Original Article

Flavonoids from tartary buckwheat induce G2/M cell cycle arrest and apoptosis in human hepatoma HepG2 cells

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The cytotoxicity and antioxidant activity on human hepatoma cell line HepG2 of three flavonoids homogenous compounds from tartary buckwheat seeds and bran, namely quercetin, isoquercetin, and rutin, were investigated. The total antioxidant competency detection results indicated that the antioxidant capacity of quercetin was the strongest in a biological response system. A [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay showed that quercetin exhibited the strongest cytotoxic effects against the HepG2 cell line. Flow cytometric analysis indicated that quercetin significantly increased the production of reactive oxygen species, and led to the G2/M phase arrest accompanied by an increase of apoptotic cell death after 48 h of incubation. Quercetin-induced cell apoptosis was shown to involve p53 and p21 up-regulation, Cyclin D1, Cdk2, and Cdk7 down-regulation. These results suggested that the induction of G2/M arrest, apoptosis, and cell death by quercetin may associate with increased expression of p53 and p21, decrease of Cyclin D1, Cdk2, and Cdk7 levels, and generation of reactive oxygen species in cells. This study will help to better understand and fully utilize medicinal resources of plant flavonoids.

Keywords buckwheat’s flavonoids; anticancer; ROS; cell cycle; p53

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. Although great progress has been made in the treatment of HCC, such as surgical management or non-surgical therapeutic modalities, these therapies are not sufficient for patients with advanced HCC. HCC is rarely curative, partly due to the comparatively small amount of antitumor drugs. Thus, to find a more effective therapeutic schedule becomes urgent. There has been much research to find novel and effective drugs for the treatment of HCC.

Recent interest has been focused on flavonoids that are now being considered as targeted anticancer drugs for the prevention and treatment of tumors.

Flavonoid compounds, also called bioflavonoids, belong to the plant secondary metabolites and exist widely in fruits, vegetables, grains, and other vegetable diets. It has been identified that there are more than 6000 kinds of natural flavonoids [1,2]. In Asian countries, flavonoids and its derivatives exist in all most medicinal plants and spices, and have often been used to prevent and treat various diseases. The flavonoids intake per person per day in China is far more than that in European and American nations.

There are many kinds of flavonoids found ubiquitously in plants and the biological activities of flavonoids cover a very broad spectrum including antioxidation, antiviral, anti-inflammatory, prevention of cardiovascular diseases, and anticancer properties [3–5]. Some researchers suggested that flavonoids are effective antioxidants that have a strong inhibitory effect on the proliferation of several human cancer cell lines [6,7]. However, there are no systematical reports on the antitumor effect of flavonoids of buckwheat. The mechanism has not been fully identified and their structure–activity relationships are still not well known.

Buckwheat is recognized as a health food, because it contains a rich supply of flavonoids, including rutin and quercetin, lipids, amino acids, and biologically active polysaccharides. Recently, it has gained more and more attention owing to its diverse activities, such as antioxidation, anti-inflammation, reducing the blood lipid, strengthening blood vessels, and regulating the blood sugar in the human body [8–10]. Previously, we obtained the extractive (mainly rutin) and its hydrolysis products (quercetin and isoquercetin) from tartary buckwheat bran and seeds [11,12]. Quercetin, isoquercetin, and rutin belong to flavones class of the flavonoids and their basic structure is quercetin as shown in Fig. 1. For quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), the molecular formula is C15H10O7, and the relative molecular mass is 302.23 Da. For its monoglycoside isoquercetin (quercetin-3-glucoside), the molecular formula is C21H20O12, and the
relative molecular mass is 464.38 Da. For diglycoside rutin (rutoside), the molecular formula is C_{27}H_{30}O_{16}, and the relative molecular mass is 610.52 Da. In this study, we evaluated the antitumor effect and the total antioxidant competency (T-AOC) of quercetin, isoquercetin, and rutin, trying to establish the foundation for further study of the antioxidant effect and antitumor mechanism of these flavonoid homologous compounds, which may provide experimental basis to fully utilize medicinal resources of plant flavonoids.

Materials and Methods

Materials

Three flavonoids, quercetin, isoquercetin, and rutin, were from tartary buckwheat bran and seeds, kept in our laboratory, and their purity is no <96%, respectively. They were dissolved in sterile dimethyl sulfoxide (DMSO) and stored at −20°C. The final DMSO vehicle concentration did not exceed 0.1% (v/v) either in control or in treated samples throughout the experiments. Quercetin, isoquercetin, and rutin were diluted to various concentrations with culture medium before each experiment.

Reagent

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and DAPI (4′, 6-diamidino-2-phenylindole 2hci) were purchased from Sigma (St Louis, USA). The annexin V-FITC apoptosis detection kit was purchased from BioVision (Mountain View, USA). Pancreatin (trypsin; 1 : 250) and DMSO were purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). The reactive oxygen species (ROS) detection kit was obtained from Beyotime Biological Technology Co., Ltd. (Shanghai, China). The T-AOC detection kit was purchased from Built Technology Co., Ltd. (Nanjing, China). The classical Total RNA isolation kit was from Bio Basic, Inc. (Ontario, Canada). Antibodies against p21, p53, Cyclin D1, Cdk2, and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Other chemical reagents were commercially available with analytical grade.

Cell proliferation assays

Effects on cell proliferation were analyzed using an MTT assay. Briefly, HepG2 cells (2 × 10^5 cells/ml) were seeded
in 96-well plates in a volume of 100 μl, and were allowed to attach for 24 h before treatment. Various concentrations (12.5, 25, 50, and 100 μM) of quercetin, isoquercetin, or rutin were added to wells and the plates were incubated for another 0–72 h. After cells were stained with 20 μl MTT (5 mg/ml) for 4 h at 37°C, culture medium was removed and 150 μl of DMSO was added and mixed for 10 min. Absorbance at 570 nm was measured with a microplate ELISA reader (Model 550; Bio-Rad, Hercules, USA). Wells containing culture medium with 0.1% DMSO only were used as controls. Mean values for three parallel experiments were calculated. Percentage of inhibition = (1 – mean absorbance of experimental sample/mean absorbance of control) × 100%.

Staining of apoptotic cells with DAPI

HepG2 cells from exponentially growing cultures were seeded in six-well plates and allowed to attach for 24 h before treatment. The cells were treated with or without (control, containing 0.1% DMSO) 100 μM quercetin, isoquercetin, or rutin for 48 h. After treatment, cells were washed with phosphate-buffered saline (PBS), and re-suspended in the fixation solution (4% paraformaldehyde) for ~10 min at 4°C. Cells were stained with DAPI (2 μg/ml) for 5 min at room temperature and then apoptotic cells were evaluated with a laser confocal microscope (FV1000; Olympus, Tokyo, Japan). Apoptotic cells were identified by nuclear condensation and fragmentation.

Flow cytometric analysis of cell apoptosis

The flow cytometric evaluation of annexin V-fluorescein isothiocyanate and propidium iodide (PI) cells was performed using the apoptosis detection kit according to the manufacturer’s protocol. Briefly, 1 × 10⁶/ml (500 μl) cells were treated with 100 μM quercetin, isoquercetin, or rutin for 48 h. Wells containing culture medium with 0.1% DMSO were used as controls. Cells were collected and washed twice with PBS. Cells were centrifuged at 1500 g for 5 min and resuspended in 500 μl of binding buffer, containing 2.5 μl of fluorescence-conjugated annexin V and 2.5 μl of PI, then incubated for 20 min in the dark at room temperature. Then cells were analyzed by flow cytometry (Beckman Coulter, Pasadena, USA) using the Cell Quest software (Becton Dickinson, San Jose, USA).

ROS assays

Intracellular ROS were measured using a DCFH (2', 7'-dichlorofluorescein) assay. Briefly, a stock solution of 1 mM DCFH-DA (2',7'-dichlorofluorescein–diacetate; Molecular Probes, Eugene, USA) was prepared in DMSO, and stored at −20°C. An amount of 100 μM DCFH-DA was freshly prepared by diluting the stock solution with medium for analysis. Cells cultured in the plates were exposed to culture medium containing 50, or 100 μM quercetin, isoquercetin, or rutin for 48 h. Cells were incubated with DCFH-DA for 20 min at 37°C and washed three times with serum-free medium to remove extracellular DCFH-DA. Then the cells were analyzed by flow cytometry. The fluorescence intensity was recorded for 30 min using a 488/525 nm excitation/emission filter.

Cell cycle analysis

A cell suspension at a density of 5 × 10⁴ per well was seeded in six-well plates. The cells were incubated at 37°C for 24 h and treated with 100 μM quercetin or isoquercetin for 48 h, respectively. Wells containing culture medium with 0.1% DMSO were used as controls. These cells were harvested and centrifuged at 1500 g for 5 min, washed with PBS, and centrifuged once again. After the treatment, adherent and floating cell populations were combined and fixed in ethanol (70% in PBS), washed with PBS, and then stained with 50 μg/ml PI and 50 μg/ml RNase A in PBS at room temperature in the dark for 30 min. Cell distribution in the different phases of the cell cycle was analyzed by flow cytometry using the ModFit LT V3.0 software (Verity Software House, Topsham, USA).

Reverse transcription–polymerase chain reaction

Total RNA in the cells after being treated with 100 μM quercetin for 48 h was extracted using a classical total RNA isolation kit according to the manufacturer’s instructions. The reverse transcription reaction was performed using oligo (dT) and AMV-RT reverse transcription enzyme (5 U/μl). The newly synthesized cDNA was amplified by polymerase chain reaction (PCR). The following primer pairs were used: for Cyclin D1 primers, 5'-CTGGATGCTGAGGTCGCG AGGA-3' (forward), 5'-CTGGCATTGGAGAGGAAG TGTT-3' (reverse); for p53 primers, 5'-GGCCACAGAGG AAGAGATC-3' (forward), 5'-GGCCAACCTTGTCGAG TGAG-3' (reverse); for β-actin primers, 5'-ATCCGCTGG ACACATG-3' (forward), 5'-TACAGTCTAGGTGGAG AACAG-3' (reverse); for Cdk2 primers, 5'-ATCCGCCCTTGG ACACACTG-3' (forward), 5'-CGCCCTTGGATGGGTGCTG TG-3' (reverse); for p21 primers, 5'-TTGATTAGCAG CGGAGAATC-3' (forward), 5'-GGCCAACCTTGTCGAG TGAG-3' (reverse); for GAPDH, as an internal control, 5'-GCCACTGTTCTCAGTGG-3' (forward) and 5'-TGTA CCACTCAAGGTGTTG-3' (reverse). Amplification cycles were: 94°C for 5 min, then 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by 72°C for 5 min. The amplified products were electrophoresed on 1.5% agarose gels, and PCR fragments were visualized by ethidium bromide staining. The gel image was taken under ultraviolet light.
Western blot analysis
To prepare the whole-cell extract, cells were washed twice with cold PBS, and lysed in cold radio immunoprecipitation assay extraction buffer [1 × PBS, 0.5% deoxycholic acid sodium salt, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethanesulfonyl fluoride, 1% leupeptin and 1% aprotnin] for 30 min on ice. The lysates were centrifuged at 12,000 g for 10 min at 4 °C, the supernatants were collected and protein concentration was determined by Bradford’s method. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis (20 μg/lane) and electroblotted onto nitrocellulose membrane. Membranes were incubated in the blocking solution consisting of 5% non-fat milk in Tris-buffered saline and tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20) for 1 h, then immunoblotted with the primary antibodies (rabbit anti-p53 antibody, anti-p21 antibody, anti-Cyclin D1 antibody, anti-Cdk2 antibody, and β-actin antibody), and subsequently incubated with an anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase. Bands were detected using an ECL enhanced chemiluminescence detection kit according to the manufacturer’s protocol.

Statistical analysis
Data were analyzed using SPSS 16.0 software (Chicago, USA). Statistical significance was determined using a one-way analysis of variance. \( P < 0.05 \) was considered statistically significant.

Results

Total antioxidant competency (T-AOC) determination
As shown in Fig. 2, the concentrations of quercetin, isoquercetin, or rutin were positively associated with the T-AOC values. With the increase of sample concentrations, the T-AOC values were also increased. The antioxidation of the same antioxidant is different in different systems. For the direct determination of quercetin, isoquercetin, or rutin solution (Fig. 2A), we found that the order of T-AOC was rutin > isoquercetin > quercetin. The antioxidant of quercetin was the lowest. After HepG2 cells were treated with quercetin, isoquercetin, or rutin for 48 h, we measured the T-AOC of cells and culture medium (Fig. 2B,C). Results showed that the order of T-AOC was quercetin > isoquercetin > rutin. In addition, results also showed that the T-AOC of cell culture medium was stronger than that of sample solution or cells, and the antioxidant capacity of quercetin was the strongest in a biological response system (Fig. 2D–F).

Inhibition of HepG2 cell growth
The effect of quercetin, isoquercetin, and rutin on cell growth was determined using the MTT assay. As shown in Fig. 3A–C, quercetin, isoquercetin, and rutin could inhibit HepG2 cell proliferation in the concentration range of 12.5–100 μM. With the increase of time and sample concentrations, the inhibition rate was increased, which was in a dose- and time-dependent manner. HepG2 cell growth was inhibited by ≥40% when incubated with 100 μM of quercetin for 72 h, while the inhibition of rutin was lower than quercetin or isoquercetin, i.e. 25%. With the same concentration and duration, quercetin showed stronger inhibition than isoquercetin or rutin. So the inhibition rate was quercetin > isoquercetin > rutin (Fig. 3D,E).

Staining of apoptotic cells with DAPI
To determine whether cell growth inhibition mediated by quercetin, isoquercetin, and rutin is associated with apoptosis, we used DAPI to investigate the changes of cell nuclei. As shown in Fig. 4, control cells showed homogeneous staining of the nuclei. In contrast, when cells were treated with 100 μM quercetin, isoquercetin, or rutin for 48 h, apoptotic cells showed irregular staining of their nuclei because of chromatin condensation and nuclear fragmentation, which were the most frequent findings in apoptotic cells.

Flow cytometric analysis of cell apoptosis
HepG2 cell apoptosis was analyzed by flow cytometry after treatment with or without 100 μM of quercetin, isoquercetin, or rutin for 48 h, respectively. Figure 5 shows the percentages of apoptotic cells that were undergoing early apoptosis and late apoptosis (including dead cells). Apoptotic HepG2 cells were 0.9% (0.3% + 0.6%) when the cells were not treated with drugs (control group), but 64.1% (30.2% + 33.9%), 38.3% (26.1% + 12.2%), and 15.8% (13.2% + 2.6%) when cells were treated with 100 μM quercetin, isoquercetin, or rutin for 48 h, respectively. It was important to note that when cells were treated with 100 μM quercetin for 48 h, most of HepG2 cells were apoptotic or dead, and the remaining living cells were only 34.1%, suggesting that quercetin showed a remarkable induction of apoptosis. Of note is that most of HepG2 cells with 100 μM quercetin or isoquercetin for 48 h were in the early stages of apoptosis, and the apoptosis rate is 30.2% or 26.1%, respectively. Late stages of apoptotic cells significantly increased after 48 h. In contrast, apoptosis rate in late stages increased obviously after for 48 h quercetin treatment. Rutin induced the apoptosis of HepG2 cells weakly. Together, these data indicated that quercetin and isoquercetin showed a remarkable induction of apoptosis, and quercetin showed a stronger apoptotic effect than isoquercetin or rutin under the same concentration and duration treatment of HepG2 cells. This result is in agreement with the data obtained by the MTT assay.

Detecting cell ROS
To investigate whether ROS is involved in flavonoid-mediated cell death, we also measured the level of ROS.
within the cells by using a ROS-sensitive fluorescence probe DCF. DCFH is oxidized in cells by ROS to form fluorescent DCF, which is an indicator of the level of ROS in cells. Thus, high fluorescence intensity is an indication of high ROS content. Figure 6 shows the ROS percentage in cells. The ROS content in control group cells (0.1% DMSO) was 14.4%. When HepG2 cells were treated with 50 \( \mu \text{M} \) or 100 \( \mu \text{M} \) quercetin, isoquercetin, or rutin for 48 h, the ROS content was 59.4% and 75.9% for quercetin, 57.1% and 67.8% for isoquercetin, and 41.8% and 63.9% for rutin, respectively. These results indicated that treatment with three flavonoids can trigger ROS production, change cell redox state, produce oxidative stress and lead to cell apoptosis of HepG2 cells. So ROS was considered to be an early signal of apoptotic cells. These results showed that quercetin, isoquercetin, and rutin could induce tumor cell apoptosis through regulating ROS content, and the ROS content in quercetin-treated cells was higher than those in isoquercetin- or rutin-treated cells. It suggested that the generation of ROS is an important mechanism that might contribute to the cytotoxicity of flavonoids in tumor cells.

**Effects on cell cycle progression**

The inhibition of cell growth could be a result of the induction of apoptosis that may be mediated by cell cycle arrest. However, conflicting results have been reported with regard to the stage-specific cell cycle arrest caused by flavonoids [13]. So we analyzed the distribution of HepG2 cells in...
different stages of the cell 24 h after exposure to certain concentration quercetin and isoquercetin. As shown in Fig. 7, treatment with 50 and 100 μM quercetin for 24 h led to an accumulation of HepG2 cells in S and G2/M phases with the concomitant decrease of the population of G1 phase. After HepG2 cells were exposed to 50 and 100 μM isoquercetin for 24 h, G2/M phase arrest was induced with the concomitant decrease of the population of G1 and S phase and increase of the population of S phase, respectively. Results of flow cytometry analysis showed that exposure of HepG2 cells to 50 or 100 μM quercetin and isoquercetin induced the G2/M phase arrest.

Effect of quercetin on the cell cycle regulation factors
First, mRNA levels of cell cycle regulation factors, including cyclin D1, Cdk2, Cdk7, p21, and p53, were detected by RT-PCR after treatment with 100 μg/ml quercetin for 24 h. As shown in Fig. 8A, Cyclin D1, Cdk2, and Cdk7 mRNA levels were decreased, p21 and p53 mRNA levels were increased, while GAPDH mRNA (as an internal control) did not change. Next, cell cycle-related protein expression in HepG2 cells was analyzed by western blot analysis. As shown in Fig. 8B, treatment with quercetin increased the expression level of p21 and p53 proteins and inhibited the expression level of cyclin D1 and Cdk2.

Discussion
Flavonoids as either simple or complex glycosides exist in many plants. Humans are estimated to consume ~1 g flavonoids per day. Several biological functions of flavonoids have been identified, however, their use in the pharmaceutical field...
and isoquercetin contains one glycosyl, so the T-AOC of rutin solution was higher than that of isoquercetin. When HepG2 cells were treated with three flavonoids for 48 h, we found that the order of T-AOC was quercetin > isoquercetin > rutin. We also showed that the T-AOC of cell culture medium was stronger than that of sample solution and cells. The reason for this may be that glycosyls are to substitute with hydrogen, which reduces the hydroxyl participating in reduction reaction. In general, hydroxyl into glycosyl brings positive and negative effects. Different experimental results may be due to its positive and negative effects of different sizes in different experimental system. So we conclude that the antioxidation of the same antioxidant is different in different systems. In biological response system, the antioxidant capacity of quercetin was the strongest.

MTT assay results showed that three flavonoids could specifically inhibit the growth of HepG2 cells in a time- and dose-dependent manner, and the inhibiting effect of quercetin was higher than that of isoquercetin or rutin. The appearance of nuclear condensation and apoptotic bodies were universal characteristics of cells undergoing apoptosis. By annexin V/PI staining, the high early apoptotic rate appeared in the quercetin- and isoquercetin-treated cells, which was higher than that of rutin-treated cells. Most of HepG2 cells with 100 μM quercetin or isoquercetin treatment for 24 h were in the early stages of apoptosis and late stages of apoptotic cells were significantly increased after 48 h. In contrast, early stages of apoptosis rate increased obviously after rutin treating for 48 h. These data indicated that quercetin and isoquercetin treatment showed a remarkable induction of apoptosis and the order is quercetin > isoquercetin > rutin.

ROS is an important intracellular signal of cell proliferation. Under normal circumstances, its production and resolution are in dynamic balance, and the redox state of the intracellular environment maintains a stable state. A great deal of research indicated that many antitumor drugs are closely related to the ROS initiation within tumor cells [19–21]. Such as antitumor drug arsenic trioxide (As2O3), polysaccharides, quinone anticancer drugs, and so on, they can produce a lot of ROS inducing tumor cell apoptosis [22]. It is worth noting that a lot of plant polyphenol compounds influencing ROS levels are similar to these antitumor drugs. In this study, we showed that three flavonoids could specifically inhibit the growth of HepG2 cell apoptosis. Therefore, increasing ROS levels and inducing HepG2 cell apoptosis may be an important mechanism of plant polyphenols playing antitumor function, which needs further study.

By reducing the intracellular antioxidant activity, flavonoid compounds could increase cell oxidative stress, lead to cell peroxidation damage, and cause the cellular peroxidation damage product to increase significantly [23,24]. The
Figure 5. Flow cytometric analysis of apoptosis  
(A) Control. (B–D) Cells were incubated with 100 μM quercetin, isoquercetin, or rutin for 48 h, respectively.

Figure 6. Flow cytometric analysis of intracellular ROS  
(A) Control. (B–D) Cells were incubated with 50 and 100 μM quercetin, isoquercetin, or rutin for 48 h, respectively.
function of antioxidase was important for the regulation of ROS and the proliferation, apoptosis, inflammatory reaction of cell life activities. ROS could selectively kill the cancer cells [25]. This may be the important mechanism of antitumor drugs to exert their effect.

In most cases, drugs exert their effects on multiple different targets. As is known to all, the cell growth and proliferation of mammalian cells are mediated via cell cycle progression. In recent years, studies have shown an association between cell cycle regulation and cancer, and inhibition of the cell cycle has become an important target for the management of cancer [26]. The data of cell distribution in the cell cycle in our study demonstrated that some procedure might be impeded to prevent the cells from entering into G2/M phase. It is suggested that quercetin might mediate growth inhibition of HepG2 cells by perturbation in G2/M phase of the cell cycle. The potency of the quercetin on the induction of cell cycle arrest was in agreement with the results of the cell growth analysis in this study. Many previous studies have also shown that flavonoids induce inhibition of growth via cell cycle arrest in the G2/M phase of different cancer cell lines, such as PC3, HL-60 leukemia, and many cell lines of digestive system cancer [27–29], which were inconsistent with the results obtained in our study. However, several studies have also indicated that the arrest of cycle in G0/G1 was induced by flavonoids in human leukemic T cells [30]. Therefore, the cellular phenomena referred to above may suggest that the cell cycle arrest caused by flavonoids is cell type and concentration-dependent.
Progression of the cell cycle is controlled by the actions of various types of cyclins and cyclin-dependent protein kinases (Cdks). Cyclin D1 is involved in the occurrence and development of tumor and is considered as a proto-oncogene. Cyclin D1 high expression is an important factor in the start and extension of RNA transcription, and also participates in the regulation of Cdk activity. Previous studies have shown that Cd7 plays a key role in the pathogenesis of tumor, inhibiting its function may inhibit the process of cell cycle. So Cd7 is regarded as a potential anti-cancer drug research target. p21 is present during G1 phase, while its levels are decreased during S phase but increased during G2 phase. Cell cycle arrest or apoptosis in response to DNA damage is mediated primarily by the p53 transcription factor. RT-PCR and western blot analysis indicated that up-regulation of p21 and p53 expression but down-regulation of cyclin D1, Cdk2, and Cdk7 expression occurred in HepG2 cells treated with quercetin. These results suggested that the induction of G2/M arrest, apoptosis, and cell death by quercetin may associate with increased expression of p53 and p21, decrease of cyclin D1, Cdk2, and Cdk7 levels, and generation of ROS in cells.

Taken together, this study has important guiding significance for screening excellent antioxidants and efficient anticancer drugs, and will help reveal the relationships between antioxidant and molecular structure, antitumor, and apoptosis. But the exact mechanism still needs further studies.

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