Research Article

Cytotoxicity and Apoptosis Induction of 7- Hydroxycoumarin on SW480 Colon Cancer Cell Line

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Abstract

Introduction: Colorectal cancer is a common disease ranked as the third-highest deathly cancer worldwide. In recent years, for the treatment of cancers scientists have preferred herbal remedies because of their better effects and fewer side effects. So, in this study, the cytotoxicity effect and apoptosis induction of 7-hydroxycycoumarin were investigated on the SW480 colon cancer cell line .

Methods: anti-proliferative effect of 7-hydroxycoumarin was investigated using Trypan blue and MTT assay at different concentrations on SW480 cell line. Additionally, apoptosis induction was examined by flow cytometry and real-time PCR .

Results: The results show a decrease in viability. IC50 concentrations were observed at the concentrations of 164.2, 80.48, and 41.97 µg/ml for 24, 48, and 72 h, respectively. Of note, IC50 concentrations were not observed in the trypan blue assay. As well, there was a 2.4-fold increase in apoptosis (P<0.001) using Annexin / PI kit by flow cytometry. as well as the increased expression of BAX/BCL2 ratio of about 13.69, which was observed at 100 µg/ml compared to the control .

Discussion: The results indicate that 7-hydroxycoumarin could inhibit cell proliferation and induce apoptosis in the SW480 cell line. Therefore, 7-hydroxycycoumarin can be considered as an anti-cancer agent in the complementary treatment of colon cancer.

***Keyword*:** Colon cancer, 7- hydroxycoumarin, cytotoxicity

Introduction

Cancer is one of the most important human diseases in most countries and is the third leading cause of death in Iran (Rafiemanesh *et al.*, 2016). Cancer usually results from defects in the regulatory mechanisms of cell growth, division, and apoptosis, which are due to genetic damage that is often caused by chemicals, hormones, and sometimes by viruses. (Giovannucci, 2002; Issa and Noureddine, 2017). In addition, epidemiological studies confirmed that colon cancer is more likely to be affected by

environmental factors, rather than genetic factors. (Issa and Noureddine, 2017). Nowadays, finding novel cancer treatment strategies is a global challenge. Despite many scientific advances in identifying the causes of cancer, still there is no specific treatment for these patients. (Weston *et al.*, 2003; Issa and Noureddine, 2017). Many chemotherapy drugs have been developed over the past two decades for the treatment of cancer patients, but they have major drawbacks as side effects such as the high toxicity that damages healthy cells and drug resistance in cancer cells (Giovannucci, 2002). Recent efforts have mostly focused on the use of high-potential drugs with no effects similar to those of chemotherapeutic drugs, among which medicinal plants are important in the treatment of cancer cells (Dillard and German, 2000; Lagoa *et al.*, 2020) and Introducing drugs used in traditional medicine, especially medicinal herbs, is a good starting point for developing research projects in this regard, in order to achieve new drugs for cancer treatment. Coumarins are a class of secondary metabolites and naturally occurring benzopyrone derivatives. Some previous studies, especially animal studies, have shown that coumarin has antioxidants and anti-cancer effects (Küpeli Akkol *et al.*, 2020; Önder, 2020). Moreover, several studies showed that coumarin 1 and 2-benzopyrene forming the basis of coumarins or -7-hydroxy coumarin, could inhibit cancer cell proliferation (Marshall *et al.*, 1994). Coumarin derivatives exert their anticancer effects by inhibiting aromatase, carbonic anhydrase, and steroids sulfatase. Additionally, these could evaluate the cytotoxic potential of these compounds in different cancer cell lines to evaluate their anticancer potential (Önder, 2020). In the present research, the cytotoxicity effect and apoptosis induction of 7-hydroxy coumarin, which is one of the phenolic compounds derived from green plants, have been studied as anticancer agents.

Materials and Methods

Chemicals and reagents: Coumarin(cat≠820668), DMEM culture medium, fetal bovine serum, penicillin, streptomycin, MTT powder, 3-(4.5-d-methyl tis-azole-2-yyl)-2.5-d-phenyltetrazolium bromide, PBS, and dimethyl sulfoxide were obtained from Sigma Co. (Sigma, St. Louis, MO, USA) and Apoptosis was examined using the Annexin-V-FLUOS PI kit (Sigma-Aldrich). Total RNA was extracted by the Super RNA Extraction kit for Tissue & Cells (cat: YT9080). cDNA was synthesized using a cDNA synthesis kit (cat: YT4500). Gene expression was assessed by the Green SYBR Master MIX qPCR kit (amplicon).

Cell culture and treatment: Colon cancer cell line SW480 was obtained from the Pasteur Institute of Iran and then transferred to the laboratory. Thereafter, the SW480 cell line was cultured in a DMEM medium with FBS 10% and incubated at 37ºC, 96% humidity, and 5% CO_2. Cell lines were treated with different concentrations of 7-hydroxy coumarin at 25, 50, 100, and 250 µg/ml, and the untreated group was selected as the control.

Trypan blue assay: Living cell membranes do not allow non-electrolyte dyes to enter the cell, but dead cells can be well-stained under this condition. To determine viable cell activity, 0.1 ml of cell suspension including control cells and treated cells were incubated with different concentrations of -7-hydroxy coumarin and 0.1 ml of trypan blue solution (0.1% w/v in 0.15 M in PBS) (Sigma USA). In a cavity of a 96-well plate, the cells were immediately stained to determine the number of the stained (dead) and non-stained cells (living cells). Thereafter, the percentage of vital activity and cytotoxicity were calculated using the formulas below:

$$\% \text{ Viability} = (\text{viable cells}/\text{Total number of cells counted}) \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \% \text{ viable cells}$$

MTT assay: MTT assay is a colorimetric assay used for the evaluation of cytotoxicity of cells. 1×10^4 cells were cultured in a 96-well plate and then treated at the defined concentrations for 24, 48, and 72 h. After the period of treatment

by removing the supernatant in each well, 80 µl DMEM without phenol and 20 µl of MTT or tetrazolium dye solution were added into each well and the plate was then incubated for 4 h at 37ºC. Following the incubation, 100 µl of DMSO was added to each well and measured at 570 nm by ELISA reader. The experiments of this study were performed in triplicate and both cell viability and cytotoxicity were calculated based on the following formulas:

%Cytotoxicity = 1- (mean absorbance of treatment / mean absorbance of control) × 100
% Viability = 100 -% Cytotoxicity

Apoptosis Assay: The percentages of apoptosis and necrosis were evaluated using the Annexin-V-FLUOS PI kit. For this purpose, SW480 cells were cultured at 1×10^5 density, then the cells were treated for 48h at concentrations IC50 selected from the results of the trypan blue staining and MTT assay. The cells were collected after the treatment and washed twice with PBS. Thereafter, PI and Annexin-V were added to the cell suspension in the binding buffer. After the incubation for 15 min at 37°C in the dark, they were analyzed using a flow cytometer (BD Biosciences FACS Calibur TM).

RNA Extraction, cDNA synthesis, and qRT-PCR Assays: Total RNA was extracted from the treated and untreated cells using Super RNA Extraction kit for Tissue & Cells (cat: YT9080).) and purity of RNA was evaluated by NanoDrop instrument (Thermofisher). Moreover, cDNA synthesis was performed using a cDNA synthesis kit (YT4500). Real-time PCR was also conducted to evaluate changes in the gene expression using the SYBER Green qPCR Master MIX (amplicon) kit by an ABI step-one system. The sequences of primers of Bax and Bcl2 (Sinaclon, Iran) and housekeeping genes are shown in Table 1. As well, the analysis of gene expression was done with $2^{-\Delta\Delta Ct}$ (Livak).

Statistical analysis: The results were analyzed by SPSS (SPSS, Inc., Chicago, IL, USA), two-way ANOVA, and Tukey's tests, and $p < 0.05$ was considered as the statistical significance level.

Table 1. Primer sequences for real-time reverse-transcription PCR analysis

Gene	Sequence	Product Size (bp)
Bax	5´TTGCTTCAGGGTTTCATC CAG3´ 5´AGCTTCTTGGTGGACGCA TCC3´	169
BCL2	5´TGTGGATGACTGAGTAC CTGAACC3´ 5´ CAGCCAGGAGAAATCAAA CAGAG3´	170
GAP DH	5´ CCACTCCTCCACCTTTGAC GCT3´ 5´ TTACTCCTTGGAGGCCATG TGG3´	139

Results

Evaluation of cell viability by trypan blue staining: The results of the trypan blue assay showed that 7-hydroxy coumarin caused cell death in SW480 colon cancer cells. As shown in Figure 1, the viability of SW480 cancer cells decreased at all the studied concentrations in the defined time. Notably, the highest viability rate was observed at 25 µg/ml 90.64±1.93% during 24 h treatment and the lowest viability was at 250 µg/ml 67.72%±1.67% after 72 h treatment with 7-hydroxy coumarin. The percentage of IC50 in SW480 cancer cell line was not detected in trypan blue by passing 24, 48, and 72 h.

Evaluation of cytotoxicity by MTT assay: The results of the MTT assay showed increased cytotoxicity induced by 7-hydroxy coumarin at the defined concentration for 24, 48, and 72h ($P<0.0001$). As shown in Figure 2, at all the studied concentrations and in the defined time, the viability rate of SW480 cancer cells has reduced. The highest viability rate was observed in 25 µg/ml as about 77.43±%2.48 after 24 h and the lowest viability was reported

at 250 µg/ml as about 16.82±1.79% after 72 h with 7-hydroxy coumarin. IC50 levels of SW480 cancer cell line in MTT assay were reported as 164.2, 80.48, and 41.97 µg/ml, after 24, 48, and 72 hours, respectively.

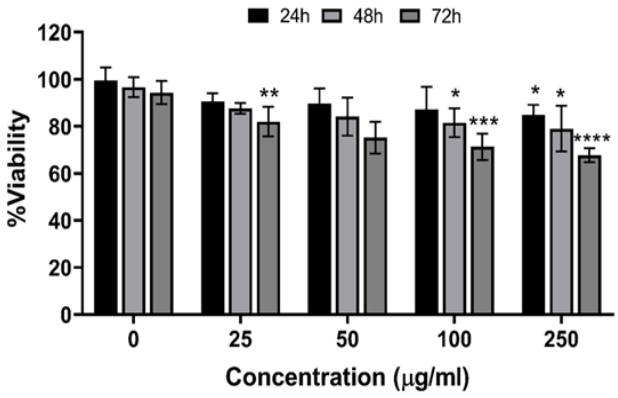

Figure 1: Comparison of viability rates of SW480 cell line following the treatment with 7-hydroxy coumarin using Trypan blue test during 23,48 and 72 h. (*P < 0.05, **P < 0. 01, and ***P < 0. 001compared with the control group).

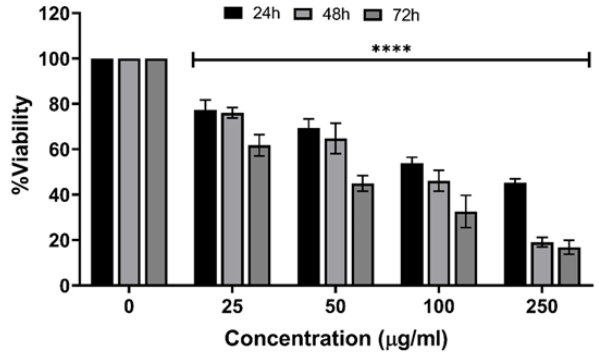

Figure 2: Comparison of viability rates of SW480 cell line following the treatment with -7-hydroxy coumarin using MTT assay during 48 h. (****P< 0.0001 compared with the control group).

Results of Apoptosis Assay with Annexin/PI Kit: According to the results of Trypan Blue and MT assay tests, IC50 concentration at 100 µg/ml for 48 hours was selected for the evaluation of apoptosis using Annexin/PI kit by flow cytometry in SW480 cancer cell line. As shown in Figures 3 and 4, there was a significant increase in apoptosis percentage as about 16.46±1.9% in the treatment group with 100 µg/ml compared to the control group 6.85±0.69 (P<0.001). As well, a significant decrease was observed in viable cells in the group treated by 100 µg/ml compared to the control group (P<0.05)

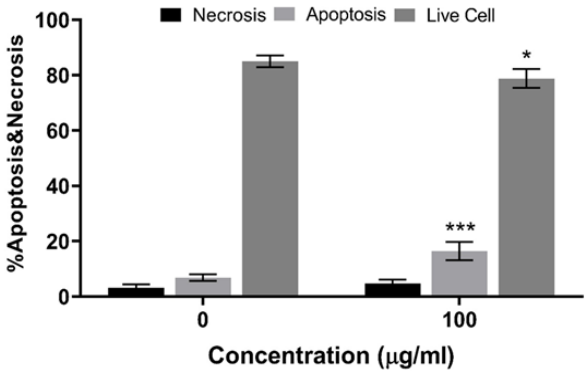

Figure 3: Comparison of apoptosis and necrosis percentages in SW480 cell line following the treatment with -7-hydroxy coumarin during 48 h. (*P < 0.05, ***P< 0.001 compared with the control group).

Gene Expression assay: As shown in Figure 5, the reduced expression of *BCL2* was observed in the 100 µg/ml group as about 0.401±0.072 compared to the control group (P=0.178). Furthermore, the increased *BAX* expression was reported in the 100 µg/ml group as about 5.5±1.03 compared to the control group (P<0.05). As shown in figure 6, the *BAX*/*BCL2* ratio has increased about 13.69±0.142 in the group treated with 100 µg/ml compared to the control group.

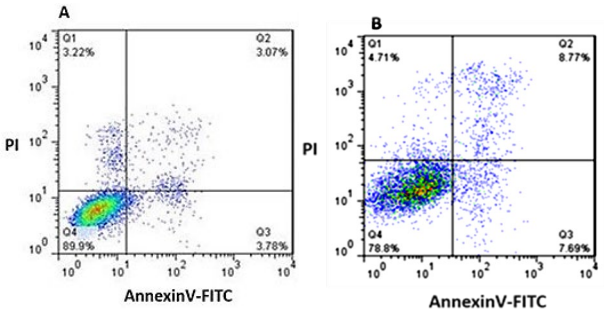

Figure 4: Percentage of cells in different phases of Annexin-V/PI test in SW480 cell line following the treatment with -7-hydroxy coumarin during 48 h. (A: control, B: 100 µg/ml). Q1 shows necrotic cells An^-/pI^+, Q2 shows delayed apoptotic cells An^+/pI^+, Q3 shows primary apoptotic An^-/pI^-, and Q4 shows the living cells of An^+/pI^-.

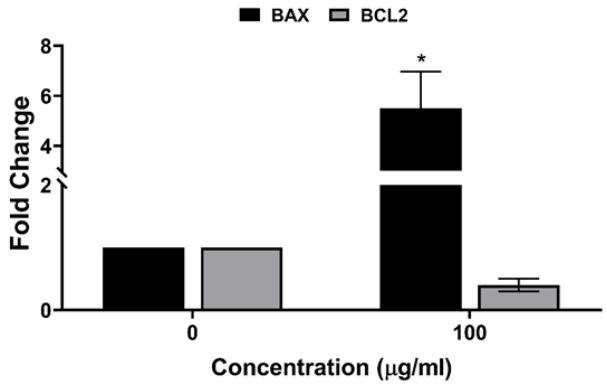

Figure 5: Changes in the expression levels of *BAX* and *BCL2* genes in SW480 cell line following the treatment with -7-hydroxy coumarin during 48 h. (*P < 0.05 compared with the control group).

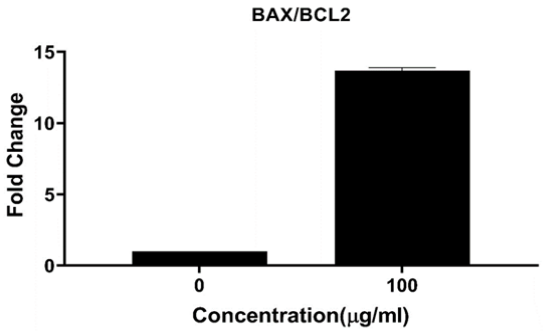

Figure 6: Expression ratio of *BAX* / *BCL2* gene expression in SW480 cell line following the treatment with -7-hydroxy coumarin during 48 h.

Discussion

Colorectal cancer is the most common malignancy associated with the gastrointestinal tract. Additionally, it is the third leading cause of death in Iran (Sung *et al.*, 2021). Approximately 50,000 new cancer cases are annually identified in Iran (Vardanjani *et al.*, 2018). Colorectal tumors with more than 90% malignancy rate have the highest rate among other tumors. Moreover, this cancer accounts for 8% of all cancer deaths. Unfortunately, most colon and rectal tumors are silent and grow slowly, and do not develop symptoms until becoming large enough and dangerous (Sung *et al.*, 2021). Several studies have shown that coumarin and 7-hydroxy coumarin could inhibit cancer cell proliferation by inhibiting aromatase, carbonic anhydrase, and steroid sulfatase (Bisi *et al.*, 2017). In the present study, 7-hydroxy coumarin has been studied as an anticancer agent. As mentioned earlier in the results section, the cytotoxicity effect of 7-hydroxy coumarin on the SW480 cancer cell line was recorded at 164.2, 80.48, and 41.97 µg/ml after 24, 48, and 72 hours, respectively. Furthermore, a significant increase of about 2.4-fold was observed in apoptosis percentage as well as the increased expression of *BAX*/*BCL2* ratio as about 13.69 in the group treated with 100 µg/ml compared to the control group.

Gonzalez *et al.* studied apoptosis induction of both coumarin and 7-hydroxycoumarin in different human lung cancer cell lines. As well, they evaluated the parameters of changes in cell morphology and cell cycle modification. Their results showed that 7-hydroxycoumarin had better overall cytotoxic effects than coumarin. Additionally, apoptotic effects were observed with hydroxycoumarin, but only at high doses. Finally, it was concluded that the use of di hydroxycoumarin along with other chemotherapy drugs could increase their efficacy in the treatment of lung cancer (Lopez-Gonzalez *et al.*, 2004). Bennett Saidu et al. in their study investigated the effects of growth inhibition and apoptosis induced by polysulfide coumarins at different concentrations on HCT116 colon cancer cell line, with IC50 at 50 µmol concentration in HCT116 cell line. The efficacy of polysulfide coumarin on cancer cell lines were recorded in a dose- and time-dependent manner (Saidu *et al.*, 2012). In addition, Musa M *et al.* evaluated the cytotoxicity and cell cycle modification of 8 derivatives of coumarin compounds at different doses of 25, 50, 75, and 100 µmol against A549 lung cancer, CRL 1548 liver cancer, and CRL 1439 normal liver cells. Correspondingly, LD50 was observed at 48.1, 45.1, and 49.6 µmol on A549, CRL1439, and CRL1548 cell lines for 48h, respectively. Besides, Cell cycle analysis showed arresting in the S and G2 phases at a concentration of 20 µm (Musa *et al.*, 2011). In another study, Beillerot *et al.*

investigated the protective effects of coumarin derivatives on the oxidative stress induced by doxorubicin. They have shown the protective antioxidant effect of coumarin derivatives with no effect on the antitumor activity of doxorubicin. Several derivatives of coumarin were synthesized and their antioxidant potencies were also evaluated using the FRAP (ferric reducing ability of plasma) method. Thereafter, the number of free radicals in MCF7 breast cancer cells was measured using flow cytometry. As a result, they reported that 4-methyl, 8-di-7-hydroxycoumarin reduced the number of free radicals by doxorubicin without altering its cytotoxicity (Beillerot *et al.*, 2008). On the other hand, Devji *et al*. synthesized a number of hydroxy coumarin derivatives and then evaluated their anticancer activity in the pancreatic cancer cell line PANC-1, as a new derivative of coumarin germanyl, which showed high cytotoxicity activity at a concentration of 6.25 µg/ml (Devji *et al.*, 2011). Mirunalini *et al*. also evaluated the cytotoxicity of coumarin on HepG2 cell line liver cancer at different doses ranging from 2.5 to 1000 g/ml over a 24-hour period, as a result, IC50 was observed at 62.5 µg/ml (Mirunalini *et al.*, 2014). According to the results, it can be suggested that coumarin diets can be considered as an inhibitor of carcinogenicity through induction of cytotoxicity and apoptosis.

Conclusion

In the present study, we observed that 7-hydroxy coumarin induced both cytotoxicity and apoptosis on SW480 cancer cells. Thus, coumarin can be used as a supplement in the optimal treatment of colon cancer.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Fund: None

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