and lung cancer [20]. It has been found that Qinglongyi has a cytotoxic effect on gastric, liver, colon, and pancreatic cancer cells [21, 22], and can induce cell cycle arrest. Qinglongyi can also induce apoptosis in human gastric cancer SGC-7901 cells through ROS production and a reduction in the Bcl-2/Bax ratio [23].

More than 130 chemical constituents have been found in Qinglongyi, including naphthoquinones, polyphenols, flavonoids, cyclic diarylheptanes, and polysaccharides, of which flavonoids are among its main components and also its main active compo-

nents [24, 25]. It has been confirmed that Qinglongyi flavonoids (QFs) can significantly inhibit the proliferation of colon cancer cells [26], induce apoptosis in colon cancer cells [27], and inhibit the metastasis of colon cancer cells [28]. However, there are no systematic studies or reports on the anti-colon cancer effect of QFs, and their potential molecular mechanism has not been clarified. Therefore, in this study, the inhibitory effect of QFs on the proliferation of CT26 colon cancer cells and their inhibitory effect on subcutaneous tumors in nude mice were studied, and their underlying mechanism was also explored, so as to provide a theoretical basis for the clinical treatment of colon cancer.

MATERIALS AND METHODS

Plant materials and preparation of QFs

Qinglongyi was collected from Jiaohe City, Jilin Province, China, and was identified by Prof. Guangxin Yuan (School of Pharmacy, Beihua University) and Dr. Yinglan Jin (Jilin Municipal Institute for Drug Control). A voucher specimen (No. 19110612) has been deposited at Beihua University, Jilin, China.

Qinglongyi was washed with water to remove impurities on its surface, then dried and crushed into powder. The powder was passed through a 40-mesh sieve. The sieved powder was heated and reflux-extracted twice with a 10-fold volume of 75% ethanol, for 1 h each time. The filtrates were mixed, and the ethanol was recovered under reduced pressure, after which the mixed filtrate was evaporated to dryness. The residue was dissolved in water. The residue solution was loaded onto a pre-treated AB-8 macroporous resin column in several batches and allowed to stand for 1 h. The column was then washed with two column volumes of water, and the eluate was discarded. The column was eluted with 1.5 column volumes of 75% ethanol, and the eluate was collected. The ethanol was recovered under reduced pressure, and the eluate was evaporated to dryness to obtain the total flavonoids of Qinglongyi, referred to as Qinglongyi flavonoids (QFs).

Animals and cells

Six-week-old, specific pathogen-free (SPF) BALB/c-nu mice weighing 20 ± 2 g, with equal numbers of males and females, were purchased from Beijing Weitong Lihua Company (Beijing, China; Permit No. SCXK(J)-2019-0017). The mice were raised in separate cages at a humidity of 45%–55% and a temperature of 20–25 °C, with free access to food and water. The experiments were approved by the Animal Ethics Committee of Beihua University (No. 202006120039).

Mouse CT26 cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China).

Chemicals and reagents

MTT reagent was purchased from Wuhan Seville Biotechnology Co., Ltd. (Wuhan, China). A BCA kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). A β-Actin antibody was purchased from Wuhan Seville Biotechnology Co., Ltd.; RAS, Akt, p-Akt, ERK1/2, and p-ERK1/2 antibodies were purchased from CST Co., Ltd. (USA). RPMI 1640 culture medium was purchased from Wuhan Procell Life Technology Co., Ltd. (Wuhan, China). Trypsin was purchased from Hangzhou Jinuo Biology Co., Ltd. (Hangzhou, China). Fetal bovine serum was purchased from Gibco (USA). The other reagents were of analytical grade, and the water used in this study was double-distilled water prepared in our laboratory.

Assessment of the inhibitory effect of QFs on the proliferation of CT26 cells *in vitro* using an MTT assay

CT26 colon cancer cells in the logarithmic growth phase were seeded in 96-well plates at a density of 5×10^4/ml, 100 µl per well, and cultured in a 5% CO_2 incubator at 37 °C for 24 h. The cells were cultured in 100 µl of culture medium containing different concentrations of QFs (0, 6.25, 12.5, 25, 50, and 100 mg/l), and those in the solvent control group were cultured in culture medium containing 0.054% DMSO for 24 h. Four replicate wells were used for each condition. The culture plates were removed from the incubator, then 20 µl of 5 g/l MTT solution was added to each well, and the cells were further incubated for 4 h. Then, 150 µl of DMSO was added to each well after the remaining incubation solution was discarded, and the plates were placed on a shaker and shaken for 10 min to completely dissolve the crystals. The optical density (OD) of the solution in each well was measured at 490 nm with a microplate reader for the calculation of the cell proliferation rate.

$$\text{Proliferation rate} = (\text{OD}_{\text{QFs}} - \text{OD}_{\text{control}})/\text{OD}_{\text{control}}$$

Detection of RAS gene expression in CT26 cells by RT-qPCR

CT26 cells collected from the control group and QFs-treated groups in cell culture dishes were treated with 1 ml of Trizol, and then thoroughly ground for RNA extraction. A reverse transcription kit was used to reverse-transcribe RNA into cDNA, and the expression of the RAS gene was detected by RT-qPCR. The relative difference in the expression of RAS mRNA between the QFs-treated groups and the control group was analyzed using the $2^{-\Delta\Delta C_t}$ method. The primers were synthesized by Dalian Bioengineering Technology Co., Ltd. (Dalian, China), and the sequences are shown in Table S1.

Modeling, grouping, and administration

BALB/c-nu mice were acclimatized to the laboratory environment for one week. The trypan blue exclusion assay showed that the viability of CT26 colon cancer cells in the logarithmic growth phase was greater than 95%. The concentration of the CT26 cells was adjusted to 4×10^6 cells/ml for the preparation of a cell suspension, and 0.2 ml of the cell suspension was subcutaneously injected into the right axilla of the mice to establish a solid tumor. When the tumor size was approximately 100 mm^3, the tumor-bearing mice were randomly divided into the model group, the 5-FU group, and the high-, medium-, and low-dose QFs groups, with 10 mice in each group. Mice in the model group were given normal saline (0.1 ml/10 g), those in the 5-FU group were given 5-FU (10 mg/kg), and those in the low-, medium-, and high-dose QFs groups were given QFs solution (50 mg/kg, 100 mg/kg, and 200 mg/kg, respectively) by gavage, once daily for 9 days.

Determination of tumor inhibition rate

The body weight of tumor-bearing mice was measured every two days, and the signs and weight changes in mice in all the groups were observed. Twenty-four hours after the last administration, the mice were sacrificed, and the tumor tissues were removed and weighed for the calculation of the tumor inhibition rate. The calculation formula is as follows:

Tumor inhibition rate = [(tumor weight of mice in model group − tumor weight of mice in drug-treated group)/(tumor weight of mice in model group)] × 100%

Hematoxylin and eosin (H&E) staining and histopathological observation

The tumor-bearing mice were weighed and sacrificed, and the tumor tissue was collected. The tumor tissue (1 cm × 1 cm) was cut and fixed in 10% formaldehyde solution. The sections were paraffin-embedded and routinely stained with H&E. The histopathological changes of the tumor tissue were observed by examining the sections under an optical microscope.

Detection of RAS, Akt, p-Akt, ERK1/2, and p-ERK1/2 protein expression in the tumor tissue by Western blot analysis

The tumor tissue (0.1 g) was cut into pieces. The tumor tissue pieces were placed into a glass homogenizer, and 1 ml of Ripa lysis buffer was added. The tissue was then ground on ice for the extraction of proteins, and the proteins were quantified using a BCA kit. After the denaturation of proteins, the proteins were subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk at room temperature for 1 h. Then, the primary antibodies, including rabbit anti-RAS (1:1000), Akt (1:1000), p-Akt (1:1000), ERK1/2 (1:1000), p-ERK1/2 (1:1000), and rabbit anti-β-actin (1:1000) were added to the membrane, and the membrane was incubated at 4 °C for 2 h. After washing the membrane with TBST, the HRP-labeled secondary antibody was added to the membrane. The membrane was washed with TBST and then developed by chemiluminescence, and the protein band intensities were analyzed. The relative expression of proteins was expressed as target protein/β-actin or phosphorylated protein/total protein.

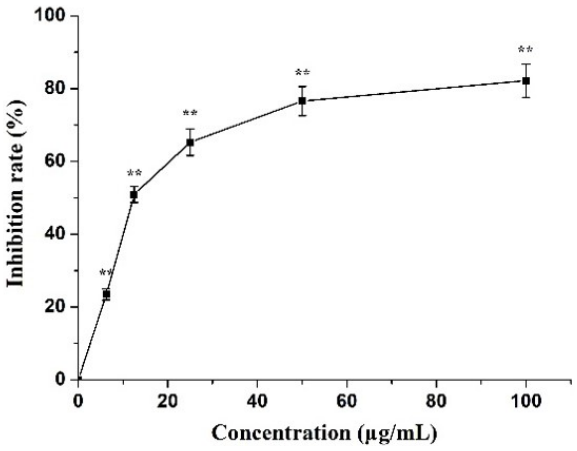

Fig. 1 Inhibition of CT26 colon cancer cell proliferation by QFs (mean ± s, $n = 3$). ** $p < 0.01$ compared with the control group.

Statistical analysis

SPSS 22.0 software was used for the statistical analysis of data, and ImageJ was used for the analysis of Western blot band intensities. The quantitative data were expressed as mean ± s, and "n" was used to indicate the number of samples. The measurement data from the two groups were compared by an independent samples t-test, and the differences with $p < 0.05$ were considered statistically significant.

RESULTS

Inhibition of CT26 colon cancer cell proliferation by QFs

After treatment with QFs for 24 h, the effect of QFs on the proliferation of CT26 cells was analyzed by the MTT method. The results in Fig. 1 showed that QFs could inhibit the proliferation of CT26 cells, and the inhibitory effect increased with increasing QFs concentration; the IC50 of QFs against CT26 cells was 12.35 ± 1.21 μg/ml after 24 h of treatment with QFs. Based on the above results, the final QFs concentrations of 6.25, 12.5, and 25 μg/ml were used to treat the cells in the QFs-treated groups, and the cells in the control group were treated with an equal volume of the solvent.

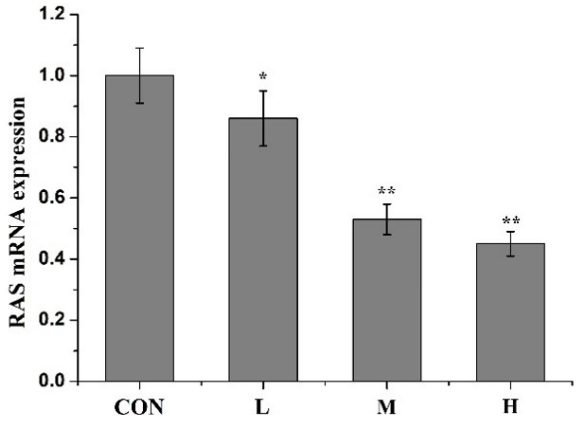

Fig. 2 Effect of QFs on RAS gene expression in CT26 colon cancer cells (mean ± s, $n = 3$). CON: blank control group; L: low-dose QFs group; M: medium-dose QFs group; H: high-dose QFs group; * $p < 0.05$ compared with the control group; ** $p < 0.01$ compared with the control group.

Table 1 Effect of QFs on tumor weight in mice (mean ± s, $n = 10$).

Group	Dose/ mg/kg	Tumor weight (g)	Tumor inhibition rate (%)
Model	–	3.513 ± 0.332	
5-FU	10	1.122 ± 0.117**	63.55 ± 6.12
Low-dose QFs	50	2.632 ± 0.257**	48.82 ± 5.03
Medium-dose QFs	100	2.053 ± 0.216**	37.66 ± 3.55
High-dose QFs	200	1.617 ± 0.169**	23.25 ± 2.59

Compared with the model group: ** $p < 0.01$.

Effect of QFs on RAS gene expression in CT26 colon cancer cells

As shown in Fig. 2, compared with the control group, RAS gene expression in CT26 colon cancer cells in the low-, medium-, and high-dose QFs groups was reduced in a dose-dependent manner ($p < 0.05$, $p < 0.01$), suggesting that the inhibition of CT26 cell proliferation by QFs may be associated with the inhibition of RAS gene expression by QFs or the expression of downstream genes in the RAS signaling pathway.

Effect of QFs on the tumor inhibition rate

As shown in Table 1, the tumor inhibition rate in the 5-FU group was 63.55%, the highest among the groups, and the tumor inhibition rates in the low-, medium-, and high-dose QFs groups were 23.25%, 37.66%, and 48.82%, respectively, showing a dose-dependent trend. These results indicate that QFs could inhibit tumor growth in mice.

H&E staining results of tumor tissues from mice treated with QFs

Tumor cell necrosis in the low-, medium-, and high-dose QFs groups, especially in the medium- and high-dose QFs groups, was more pronounced than that in the model group, indicating that QFs could promote apoptosis in colon cancer cells (Fig. 3). The results showed that most of the nuclei were darkly stained, and the cytoplasm was dense and showed increased eosinophilic staining in the QFs-treated groups, suggesting apoptosis.

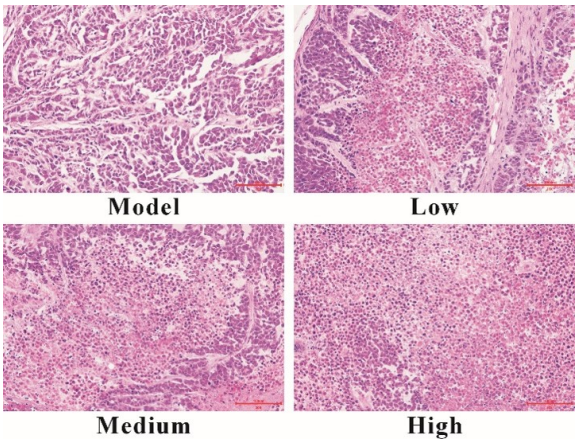

Fig. 3 H&E staining of tumor tissues from mice treated with QFs. Scale bars = 100 μm.

Inhibition of RAS-related protein expression by QFs in tumor tissues

In order to explore the mechanism underlying the anti-colon cancer effects of QFs, their effects on the RAS/Akt/ERK signaling pathway in mouse tumor tissues were further studied. As shown in Fig. 4, the expression levels of RAS, phosphorylated Akt, and phosphorylated ERK1/2 decreased in a dose-dependent manner in the low-, medium-, and high-dose QFs groups, suggesting that the inhibition of tumor growth by QFs may be related to the inhibition of the RAS/Akt/ERK signaling pathway by QFs.

DISCUSSION

The incidence rate of colon cancer has been increasing year by year, and it has become one of the most common malignancies in the digestive system, with a high mortality rate. Commonly used treatments for colon cancer are not entirely satisfactory, and all commonly used drugs can cause severe side effects, so there is an urgent need to find new drugs for the treatment of colon cancer. Qinglongyi, the immature exocarp of walnut fruits during their development, contains a variety of flavonoids. However, the exocarp will fall off, and the flavonoid content will decrease when the fruit matures [29]. In this study, the *in vitro* cell experiment showed that QFs could inhibit the proliferation of CT26 colon cancer cells cultured *in vitro*, and the IC_{50} value of QFs 24 h after administration was 12.35 ± 1.21 μg/ml, indicating that QFs may be an

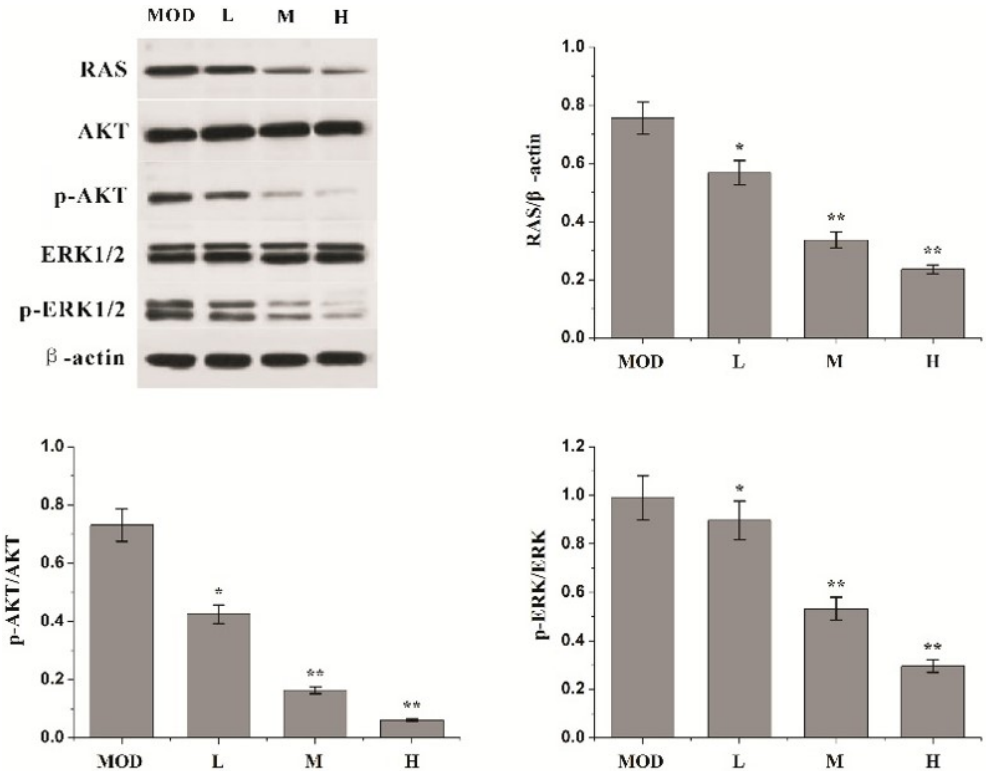

Fig. 4 Effects of QFs on the expression levels of RAS-related proteins in mouse tumor tissues (mean $\pm s$, $n = 3$).

effective component against colon cancer in mice. The *in vivo* experiment showed that QFs could significantly inhibit the growth of subcutaneous tumors in nude mice, and the tumor inhibition rates of mice in the low-, medium-, and high-dose QFs groups were 23.25%, 37.66%, and 48.82%, respectively, showing a dose-dependent trend (Table 1), indicating that QFs could inhibit tumor growth *in vivo*, which was consistent with the results of the *in vitro* cell experiment.

The RAS gene plays an important role in the occurrence and development of many tumors, and is involved in cell growth, proliferation, development, and differentiation, as well as other important physiological processes, such as malignant regulation. In addition, the RAS protein is also an important component of the epidermal growth factor receptor (EGFR) signaling pathway [30, 31]. The EGFR signaling pathway plays an important role in the formation of primary colorectal cancer foci and can transfer extracellular signals to the nucleus mainly through the mitogen-activated protein kinase (RAS-RAF-ERK) and phosphatidylinositol kinase (PI3K-PTEN-AKT), thus increasing the transcription levels of genes in the nucleus and subsequently regulating the important physiological processes, such as cell proliferation, differentiation, transformation, and apoptosis [32, 33]. The RAS/AKT/ERK signaling cascade is canonically activated by upstream receptor tyrosine kinases, which trigger RAS membrane recruitment and subsequent phosphorylation of both AKT and ERK effector arms. This dual-axis activation converges on shared downstream substrates that regulate c-Myc translation, cell cycle progression, and adjustment of the apoptotic threshold. Recent cancer studies have further clarified that the RAS/AKT/ERK cascade does not act in isolation: its sustained activation can reshape the tumor immune microenvironment by polarizing tumor-associated neutrophils toward a pro-tumor phenotype, while also accelerating epithelial-mesenchymal transition to support circulating tumor cell cluster formation [34]. These newly documented upstream-downstream links extend beyond intracellular signaling, revealing how oncogenic pathway rewiring drives intercellular and microenvironmental changes that promote post-treatment recurrence and distant metastasis, offering a new rationale for combinatorial therapies targeting both intracellular kinases and the surrounding tumor stroma. Currently, many targeted drugs are available that target the RAS/RAF/MEK/ERK signaling pathway [35, 36]. Accumulating evidence from recent pharmacological studies has demonstrated that natural flavonoids exert potent anti-colorectal cancer effects, with their pharmacological activity closely linked to targeted regulation of the MAPK/AKT signaling pathway [37, 38]. Jeong et al [39] proposed a series of new strategies to intervene in the RAS-ERK signaling pathway in the treatment of colon cancer by reducing the activity of proteins. Therefore, in this study, it

was important to clarify whether QFs could exert their anti-colon cancer effect by interfering with the RAS signaling pathway.

The occurrence of colon cancer is related to the sustained activation of the RAS protein. Therefore, the *in vitro* effect of QFs on RAS gene expression in CT26 colon cancer cells was examined. The results showed that the expression level of RAS mRNA in CT26 colon cancer cells decreased significantly in a dose-dependent manner in the low-, medium-, and high-dose QFs groups. Therefore, it was speculated that the inhibitory effect of QFs on the proliferation of CT26 colon cancer cells might be related to their inhibition of RAS gene expression or the expression of genes in its downstream signaling pathways. Subsequently, the expression levels of RAS, Akt, and ERK1/2 proteins and their phosphorylated forms in mouse tumor tissues were examined by Western blot analysis to further investigate the anti-colon cancer mechanism of QFs. It was found that the expression levels of RAS, phosphorylated Akt, and phosphorylated ERK1/2 proteins decreased significantly in a dose-dependent manner in the QFs-treated groups, indicating that QFs could downregulate the expression of downstream molecules of the RAS signaling pathway during activation of the RAS signaling pathway. Furthermore, the results of H&E staining also suggested that QFs could promote apoptosis in colon cancer cells.

CONCLUSION

QFs exhibit pharmacological activity against colon cancer *in vivo* and *in vitro*, and their mechanism may involve the RAS/AKT/ERK signaling pathway in regulating the expression of its downstream proteins. As for their specific targets, further research is needed. This study is expected to provide new insights for the development of anti-colon cancer drugs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2026.096.

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Appendix A. Supplementary data

Table S1 Primer sequences.

Gene name	Primer sequence ($5' - 3'$)
RAS	Forward: ATGACTGAGTATAAACTTGT Reverse: TCACATAACTGTACACCTTG
β-actin	Forward: TGTTACCAACTGGGACGACA Reverse: CTGGGTCATCTTTTCACGGT