

RESEARCH ARTICLE

Luteolin Induced-growth Inhibition and Apoptosis of Human Esophageal Squamous Carcinoma Cell Line Eca109 Cells in vitro

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Abstract

Luteolin is a plant flavonoid which exhibits anti-oxidative, anti-inflammatory and anti-tumor effects. However, the antiproliferative potential of luteolin is not fully understood. In this study, we investigated the effect of luteolin on cell cycling and apoptosis in human esophageal squamous carcinoma cell line Eca109 cells. MTT assays showed that luteolin had obvious cytotoxicity on Eca109 with an IC_{50} of $70.7 \pm 1.72 \mu\text{M}$ at 24h. Luteolin arrested cell cycle progression in the G0/G1 phase and prevented entry into S phase in a dose- and time-dependent manner, as assessed by FCM. Luteolin induced apoptosis of Eca109 cells was demonstrated by AO/EB staining assay and annexin V-FITC/PI staining. Moreover, luteolin downregulated the expression of cyclin D1, survivin and c-myc, and it also upregulated the expression of p53, in line with the fact that luteolin was able to inhibit Eca109 cell proliferation.

Keywords: Luteolin - esophageal cancer - Eca109 - cell cycle arrest - apoptosis

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Introduction

Esophageal cancer (EC) is one kind of high incidence of upper digestive tract cancers worldwide. GLOBOCAN 2008 has reported an estimated 481645 new cases and 406533 deaths occurred for both sexes (Globocan, 2008). Esophageal squamous cell carcinoma (ESCC) is relatively rare in western world but is the most common type of EC in individuals with East Asian or African ancestry. Dietary habit and lifestyle play important roles in developing EC. Most epidemiological evidence has shown tobacco and alcohol have roles in this cancer (Oze et al., 2010; Gao et al., 2011), however, high consumption of vegetables and fruits could reduce the risk of EC (Mayne et al., 2001; Veugelers et al., 2006; Fan et al., 2008).

Flavonoids are important phytochemicals characterized by a common benzo- γ -pyrone chemical structure and found widely in different plants, especially in genus *Citrus* (Nogata et al., 2006; Rutaceae et al., 2006; Uehara et al., 2012). Flavonoids exhibit various beneficial properties in a multitude of disease states, including cardiovascular disease, neurodegenerative disorders and cancer (Gao et al., 2012; Master et al., 2012; McCullough et al., 2012). Luteolin, 3',4',5,7-tetrahydroxyflavone, is a common dietary flavonoid can be found in a variety of vegetables, fruits, and medicinal herbs (Sasaki et al., 2003). Luteolin has been shown to possess multiple biological activities such as anti-inflammation, anti-oxidant and anti-cancer

(Ashokkumar et al., 2008; Park et al., 2012). Among these activities, the anti-cancer effect of luteolin has been studied in vitro. Luteolin inhibits the proliferation of many types of cancer cells through regulating the cell cycle (Fotsis et al., 1997; Knowles et al., 2000; Ong et al., 2010; Cai et al., 2011). Moreover, luteolin also could induce tumor cells apoptosis including epidermoid carcinoma, leukemia, pancreatic tumor, lung cancer and hepatoma (Huang et al., 1999; Lee et al., 2002; Cheng et al., 2005; Lee et al., 2005; Chen et al., 2012). However, the vitro experiment of luteolin affected on human esophageal cancer cell line has not been intensely investigated. In the present study, we will investigate the anti-cancer potential of luteolin on human esophageal carcinoma cell line Eca109 cells and explore the possible molecular mechanisms. Data from our study expand the spectrum of the anti-cancer effect of luteolin and offer valuable clues to the in vivo study, which was necessary to be carried out in future.

Materials and Methods

Materials and reagents

Human esophageal squamous carcinoma cell line Eca109 was purchased from Shanghai cell bank of Chinese academy of sciences. Fetal bovine serum (FBS) and RPMI 1640 medium were purchased from Gibco, USA. Luteolin (Figure 1) was provided by the National Institute for the Control of Pharmaceutical and

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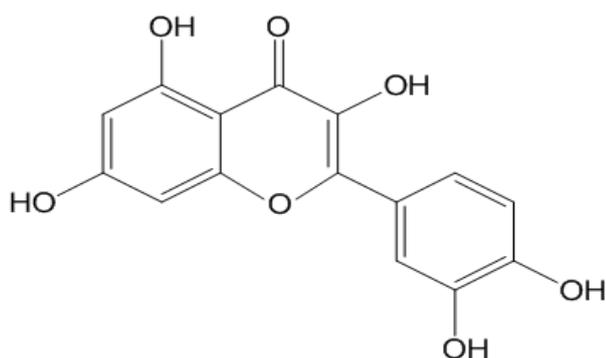


Figure 1. Chemical Structure of Luteolin (CAS No: 491-70-3)

Biological Products (Beijing, China) and dissolved in dimethylsulfoxide (DMSO) diluted with RPMI 1640, then filtered through a 0.22 μ m microfiltration membrane. MTT ([3-(4,5)-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 5-fluorouracil (5-FU) as positive control drug were both obtained from Sigma (St Louis, MO, USA). Cell cycle detection kit and Annexin V-FITC/PI apoptosis detection kit and Acridine Orange/Ethidium Bromide (AO/EB) staining kit were purchased from KeyGEN Biotechnology Co. Ltd. (Nanjing, China). M-MLV Reverse Transcriptase kit and primers were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). Human cyclin D1, survivin, c-myc and p53 ELISA Kit were provided by Science Biotechnology Co. Ltd. (Yantai, China).

Cell culture

The ESCC cell line Eca109 was cultured in RPMI 1640 medium supplemented with 10% FBS, 100U/mL penicillin and 100 μ g/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO₂.

MTT assay

Ten thousand Eca109 cells at logarithmic phase per well were seeded into a 96-well plate. After 24h incubation, when Eca109 were adhesive, the cells were exposed to a range of concentration of luteolin from 20 to 240 μ M for 24h, 48h, 72h respectively. The Eca109 cells treated with routine medium containing 0.1%DMSO served as the negative control group and 240 μ M 5-FU as positive control group. All exposures were performed in five wells. After treatment, 20 μ L MTT (5 mg/mL) was added into each well and the cells were incubated under standard condition for 4h. Then supernatant was discarded and 150 μ L DMSO was added to each well. The inhibitory effect was quantified using OD₄₉₀, which was read from a microplate reader. Triplicate parallel experiments were performed for each concentration. The rate of inhibition was calculated by the following equation: rate of growth inhibition (%) = $(OD_{\text{control}} - OD_{\text{treated}}) / OD_{\text{control}} \times 100\%$

The half maximal inhibitory concentration (IC₅₀) of luteolin on Eca109 cells was measured by MTT assay, which was conducted as described before (Sun et al., 2007), and was calculated by SPSS 19.0.

Cell morphological assessment

Eca109 cells were cultured in six-well plate with

RPMI-1640 medium until adherence. 0.1%DMSO, 40, 80, 160 μ M luteolin was added to different wells. The morphology of cells was observed under an inverted microscope after treated 24h.

Cell cycle analysis

Flow cytometry (FCM) was used to conduct cell cycle analysis. Eca109 cells treated with 0.1%DMSO and different concentration of luteolin for 24h was trypsinized and washed twice with PBS. Then cell density was adjusted at 1 \times 10⁶/mL with 1mL PBS and fixed in 70% cold ethanol overnight at 4°C. Adding 100 μ L RNase A (1 mg/mL) into the Eca109 cells, and keeping at 37°C for 30min. Before the analysis, 400 μ L of 10 μ g/mL propidium iodide (PI) was added into cells and then kept in dark for 30min. The percentage of the cells in each phase of cell cycle was measured by FACS420 and the results were analyzed using the software ModFit.

AO/EB staining

AO/EB staining was used to visualize nuclear change and apoptotic body formation. At the end of luteolin treatment for 24h, attached cells were trypsinized and washed with PBS. The concentration of cells was adjusted to 1 \times 10⁵/mL with PBS. AO and EB were mixed for 1:1 and added total 1 μ L into 25 μ L cells. Then, the Eca109 cells were observed under a fluorescence microscope at 510 nm.

Quantification of apoptosis analysis

Annexin V-FITC/PI apoptosis detection kit was used to detect and quantify the presence of apoptotic cells. The same groups of Eca109 cells as cell cycle analysis were harvested by trypsin without EDTA and washed twice with PBS. Each 5 \times 10⁵ cells were suspended by 500 μ L Binding Buffer, then mixed with 5 μ L Annexin V-FITC and 5 μ L PI. All the experimental groups were kept in dark for 5-15min at room temperature until detected and quantified by FCM.

RNA extraction and semi-quantitative RT-PCR

Total RNA was isolated from Eca109 treated with 0, 40, 80, 160 μ M luteolin using Trizol Reagent according to the manufacturer's recommended protocol. The RNA purity and concentration was determined by eppendorf Biophotometer and RNA quality was identified using electrophoresis. Two micrograms total RNA was used as template to synthesis cDNA with M-MLV Reverse Transcriptase kit. PCR was started by heating at 94°C for 5 min, followed by 35 cycles at 94°C for 30s, 53°C (for cyclin D1) or 56°C (for c-myc) or 58°C (for survivin) or 57°C (for p53) for 30s, and 72°C for 45s, with a final extension at 72°C for 10 min. Forward and reverse primer sequences for cyclin D1 were 5'-CTTCAAATGTGTGCAGAAGAG-3', 5'-GCATTTTGGAGAGGAAGTGTTTC-3' respectively. Forward and reverse primer sequences for c-myc were 5'-GGCTCCTGGCAAAGGTCA-3', 5'-AGTTGTGCTGATGTGTGGAGA-3' respectively. Forward and reverse primer sequences for survivin were 5'-GGACCACCGCATCTCTACAT-3', 5'-TCTCCGCAGTTTCCTCAAAT-3' respectively.

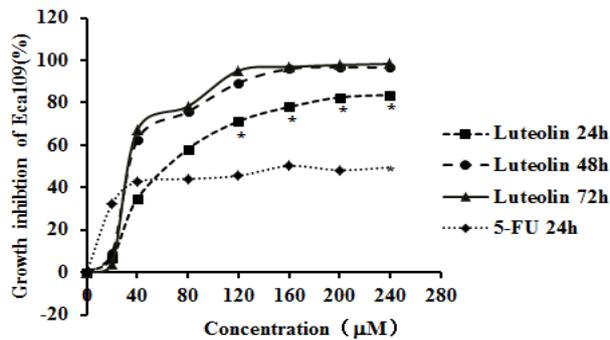


Figure 2. Luteolin Inhibited Growth of Eca109 Cells.

1×10^5 cells seeded into per well of 96-well plates were treated with 0, 20, 40, 80, 120, 160, 200, 240 μM luteolin for 24, 48h, 72h and the same concentrations of 5-FU for 24h. The results expressed as means \pm S.D. The mean of tree parallel experiments (quintuplicate wells) for each group were shown. Statistical differences between the 5-FU and luteolin at 24h were determined by Student's two-tailed non-paired t-test. Compared to 5-FU, luteolin could significantly inhibited growth of Eca109 at the concentration of 120 μM , 160 μM , 200 μM , 240 μM . * $P < 0.05$ versus control

Forward and reverse primer sequences for p53 were 5'-AAGTCCAAAAGGGTTCAGTCT-3', 5'-CCCAAACATCCCTCACAGTAA-3' respectively. Forward and reverse primer sequences for β -actin were 5'-GGCGGCACCACCATGTACCCT-3', 5'-AGGGGCCGGACTCGTCATACT-3' respectively. The lengths of the PCR products of cyclin D1, c-myc, survivin, p53 and β -actin were 374bp, 113bp, 344bp, 328bp and 202bp respectively. β -actin was used as an internal control, and the expression status of and the expression status of cyclin D1, c-myc, survivin and p53 c-myc, survivin in different groups would be known by gray ratio.

ELISA assay

ELISA assay was used to detect the concentration of cyclin D1, survivin, c-myc and p53. After treated with luteolin for 24h, attached Eca109 cells were trypsinized and washed with PBS. The concentration of cells was adjusted to $5 \times 10^5/\text{mL}$ with PBS and cells were lysed by tissue crushing equipment at 70Hz for 90s. The next experimental procedure was carried out according to the instruction of ELISA kit.

Statistical analysis

The results were expressed as mean \pm S.D. Statistical differences between the control and treated samples were determined using one-way ANOVA followed by Dunnett-t test using SPSS 19.0 software. For PCR, Quantity one software was used to determine gray value between control and treatment groups. $P < 0.05$ was considered statistically significant. Figure 2, 4C and 5 were drawn by Excel 2010.

Results

Luteolin inhibited the growth of Eca109 cells

According to the results of MTT assay, the mean IC_{50} of luteolin was $70.68 \pm 1.72 \mu\text{M}$. From 20 to 240 μM of luteolin, the effect of growth inhibition was enhanced along with the increasing concentration. Moreover,

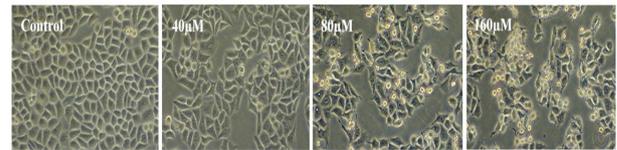


Figure 3. Micrograph of Eca109 Cells Treated with Different Concentrations of Luteolin. The morphology of Eca109 cells was dramatically altered with increasing concentrations (0, 40, 80, 160 μM) of luteolin treatment for 24h. Morphological characteristics of apoptotic cells, such as chromatin condensation and membrane blebbing. Cells were observed using inverted microscopy (100 \times)

Table 1. The Cell Cycle Distribution of Eca109 Treated with Luteolin

Cell cycle(%)	Groups			
	Control	Luteolin (40 μM)	Luteolin (80 μM)	Luteolin (160 μM)
G0/G1	56.57 \pm 4.79	61.29 \pm 1.18	65.27 \pm 2.79*	72.27 \pm 2.81*
S	34.59 \pm 2.68	28.16 \pm 1.10*	25.87 \pm 0.91*	18.61 \pm 0.79*
G2/M	8.77 \pm 2.10	10.55 \pm 1.15	8.86 \pm 2.00	9.11 \pm 2.05

Luteolin increased the percentage of G0/G1 phase and decreased the cells in S phase accordingly; * $P < 0.05$ versus control

the inhibition ratio was increasing with the extended intervention time. The exhibition of dose-response relationship could be observed (Figure 2). 5-FU used as positive control, the same density of cells exposed 5-FU and luteolin after 24h respectively, the proliferation inhibitory effect of luteolin was more greater than 5-FU.

Luteolin influence the morphological character

Compared to the control cells, Eca109 cells exposed to increasing concentration (0, 40, 80, 160 μM) of luteolin treatment for 24h presented typical apoptotic morphology with cell shrinkage, nuclear condensation and pseudopodia. Budding shape also could observed on cells partially treated with high concentration luteolin and the structure of internal cells was fuzzy (Figure 3).

Luteolin interrupted the progression of cell cycle

The effects of luteolin on the cell cycle progression in Eca109 cells were determined by flow cytometry with PI staining. The phases distribution of cell cycle indicated that luteolin could increase the percentage of Eca109 cells in G0/G1 phase and prevent cells from entering into the S phase. As shown in Table 1, Eca109 cells treated with 80 μM , 160 μM luteolin resulted in a statistically significant increase in the G0/G1 phase that was accompanied by a decrease in the S phase. The percentage of S phase decreased by 46.2% on treatment of Eca109 cells with 160 μM luteolin compared with control.

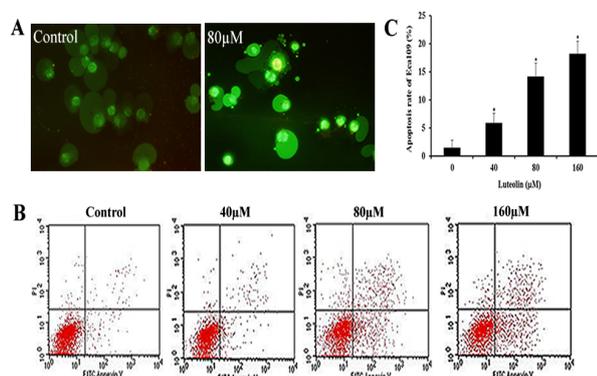
Luteolin induced cellular apoptosis in a dose dependent manner

To examine the type of cell condition induced by luteolin, Eca109 cells were stained with AO/EB, which could identified viable, apoptotic, and necrotic cells based on color and appearance. Under this circumstances, viable cells appear green with intact nuclei, early apoptotic cells appear green with bright green dots as a consequence of chromatin condensation and fragmentation, late apoptotic

Table 2. The Concentration of Cyclin D1, Survivin, c-myc and p53

Genes	Groups			
	Control	Luteolin (40 μ M)	Luteolin (80 μ M)	Luteolin (160 μ M)
cyclin D1(ng/mL)	4.38 \pm 0.10	3.98 \pm 0.25	3.96 \pm 0.20	3.86 \pm 0.19*
survivin(ng/L)	232.03 \pm 10.06	229.38 \pm 22.41	219.77 \pm 8.67	210.16 \pm 24.88
c-myc(pg/mL)	698.80 \pm 69.20	591.18 \pm 45.94	533.09 \pm 93.94	481.84 \pm 72.30*
p53(pg/mL)	1150.5 \pm 173.17	1114.96 \pm 67.50	1389.95 \pm 175.50	2239.11 \pm 80.35*

The gene expression was altered by luteolin; Except survivin, luteolin decreased the concentration of cyclin D1 and c-myc, however, it increased the concentration of p53; Data represent means \pm SD of triplicate experiments, *P<0.05 versus control

**Figure 4. The Luteolin Induced Apoptosis of Eca109.**

(A) After AO/EB staining, viable cells appear green with intact nuclei, early apoptotic cells appear green with bright green dots, late apoptotic cells nuclei stain orange with condensed and fragmented ($\times 200$). (B) The FCM of luteolin induced apoptosis of Eca109. The right lower quartile represent the viable apoptotic cells, the right upper quartile represent the non-viable apoptotic cell and non-viable non-apoptotic cells. (C) The percentage of apoptosis cells with treated luteolin. The data shown represent averages of three fields, means \pm S.D. *P<0.05 versus control

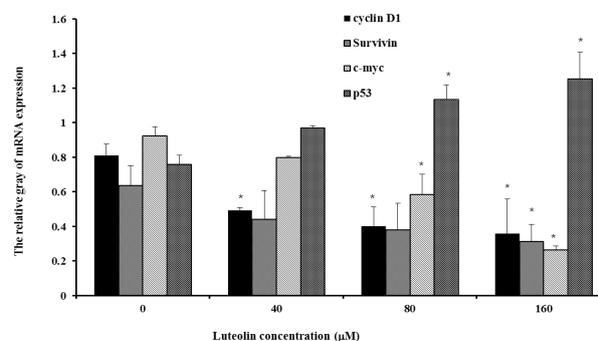
cells are easily distinguished from other cells because the nuclei stain orange with condensed and often fragmented nuclei. In contrast, necrotic cell nuclei stain orange but without condensation or fragmentation. Staining with AO/EB of the samples treated with luteolin for 24h (Figure 4A) showed normal and apoptotic cell cells.

Apoptosis induced by luteolin was further evaluated using the Annexin V-FITC/PI staining assay. Results showed luteolin appeared to be potent apoptosis on Eca109 cells, and the apoptotic effects was found to be dose-dependent (Figure 4B). The percentage of apoptosis cells increased with the concentration of luteolin (Figure 4C).

Luteolin led to alterations in the expressions of genes

After establishing luteolin led to cell cycle arrest at G0/G1 and induced apoptosis, we sought to investigate the underlying molecular mechanism. To address whether the alteration of cyclin D1, survivin, c-myc and p53 protein levels were due to the suppression of gene expression, we first checked the mRNA level of cyclin D1 survivin, c-myc and p53 using semi-quantitative RT-PCR. Compared to the control, the cyclin D1, survivin, c-myc mRNA levels of cells were decreased and the p53 mRNA level was increased suggesting that luteolin could regulate relevant genes.

To establish whether luteolin-altered expression of genes, Eca109 cells were pretreated with 40, 80,160 μ M for 24h. As shown in Table 2, the high-dose groups of

**Figure 5. Effect of Luteolin on Expression of Survivin, Cyclin D1, c-myc and p53 mRNA in Eca109 Cells.**

The relative gray values of cyclin D1/ β -actin, survivin/ β -actin, c-myc/ β -actin were decreased and p53/ β -actin was increased after treated with 40, 80, 160 μ M luteolin. The data shown represent averages of three fields, means \pm S.D. *P<0.05

luteolin decreased the expression of cyclin D1 and c-myc to the control level. The concentration of cyclin D1 and c-myc was significantly reduced to 3.86 \pm 0.19ng/mL and 481.84 \pm 72.30pg/mL, respectively. Also, the group of 160 μ M luteolin increased the protein level of p53. The concentration of p53 were significantly increased to 2239.11 \pm 80.35 pg/mL.

Discussion

Carcinogenesis, a long-lasting and multi-stage process, is characterized by clonal expansion of mutated cells. It's necessary to discover efficient pathway to impede the ectopic proliferation and accelerate death of tumor cells. Although, broad-spectrum anticancer drug such as 5-FU (a pyrimidine analogue) is widely used for clinical therapy (Gehoff et al., 2012; Pera et al., 2012; Su, 2012), the large side effects which it has been shown are a still limit aspect for use. Luteolin, a natural dietary flavonoid isolated from various plants has the potential role to slow or prevent the growth of tumor cell lines (Ko et al., 2002; Ju et al., 2007; Lim, et al., 2007; Chiu et al., 2008). In our in vitro study, we found luteolin could set back the proliferation of esophageal cancer cell line Eca109 and meanwhile it made the tested cells to be apt to apoptosis.

MTT assay (methyl thiazolyl tetrazolium) was a simple and rapid method widely used for assessment of cytotoxicity, viability, and proliferation studies in cell biology (Mosmann, 1983). In our study, the results of MTT assay indicated that luteolin could inhibit proliferation of Eca109, which exhibited dose-response and time-dependent relations in the specific scope (Figure 2). In addition, the cytotoxicity of luteolin was more intensively than 5-FU when cells treated with luteolin and 5-FU for

24h respectively.

It is well known that the growth and proliferation of cancer cells are mediated via cell cycle progression which consists of four distinct phases, G1, S, G2, and M (Massague, 2004). The inhibition of the cell cycle has become an appreciated target for management of cancer (Schwartz et al., 2005). Data in our study showed luteolin could inhibit the proliferation of Eca109 by arresting the G1/S phase (Table 1). Many published studies have also shown that luteolin induce inhibition of growth via cell cycle arrest in the G1/S phase of different cancer cell lines, such as HK1 and CNE2 nasopharyngeal carcinoma cells (Ong et al., 2010), HT-29 human colon cancer cells (Lim et al., 2005) and OCM-1 melanoma cells (Casagrande et al., 2001). Cell cycle progression is timely regulated by cyclin-dependent kinases (CDKs) (Massague, 2004). It has been well established that cyclin D plays a crucial role in the progression of cell cycle from G1 to S phase and the down-regulation of cyclin D will lead to cell cycle arrest at G1 (Malumbres et al., 2009). In the present study, we defined the critical role of cyclin D1 in luteolin-induced cell cycle arrest.

The decrease in cell proliferation and cell viability after treated with luteolin can be related not only to the effect of arresting cell cycle, but also to the induction of apoptosis. Therefore, we determined the capacity of luteolin to induce apoptosis of Eca109. Firstly, apoptosis identification was done by analysis of morphologic alterations, since its advantages such as simplicity, low cost and precision (Doonan et al., 2008). AO/EB are vital dyes that distinguish live, apoptotic and dead cells. AO could stain the nuclei of viable and apoptotic cells, however, EB stains only cells that have lost membrane integrity (Sivagami et al., 2012). The results of staining showed in our study live cells appeared uniformly green and apoptotic cells stained yellow-green with bright dot in the nuclei as a consequence of chromatin condensation (Figure 4A). However, death cells which have not appeared in graph because they only account for 3.15% in total. Secondly, apoptosis was further evaluated using the Annexin V-FITC/PI staining by FCM. Compared to the control, the number of apoptotic cells which treated with luteolin was increased. Our study demonstrated that the treatments with luteolin resulted in the apoptosis of Eca109 cells. These results corroborate the findings of many published studies in several other types of human carcinoma cells (Choi et al., 2011; He et al., 2012; Kim et al., 2012).

Survivin is an inhibitor of apoptosis, expressed in nearly all human cancers but not in most normal tissues (Altieri, 2003). Overexpression of survivin is a common event in esophageal cancer (Kato et al., 2001). It has reported that survivin mRNA expression can be considered a biomarker, reflecting the prognosis of squamous cell carcinoma but not adenocarcinoma in esophageal cancer, independent of tumor stage (Ikeguchi et al., 2002; Ikeguchi et al., 2003; Rosato et al., 2006). Our results showed that luteolin could downregulate the mRNA expression of survivin in Eca109 which indicated luteolin may improve the prognosis of ESCC. However, the protein levels of survivin are comparable between the luteolin treated and

control groups (Table 2). It is possible because 24 hours for intervention time are not long enough to see a change in protein level. Previously, other researchers have found that baohuoside-I which is one of flavonoids could inhibit cell growth and downregulate survivin expression in esophageal carcinoma via β -catenin-dependent signaling (Wang et al., 2011).

Overexpression of the c-myc gene has been found to be associated to the carcinogenesis, development and bad prognosis in a variety of tumors (He et al., 1995; Komiya et al., 2010; Joensuu et al., 2011). Moreover, previous reports have shown c-myc gene was amplification and overexpression in esophageal cancer cell lines (Jones et al., 1993; Kanda et al., 1994). Although, it has reported luteolin could not inhibit myc expression in both cervical and colorectal cancer cells (Shi et al., 2004), in our study, luteolin downregulated the mRNA level of c-myc and decreased the protein expression of c-myc on Eca109 cells.

As a tumor suppressor gene, p53 plays a critical role in the maintenance of genomic stability and regulation of cell proliferation and apoptosis (Levine, 1997; Vogelstein et al., 2000). The level of p53 protein increases in condition of cellular stresses such as DNA damage, nucleotide imbalance or oxidative damage, as well as in relation to various forms of oncogene imbalance (Gorgoulis et al., 1998; Prives et al., 1999). In our study, increased mRNA levels of p53 and overexpression of p53 protein indicated luteolin could induce apoptosis of Eca109 cells. It is reported p53 was necessary for other factors such as c-myc. Therefore, the downregulation of c-myc in our research might be associated with overexpression of p53 which exhibit anti-tumor activity on Eca109 cells. Ma et al has shown isorhamnetin, another member of the flavonoids, could inhibit proliferation and enhance apoptosis of Eca-109 cells by expressions of c-myc downregulated whereas p53 upregulated (Ma, 2007). It strengthened the evidence that flavonoid components possess antiproliferative potential.

In conclusion, our study showed that luteolin inhibited proliferation of human esophageal carcinoma cell line Eca109 by arresting cell cycle and inducing apoptosis in vitro. Moreover, luteolin downregulated or upregulated the expression of the cell cycle, apoptosis and tumor related gene such as cyclin D1, survivin, c-myc and p53 respectively. Although the deep mechanism of the effects of luteolin are still poorly understood, the in vitro findings in our study provide a significant basis for future study in vitro and in vivo.

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