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Anti-Cancer Effects of Tyrosol on Breast Cancer Cell Line- Exploring the Action Mechanisms

Rana khosravi[1], Behdokht Jamali[2*] Morvarid Heidari[3]

1Department of microbiology, Kherad Institute of Higher Education, Bushehr, Iran.
2Department of microbiology, Kherad Institute of Higher Education, Bushehr, Iran. 3Department of Cellular and Molecular Biology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.
3Department of Microbiology, Tonekabon Branch, Islamic Azad University, Tonekabon, Iran.

*Corresponding author: Department of microbiology, Kherad Institute of Higher Education, Bushehr, Iran Behdokht.jamali2023@gmail.com.

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Abstract

Background: Breast cancer is one of the most common malignancies in women for which no suitable treatment has been found yet. Therefore, the present study studied the cytotoxicity effects of tyrosol (TRY) on the Michigan Cancer Foundation-7 (MCF7) breast cancer cell line and L929 normal cells.
Methods: MCF7 and L929 cells were cultured red in DMEM-F12 culture medium after preparation and then exposed to 0, 100, 200, and 300 µM of TRY for 72 hours. Cell viability was evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, and apoptotic and necrotic cell percentages were determined by flow cytometry. After designing specific primers, the expression levels of bax, p53, and bcl-2 genes were evaluated by RT-PCR. GraphPad Prism software was used for analyzing the data.
Results: TRY-treated MCF7 cells showed significantly decreased cell viability in a dose-depended manner. Also, the cell treated with high concentrations of TRY (200 and 300 µM) had a high rate of apoptosis and necrosis ($P<0.0001$). Reactive oxygen species (ROS) content increased in TRY-treated MCF7 cells. Moreover, the overexpression of bax and p53 and downregulation of bcl-2 was seen in TRY-treated MCF7 cells.
Conclusion: TRY has anticancer effects on breast cancer cells by the induction of oxidative stress and apoptosis, as well as the regulation of genes involved in the process of mitochondrial apoptosis.

Keywords: Breast cancer, Gene, miRNA, Doxorubicin

1. Introduction

Breast cancer is one of the most common malignancies in women and has a high mortality rate (1). This disease is one of the most critical factors threatening women's health worldwide, and two million women were diagnosed with breast cancer in 2018 (2). Treatment approaches include surgery, chemotherapy, hormone therapy, and radiotherapy (3). However, over time, breast cancer cells resist chemotherapy drugs, including adriamycin and cyclophosphamide, which significantly reduces the effectiveness of the drugs (4). Therefore, finding new treatment approaches for breast cancer is of significant importance.

Medicinal plants and natural compounds (NPs) derived from them have received much attention from researchers due to their antioxidant, anti-inflammatory, and antitumor effects (5, 6). One of these NPs is Tyrosol (TYR), whose antimicrobial and antitumor effects have been proven (7, 8). TYR is a phenolic compound found in virgin olive oil and wine (8). Its antitumor activity has been shown in malignancies such as the prostate (9), colon (10), liver (11), lung (12), etc. Despite its antitumor effects, the mechanism of action of this natural compound remains unknown. Nevertheless, some authors have stated that oxidative stress is induced by the overproduction of reactive oxygen species (ROS) (13), arrest of the cell division cycle in the G1/S phase, inhibition of p21/p27, and induction of apoptosis by activation of CASP3 (9) are among the mechanisms of antitumor activity of TRY. Interestingly, this compound did not show cytotoxic activity on normal cells (9), which indicates specific cytotoxic activity against tumor cells. Therefore, TRY has excellent potential in treating various types of cancers, and it is necessary to study its effect on various types of tumors as well as the mechanism of action of antitumor effects. This research aimed to evaluate the cytotoxic effects of TYR on breast adenocarcinoma cell lines (MCF7) and normal cells (L929). Moreover, cells' ROS content, apoptosis, necrosis, and Bax, p53,

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and bcl-2 gene expressions were evaluated in TYR-treated cells.

2. Materials and Methods

2.1. Cell preparation and culture

MCF7 (breast adenocarcinoma) and L929 (normal) cell lines were purchased from Institute Pasteur, Iran-Tehran. Cells were cultured in DMEM-F12 culture medium and incubated at 35°C and 5% CO2 to proliferate.

2.2. Cell viability

MTT test was used to investigate the cytotoxicity effects of TRY on cancer and normal cells. For this purpose, 105 cells (10 mL of cell suspension) were placed in each well of a 96-well plate, and then different concentrations of TRY (0, 100, 200, and 300 µM) were added and incubated for 72 hours at 37°C and 5% CO2. After 3 days, 10 µL of 5 mg/mL MTT solution was added to each well, and after 3 hours, 100 µL of DMSO was added, and the absorption intensity of the wells was read at a wavelength of 540 nm by an ELISA reader (14). The experiment was repeated three times.

2.3. Apoptosis, necrosis and reactive oxygen species

To measure the amount of apoptosis and necrosis, the cells were collected after 72 hours of treatment with different concentrations of TRY, and the cell sediment was dissolved entirely in 5 µL of annexin-V solution, and after 10 minutes, 5 µL of the PI solution was added. Untreated cells were considered as control. The samples were then placed in the flow cytometry device. The 2', 7' dichlorofluorescein diacetate method was used to measure ROS content of cells (15).

2.4. Gene expression analysis

For RNA extraction, we used an RNA extraction kit (Qiagen, Germany) based on the manufacturer's instructions. For cDNA synthesis, 10 µl of RNA extracted with 4 µl of 5X buffer, 2 µl of dNTP (Roche, Germany), 1 µl of random hexamer, 1 µl of reverse transcriptase enzyme and 2 µl of deionized water were used. The reaction mixture was placed in the PCR machine at 42°C for 1 hour and then at 72°C for 10 minutes.

The sequence of primers was designed in Primer3 software and blasted in the NCBI database (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequence of primers is shown in Table 1.

Table 1. The sequences of primers used in the current study to measure the expression levels of *Bax*, *p53*, and *bcl-2* genes

Genes	Sequences [5'-3']
bax	F-5'- TCAGGATGCGTCCACCAAGAAG-3
	R-5'- TGTGTCCACGGCGGCAATCATC-3'
p53	F-5'- CCTCAGCATCTTATCCGAGTGG-3'
	R-5'- TGGATGGTGGTACAGTCAGAGC-3'
bcl-2	F-5'- ATCGCCCTGTGGATGACTGAGT-3'
	R-5'- GCCAGGAGAAATCAAACAGAGGC-3'
GAPDH	F-5'- ACACCCACTCCTCCACCTTTG-3'
	R-5'- TCCACCACCCTGTTGCTGTAG-3'

F: Forward; R: Reverse.

The RT-PCR reaction mixture included 2.5 µl of 10X buffer, 2.5 µl of MgCl2, 1.5 µl of dNTP, 1 µl of forward primer, 1 µl of reverse primer, 0.2 µl of Taq polymerase enzyme, 15.3 µl of deionized water and 1 µl of cDNA sample. The time-temperature program of the device included 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 72°C for 30 seconds, and at the end of a cycle of 72°C for 7 minutes.

2.5. Statistical analysis

The $2^{-\Delta\Delta CT}$ method was used to analyze gene expression data. Also, a one-way analysis of variance was used to analyze the data. The mean comparison was made based Tukey test at a probability level of $P<0.05$.

3. Results

3.1. Cell viability

TRY reduced MCF7 cell viability in a concentration-depend manner. Exposure to MCF7 cell line with 100, 200, and 300 µM TRY reduced cell viability by 15, 46, and 80 %, respectively, compared with untreated control cells (0 µM). However, TRY did not affect

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L929 cell viability, indicating its specific effect on breast adenocarcinoma cancer cells (Figure 1).

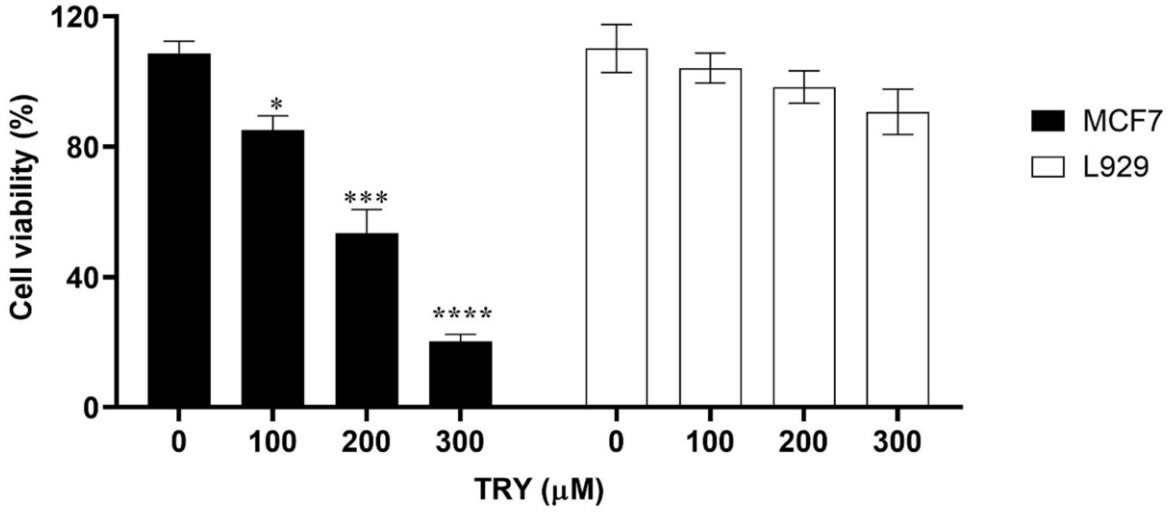
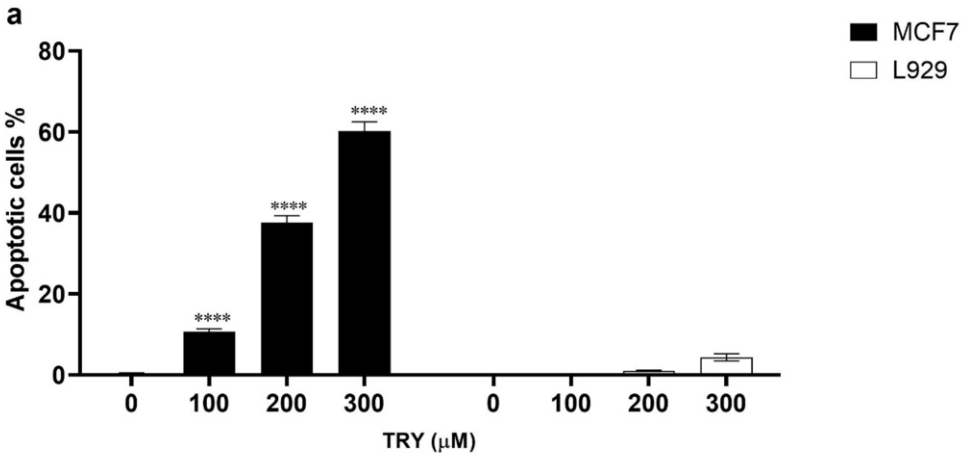


Figure 1. MCF7 and L929 cell line viability after treatment with 100, 200, and 300 μM of TRY for 72 hours. *, *** and *** indicated significant differences (P<0.05, P<0.001 and P<0.0001, respectively) compared untreated cells (0μM).

3.2. Apoptosis and necrosis

TRY-induced apoptosis and necrosis in MCF7 cell line. Although no apoptotic cells were detected in untreated MCF7 cells (0μM), cells treated with 100, 200, and 300 μM of TRY 10.68, 37.65, and 60.16% apoptosis were measured (Figure 2a). This means that this compound causes the death of cancer cells by inducing apoptosis. The same trend was seen for TRY-treated MCF7 cells, and the high concentration of TRY induced more necrosis. L929 cells treated with 300 μM TRY showed 5% apoptosis (P<0.01).



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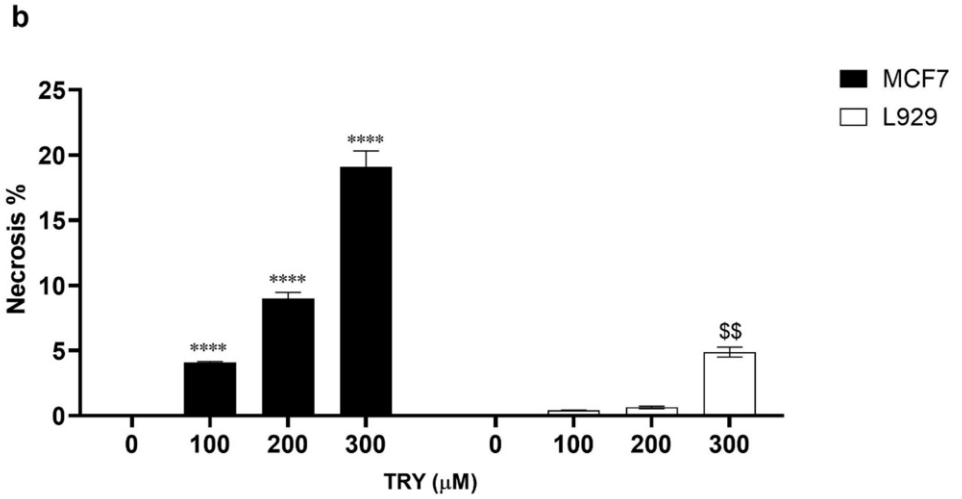


Figure 2. The apoptosis (a) and necrosis (b) percentages of MCF7 and L929 cell line after treatment with 100, 200, and 300 μM of TRY for 72 hours. *** indicates significant differences (P<0.0001) compared untreated MCF7 cells (0μM). $$ shows significant differences (P<0.01) compared untreated L929 cell line (0μM).

3.3. Reactive oxygen species

Reactive oxygen species (ROS) contents of TRY-treated MCF-7 were significantly increased. The highest ROS content was seen in cells treated with 300 μM TRY (Figure 3). However, this compound did not significantly affect the ROS content of the L929 cell line. It seems that TRY induced oxidative stress in cancer cell lines, which can explain TRY's specific cytotoxic effects on MCF7.

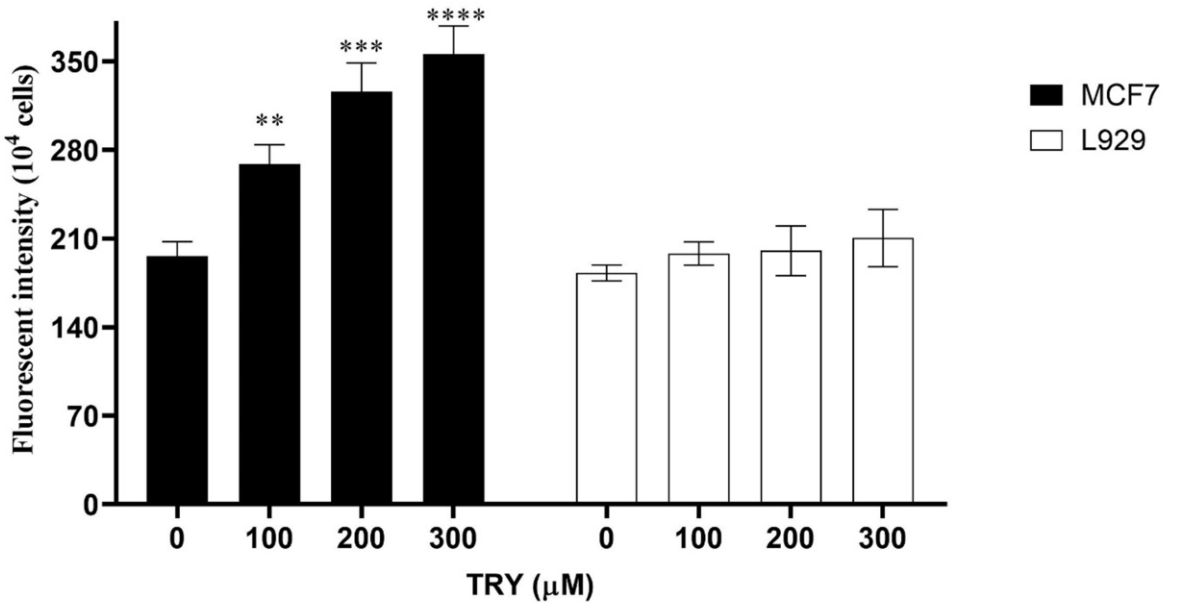


Figure 3. The reactive oxygen species (ROS) contents of MCF7 and L929 cell line after treatment with 100, 200, and 300 μM of TRY for 72 hours. **, *** and *** indicated significant differences (P<0.01, P<0.001 and P<0.0001, respectively) compared untreated cells (0μM).

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3.4. *bax*, *p53* and *bcl-2*

bax and *p53* overexpressed in TRY-treated MCF7 cell line in dose depend manner (Figure 4a, b). The highest expressions of both genes were seen in MCF7 cells treated with 300 µM TRY. *bcl-2* downregulated significantly in TRY-treated MCF7 cell line by increasing TRY dose (Figure 4c). These results indicate the induction of the mitochondrial apoptosis pathway by TRY. However, the expressions of *bax* and *bcl-2* genes were not changed in the TRY-treated L929 cell line, and only p53 overexpression was seen in cells treated with 300µM TRY ($P<0.05$) (Figure 4b).

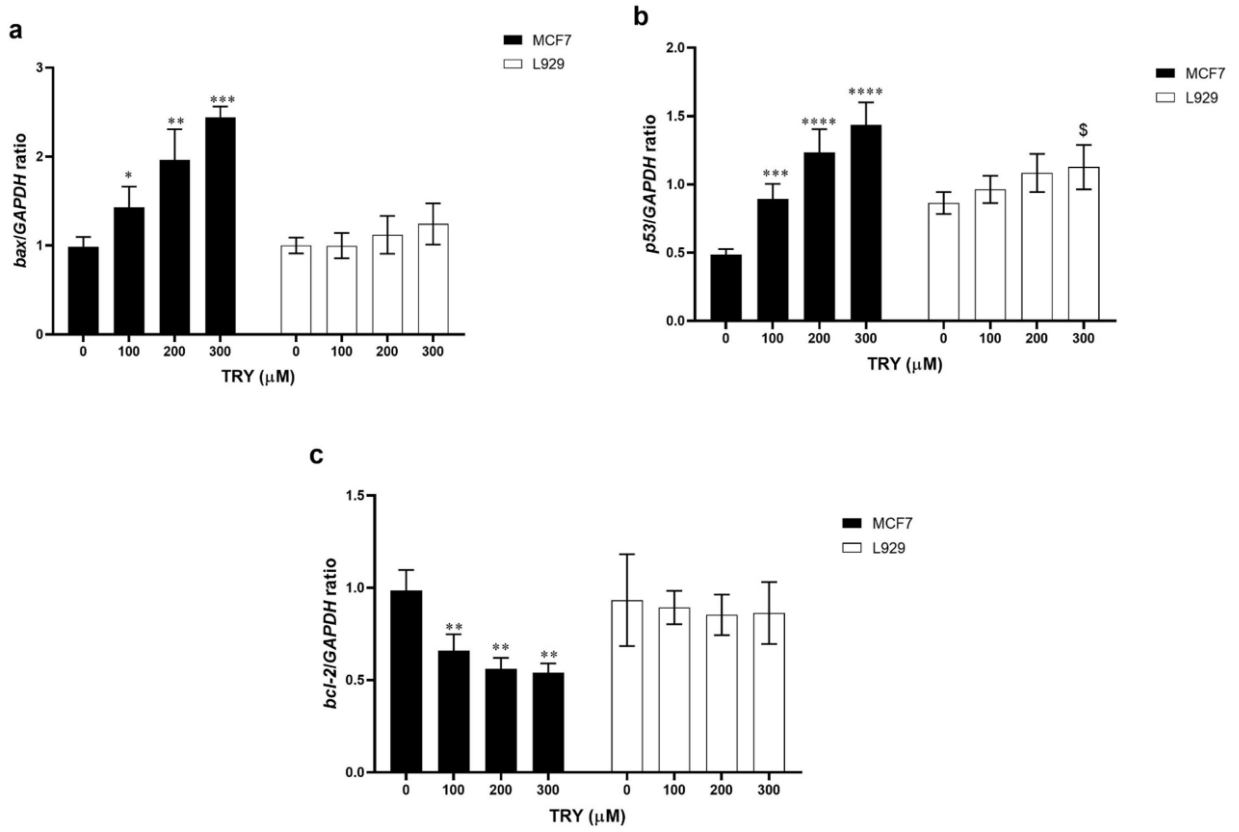


Figure 4. The expression levels of *bax* (a), *p53* (b), and *bcl-2* (c) genes in MCF7 and L929 cell lines after treatment with 100, 200, and 300 µM of TRY for 72 hours. *, **, *** and *** indicate significant differences ($P<0.05$, $P<0.01$, $P<0.001$ and $P<0.0001$, respectively) compared untreated cells (0µM). $ shows significant differences ($P<0.05$) compared untreated L929 cell line (0µM).

4. Discussion

In the present study, it was shown that TRY had cytotoxic effects on MCF7 breast cancer cells, and the mechanism of these anticancer effects was attributed to the induction of oxidative stress in the cells, followed by the induction of apoptosis and necrosis and the activation of the mitochondrial apoptosis pathway. However, this compound did not affect L929 cell viability, indicating specific cytotoxic effects on cancer cells. These results indicate the anticancer effects of TRY, which shows its high potential in treating breast cancer.

The main characteristic of cancer cells is uncontrolled cell proliferation and resistance to programmed cell death through apoptosis (16). Therefore, compounds that induce apoptosis in cancer cells can be considered anticancer compounds (17). The present study also showed that TRY has cytotoxic effects on breast cancer cells, and therefore, it can be considered an anticancer compound. The anticancer effects of this compound have been shown in cancers such as prostate (9), colon (10), liver (11), lung (12). Therefore, TRY has anticancer effects on various malignancies, which need to be studied in animal and clinical studies.

ROS content in MCF7 cancer cells treated with TRY increased concentration-dependent, indicating the induction of oxidative stress in these cells. Induction of oxidative stress is related to apoptosis and cell death (18), which can explain the increase in

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apoptosis in MCF7 cells treated with TRY. Therefore, increasing the content of ROS and induction of apoptosis by TRY in breast cancer cells can be considered as the mechanism of action of the anticancer effects of this compound.

One of the mechanisms of anticancer drugs is to change and regulate the expression of essential genes that can induce a cascade of molecular processes. Therefore, examining the expression of genes can provide a good understanding of the mechanism of action of drugs. Among the essential genes in the path of cancer protection, we can mention the p53, bax, and bcl-2 genes (19). Both p53 and bax genes have pro-apoptotic functions and induce apoptosis and cell death (20, 21). However, bcl-2 has anti-apoptotic action, and its overexpressions reduce cell apoptosis (22). Therefore, in this study, the effect of TRY on the expression of these genes was studied, and the results indicated an overexpression of both p53 and bax and downregulation of bcl-2 in MCF7 cells treated with TRY. The overexpression of bax and p53 and downregulation of bcl-2 show that TRY exerts anticancer effects by regulating these genes.

5. Conclusion

In general, according to the findings of the present study, it can be concluded that TRY has anticancer effects on breast cancer cells, and its mechanism of action can be attributed to the induction of oxidative stress and apoptosis, as well as the regulation of genes involved in the process of mitochondrial apoptosis. More research is needed on animal models and clinical settings.

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