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ORIGINAL RESEARCH

Effects of Hydroxytyrosol on Expression of Apoptotic Genes and Activity of Antioxidant Enzymes in LS180 Cells

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Purpose: Colorectal cancer is the third–most commonly occurring cancer in developed countries. Hydroxytyrosol is a potent antioxidant that has several activities, such as oxidative-stress control, inhibition of cell proliferation, and induction of apoptosis. In this study, the effect of hydroxytyrosol on the expression of genes effective in apoptosis — *BAX*, *BCL2*, *CASP3*, *P53*, *PPARG*, and *NFE2L2* — and antioxidant-enzyme activity in LS180 cells of human colorectal cancer was investigated.

Methods: The human colorectal cancer cell line LS180 was treated with different concentrations of hydroxytyrosol for 24 hours. Expression of *BAX*, *BCL2*, *CASP3*, *NFE2L2*, *PPARG*, and *P53* was investigated using real-time PCR. The activity of antioxidant and malondialdehyde enzymes was measured by calorimetric methods.

Results: Analysis of gene expression showed that hydroxytyrosol significantly increased the expression of *CASP3* and the BAX:BCL2 ratio in treatment groups compared to the control ($P<0.05$). Also, hydroxytyrosol significantly reduced the expression of the *NFE2L2* gene ($P<0.05$). Calorimetric analysis showed that hydroxytyrosol increased activity of the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase in treatment groups significantly more than the control group and reduced thiobarbituric acid–reactive substances on an oxidative stress index ($P<0.05$).

Conclusion: Hydroxytyrosol may induce apoptosis in colorectal cancer cells by increasing the expression of *CASP3* gene and increasing the *BAX*:*BCL2* ratio. Also, hydroxytyrosol may increase the activity of antioxidant enzymes and reduce the proliferation of LS180 cells by changing the antioxidant-defense system in cancer cells.

Keywords: colorectal cancer, hydroxytyrosol, apoptosis, antioxidant enzymes, calorimetric analysis, malondialdehyde enzymes

Introduction

Colorectal cancer is the third–most common cancer in men (after lung and prostate cancer) and the second–most common in women (after breast cancer).[1] The most important risk factors for colorectal cancer, which can be controlled and prevented by the individual, include environmental factors, lifestyle, and nutritional factors, such as low-fiber and high-fat diets, smoking, alcohol intake, inadequate activity, and lack of exercise. Inevitable risk factors include age, genetics, and inheritance.[2]

The most important risk factor for colorectal cancer is nutritional. It is known that the type of diet can affect the distribution of gastrointestinal cancers, and in the case of lifestyle changes and nutritional habits, up to 30% of cases of colorectal cancer can be

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prevented.[3] A diet high in fat and saturated fatty acids is a major risk factor for colorectal cancer. Saturated fatty acids cause inflammation through the production of prostaglandin E_2. The consumption of red meat and processed meat is also associated with the development of colorectal cancer. Some meat is cooked at high temperatures, resulting in heterocyclic amines and aromatic polycyclic hydrocarbons that have carcinogenic properties. On the other hand, a diet containing vegetables and fruit can protect against colorectal cancer, due to its richness in fiber and such nutrients as calcium, selenium, vitamins A, E, C, and D, folic acid, carotenoids, and plant phenols.[4,5] Hydroxytyrosol is one of the most potent antioxidant phenolic compounds in olives, and is able to transfer the hydroxyl electron group to its *ortho* position and form a hydrogen-bonding molecule with radical phenoxylic stability.[6,7]

Research has shown that the incidence of cancer in the Mediterranean region is lower than in other countries. Much empirical evidence have shown that in addition to antioxidant and anti-inflammatory properties, hydroxytyrosol carries out its anticancer activity by activating molecular signaling pathways, leading to apoptosis and cessation of the cell cycle in various tumor-cell lines, including colon cancer.[8,9] The antitumor effects of hydroxytyrosol are characterized by suppression in the expression of EGFR, eg, in tumor cells and human colorectal cancer, hydroxytyrosol degrades urinocationic receptors and reduces cell proliferation.[10] In addition, hydroxytyrosol has been shown to inhibit cell proliferation in MCF7 breast cancer cells and induce apoptosis.[11,12] Also, hydroxytyrosol induces the expression of antioxidant enzymes by activating Nrf2.[13] Therefore, this substance can be considered an important agent in the treatment of cancer.[14] These effects of hydroxytyrosol have been reported at different concentrations, wherein maximum activity was seen at 100 µM.[15] Additionally, this agent has also shown dose-dependent inhibitory effects on pancreatic cancer–cell growth.[16] The aim of this study was to evaluate the effect of hydroxytyrosol on expression of *BAX*, *BCL2*, *CASP3* (genes affecting apoptosis), *P53*, *NFE2L2*, and *PPARG*, and antioxidant enzymes (superoxide dismutase, catalase, and glutathione reductase) and malondialdehyde in a colorectal cancer (LS180) human cell line.

Methods

Cell Culture and Treatment with Hydroxytyrosol

In this study, the human colorectal cancer cell line LS180 was obtained from the cell bank of the Pasteur Institute of Tehran and grown in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO_2. After treatment of cells with 50, 100, and 150 µM hydroxytyrosol (Sigma) for 24 hours, cells were collected by trypsinization.

RNA Extraction and cDNA Synthesis

RNA extraction was performed using an RNA-extraction kit (Gena Bioscience, Germany) using the protocol provided by the manufacturer. Quantitative RNA extracted from electrophoresis was viewed on 1.5% agarose gel and measured with a NanoDrop device. cDNA was extracted using a cDNA-synthesis kits (Gena Bioscience, Germany) and synthesized by cDNA.

Evaluation of Gene Expression Using Real-Time PCR

Expression levels of *P53*, *NFE2L2*, *PPARG*, *BAX*, *BCL2*, and *CASP3* were assessed using real-time PCR (Gena Bioscience and Corbett) with the relevant primers (Table 1).

Cell Protein–Content Evaluation

In order to investigate the activity of antioxidant enzymes and oxidative indices, after treatment and collectiion of cells using cell-lysis buffer, protein was extracted and the content of each sample measured using the Bradford assay.

Activity of Antioxidant and Malondialdehyde Enzymes

The calorimetric method was used to evaluate the activity of catalase in the enzyme reaction with methanol in the presence

Table 1 Forward- and Reverse-Primer Sequences

Gene	Sequences	Product size
BCL2-F	TCGCCCTGTGGATGACTGA	134 bp
BCL2-R	CAGAGACAGCCAGGAGAAATCA	
BAX-F	TGGCAGCTGACATGTTTTCTGAC	195 bp
BAX-R	TCACCCAACCACCCTGGTCTT	
CASP3-F	TACCTGTGGCTGTGTATCCG	134 bp
CASP3-R	TCAGTGTTCTCCATGGATACCT	
NFE2L2-F	ACGGTCCACAGCTCATCAT	147 bp
NFE2L2-R	TCCGTCGCTGACTGAAGT	
P53-F	GGACATTTGCGTTCGGG	118 bp
P53-R	CTAGGATCTGACTGCGGCTC	
PPARγ-F	TAAAGTCCTTCCCGCTGACC	132 bp
PPARG-R	GGGGTGATGTGTTTGAACTTGA	
B2M-F	ACTGAATTCACCCCCACTGA	167 bp
B2M-R	AAGCAAGCAAGCAGAATTTGGA	

of oxygenated water, and the specific activity of the enzyme was calculated in terms of unit/mg protein.[17] The activity of superoxide dismutase was analyzed by a Pyrogallol radical superoxide reaction using a spectrophotometer. The specific activity of the enzyme was calculated in terms of unit/mg protein.[18] Measurement of glutathione peroxidase activity was performed based on calorimetry and glutathione oxide formation. Specific activity was also calculated in terms of unit/mg protein.[19] Malondialdehyde assays were performed based on reactions with a thiobarbituric acid–reactive substrate.[20]

Data Analysis

REST software was used to analyze the data obtained on gene expression.[21] SPSS software was used to analyze the oxidative-stress index and antioxidant-enzyme activity. To describe the data, means, SD, frequency tables, one-way ANOVA, and Tukey's post hoc test were used.

Results

Evaluation of Gene Expression

The results of this study showed that hydroxytyrosol at all concentrations significantly increased *BAX* expression ($P<0.05$, Figure 1A). Expression of *BCL2* increased in the treatment group compared to the control, but this increase was not statistically significant ($P>0.05$, Figure 1B). Hydroxytyrosol increased the BAX:BCL2 ratio at all concentrations compared to the control group ($P<0.05$, Figure 1C). Hydroxytyrosol increased *CASP3* expression at all concentrations compared to the control group ($P<0.05$) (Figure 1D). Hydroxytyrosol did not significantly affect expression of *P53* compared to the control group ($P>0.05$, Figure 2A). Furthermore, hydroxytyrosol reduced the expression of *NFE2L2* in the treated group compared to the control group, which was statistically significant at a concentration of 50 μM ($P<0.05$, Figure 2B). The treatment group also had a significant reduction in the

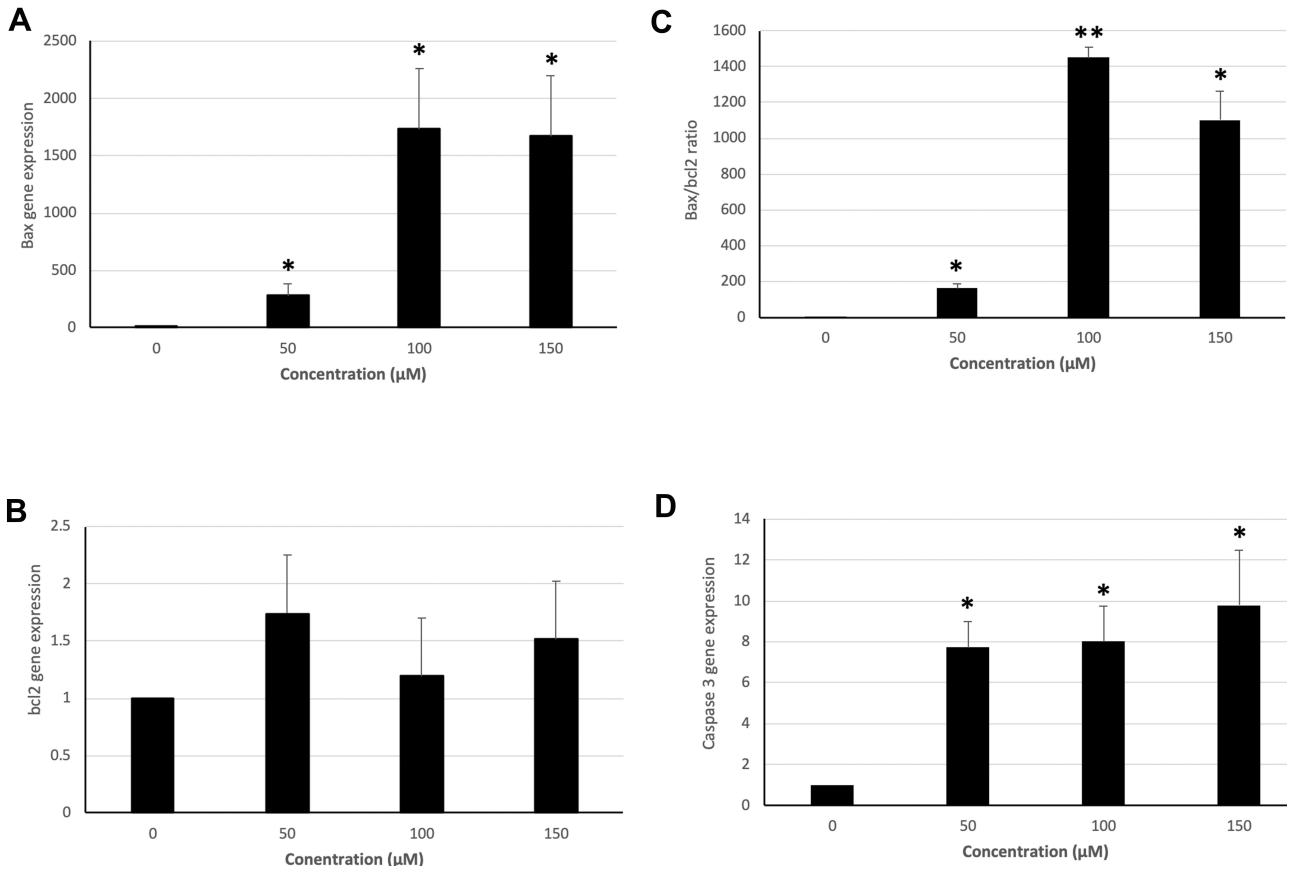

Figure 1 Expression of *BAX*, *BCL2*, and *CASP3* and BAX:BCL2 ratio in groups treated with 50, 100, and 150 μM hydroxytyrosol and controls in the LS180 cell line.
Notes: **P*<0.05; ***P*<0.01. BAX:BCL2 ratio was greatest at 100 μM concentration whereas, *CASP3* gene activity increased in a dose-dependent manner. (**A**) *BAX* expression was significant at all concentrations. (**B**) *BCL2* -expression increase was not significant. (**C**) The BAX:BCL2 ratio was increased at all concentrations. (**D**) Hydroxytyrosol increased *CASP3* expression at all concentrations compared to control group, which was statistically significant.

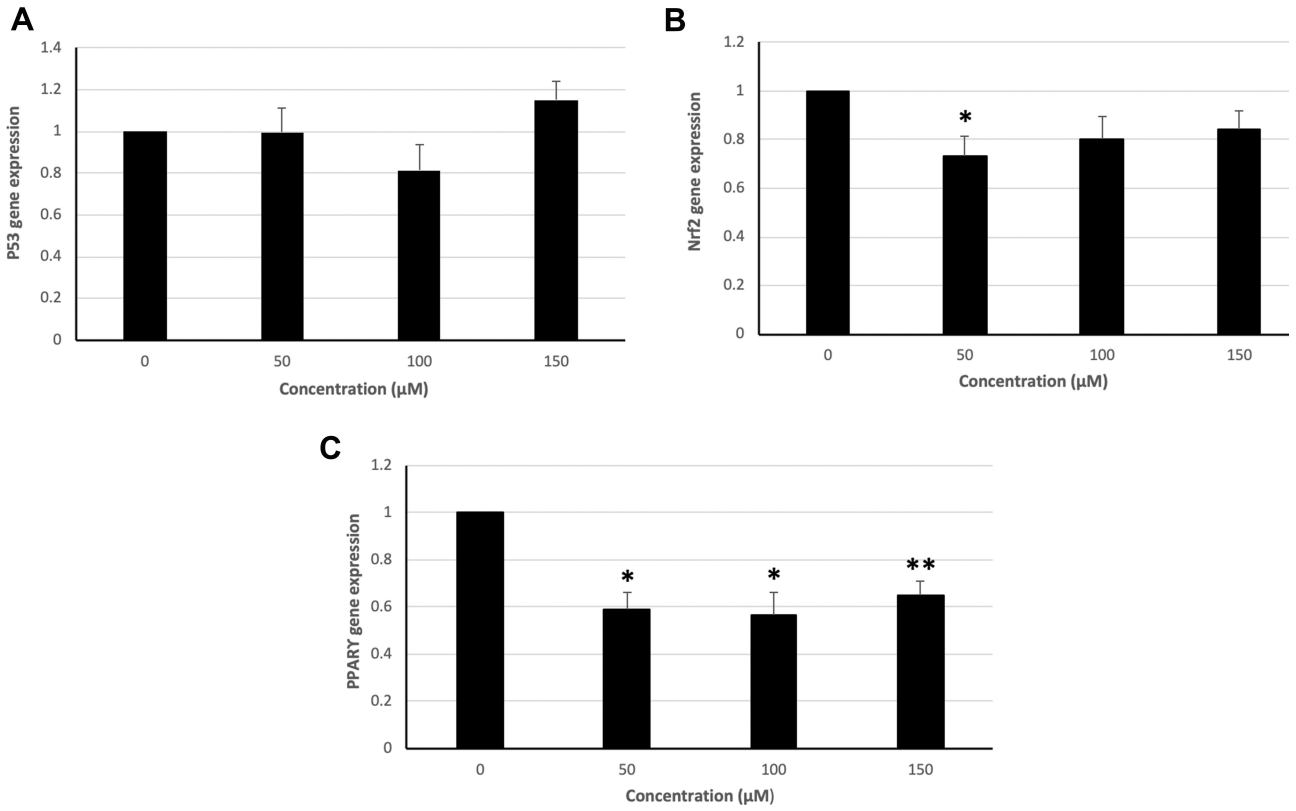

Figure 2 Expression of *P53*, *NFE2L2*, and *PPARG* genes in groups treated with 50, 100, and 150 μM hydroxytyrosol and controls in the LS180 cell line.
Notes: **P*<0.05; ***P*<0.01. *NFE2L2* expression was significant only at 50 μM. (**A**) The expression of *P53* gene compared to control group did not increase significantly. (**B**) The expression of *Nrf2* gene was significant at a concentration of 50 μM. (**C**) Significant reductions in the of PPARγ expression at all concentrations were found.

expression of *PPARG* at all concentrations by 1.7 ($P<0.05$, Figure 2C).

Effect of Hydroxytyrosol on Activity of Antioxidant Enzymes and Thiobarbituric Acid–Reactive Substances

Catalase-enzyme activity at 50, 100, and 150 μM hydroxytyrosol increased in comparison with the control group ($P<0.05$, Figure 3A). Similarly, all treatment groups had significantly increased activity of glutathione peroxidase and superoxide dismutase compared with the control group ($P<0.05$,. Figure 3B and C). At all concentrations, hydroxytyrosol decreased levels of thiobarbituric acid–reactive substances compared to the control group (P<0.05, Figure 3D).

Discussion

Colorectal cancer is the most common cancer of the digestive tract. In recent years, efforts have been made to find natural products that can fight colorectal cancer. Among these compounds, polyphenols, secondary metabolites of plants that have beneficial effects on human health and reduce the risk of most cancers, have been widely recognized.[22,23] Hydroxytyrosol is a natural polyphenol compound and a strong antioxidant. Recent studies have shown that this potent antioxidant has several biological activities, such as oxidative-stress control, cellular efficacy, and induction of apoptosis in several tumor-cell lines.[9,24] Our study evaluated the effects of three doses of hydroxytyrosol — 50, 100, and 150 μM — on colorectal cancer cells. Various concentrations of hydroxytyrosol have been reported to have antioxidant,[25] antiangiogenic,[26] and apoptotic[27] effects in different cancer-cell lines.

Several studies have confirmed the antitumor effects of hydroxytyrosol on several cancers, such as breast cancer, leukemia, and melanoma.[28] Studies have shown that hydroxytyrosol induces G_2/M cell-cycle end points and inhibits cell proliferation. It also induces apoptosis in cancer cells and inhibits Akt activity and the NFKB pathway.[29] In a study on HL60 (promyelocytic leukemia) and HT29 (colon adenocarcinoma), it was shown that hydroxytyrosol slowed cell growth and proliferation and stopped the cell cycle and apoptosis in these cells.[30] Various proteins are involved in the regulation of apoptosis. The family of BCL2 proteins,

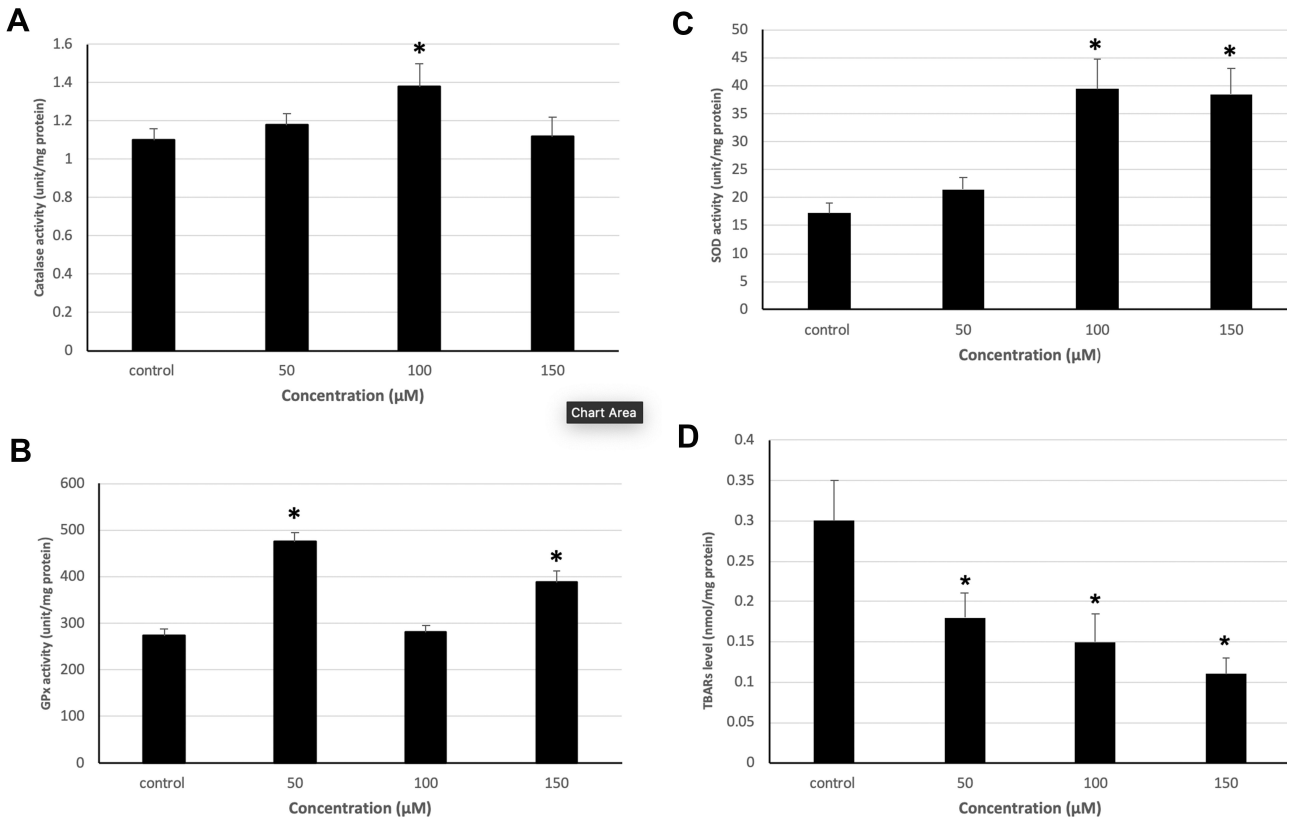

Figure 3 Enzymatic activity of catalase, GPx, SOD and TBARS in groups treated with 50, 100, and 150 μM hydroxytyrosol and controls in the LS180 cell line.
Notes: **P*<0.05. (**A**) Maximum activity of catalase was seen at 100 μM. (**B**) GPx activity was greatest at 50 and 150 μM. (**C**) SOD activity increased in a dose-dependent manner, but at 50 μM did not show a significant increase. (**D**) TBARS reduced in a dose-dependent manner.

which include proapoptotic and antiapoptotic proteins, is one of the major regulators of this process.[31] Our study showed that hydroxytyrosol at all concentrations significantly increased expression of *BAX* compared to the control group. Also, hydroxytyrosol increased *BCL2* expression at all concentrations compared to the control. Similarly, the BAX:BCL2 ratio seems to be a more appropriate indicator for inducing apoptosis and determining the cell-death rate toward apoptosis after exposure to cytotoxic agents. Our study showed that hydroxytyrosol at all concentrations significantly increased expression of *CASP3* in the LS180 cell line compared to the control group. In a study conducted on uterine (FB2 and TPC1) and follicular thyroid cancer (WRO) cancer-cell lines in 2017, hydroxytyrosol reduced the expression of mRNA and cyclin D1 protein and increased the expression of p21 in uterine cancer cells. Hydroxytyrosol also increased the expression of caspase 3 and PARP1 in these cell lines.[27]

P53 is the most widely known tumor-suppressor gene, and plays a crucial role in genomic stability and tumor suppression by inducing apoptosis, halting the cell cycle, aging, and angiogenesis.[32] Studies have shown that hydroxytyrosol induces apoptosis by effecting the expression of *P53*. The results of this study showed increased expression of *P53* at different concentrations of hydroxytyrosol in the LS180 cell line. In a study on the breast cancer–cell line MCF7, it has been shown that olerupein (from hydrolysis of oleuropein and hydroxytyrosol) increases the expression of *P53* in treated groups compared to controls and resulted in apoptosis. It has also been shown in another study on uterine cancer cells (FB2 and TPC1) and thyroid cancer cells (WRO) that hydroxytyrosol increased the expression of p53 and BAD and induced apoptosis.[27]

Nrf2 is a key factor in mechanisms of cell defense against oxidative stress.[33] The results of our study showed that hydroxytyrosol at all concentrations reduced the expression of Nrf2 compared to the control group. A study in 2011 showed that hydroxytyrosol significantly increased the expression of Nrf2 in the normal epithelial cell line MCF10A, but did not increase in the breast cancer–cell lines MDA-MB231 and MCF7 or was not effective.[33] Our study results were consistent with this study.

During normal cell activity, reactive oxygen species (ROS) are produced, which also play an important role in apoptosis. Active oxygen species act as a redox messenger in low physiological levels in signaling and intracellular regulation, while at high levels they produce oxidative stress and induce oxidative changes in macromolecules. Each cell is equipped with an antioxidant defense system to prevent the excessive production of active radicals.[34] In vitro studies have shown that the beneficial effects of phenolic compounds, such as hydroxytyrosol, on human health are due to their antioxidant properties. In a study on the CACO2 cell line, it was shown that hydroxytyrosol inhibited ROS-induced cytotoxicity in these cells and reduced malondialdehyde levels in dose-dependent groups.[35]

The results of this study showed that hydroxytyrosol significantly increased the activity of cellular antioxidant enzymes, including catalase, superoxide dismutase, and glutathione peroxidase, in all treatment groups. Also, malondialdehyde concentrations in the treated groups were significantly lower than controls. Studies have shown that anticancer compounds that have antioxidant activity may have beneficial effects by balancing ROS levels, which not only prevent the proliferation of cancer cells but also lead to apoptosis. In a similar study, the semisynthetic derivative of an anticarcinogenic alkaloid, through the production of ROS and increased oxidative stress, led to toxicity to the MCF7 breast cancer–cell line, and upregulated the activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase. The study concluded that this increase in activity of antioxidant enzymes could be a response to increased oxidative stress in treated cells.[36] On the other hand, studies have shown that phenolic compounds are not only antioxidants but can also be prooxidants and result in the production of ROS.[37] Based on the results of this study, hydroxytyrosol seems to act as a prooxidant in the LS180 cell line of human colorectal cancer and mediates the production of ROS, and thereby the increased activity of the antioxidant enzymes observed in this study could be a response to increased oxidative stress in treated cells.

Conclusion

Hydroxytyrosol can induce apoptosis in the LS180 colorectal cancer–cell line by increasing the expression of proapoptotic genes, such as *BAX*, *CASP3*, and *P53*, increasing the BAX: BCL2 ratio, and downregulating *NFE2L2* expression. Also, the treatment of cells with hydroxytyrosol upregulates antioxidative activity in the colorectal cancer–cell line, marked by increased antioxidant enzymes. In vivo studies on the therapeutic effects of hydroxytyrosol on colorectal cancer in animal models can give better conclusions.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

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