Anatolian Journal of Botany
6(2): 62-68 (2022)
Research article *doi: 10.30616/ajb.1103463*

Differential regulation of antioxidant enzymes by resveratrol in healthy and cancerous hepatocytes

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Received : 15.04.2022
Accepted : 21.06.2022
Online : 10.08.2022

Sağlıklı ve kanserli hepatositlerde antioksidan enzimlerin resveratrol tarafından farklı düzenlenmesi

Abstract: Resveratrol, mainly found in grapes, blueberries, raspberries, mulberries, and peanuts, is a naturally synthesized polyphenol that protects against several pathological issues. Increased oxidative stress in cancer cells and suppressed cellular antioxidant systems have been investigated and therefore herbal antioxidants could be used as an auxiliary substance in the treatment of several types of cancer. In this study, we inspected the effects of resveratrol, a powerful antioxidant, in the gene expression and activities of antioxidant enzymes in healthy and cancerous liver cells. After the liver cancer cells (HepG2) and healthy hepatocytes (THLE2) were treated with resveratrol at different doses (25-, 50-, 75- µM) for 48-hours, the gene expression levels of antioxidant enzymes were measured by qRT-PCR and their activities were measured spectrophotometrically. The results showed the suppression of all antioxidant enzymes in the THLE2 cells at all doses of resveratrol but conversely up-regulation of those in HepG2 cells. The reduction in gene expression in healthy cells was in line with the suppression of enzymatic activities of catalase and glutathione S-transferase, however, the activity changes in cancer cells were not significant. This differential regulation of antioxidant enzyme systems in healthy and cancerous hepatocytes demonstrates the stimulation of antioxidant enzymes in cancer cells as a protective function against oxidative damage, while decreased expression in healthy cells signifies the reduction of requirement for antioxidants, conversely. Therefore, resveratrol might be effective in cancer treatment through affecting the molecular mechanisms that can reduce cellular stress by activating antioxidant enzymes in cancer cells.

Key words: Resveratrol, antioxidant enzymes, gene expression, oxidative stress

Özet: Üzüm, yaban mersini, ahududu, dut ve yer fıstığı gibi bitkilerde bulunan resveratrol, çeşitli patolojik sorunlara karşı koruma sağlayan doğal olarak sentezlenmiş bir polifenoldür. Kanser hücrelerinde oksidatif stresin artışı ve hücresel antioksidan sistemlerin baskılanması belirlenmiş olup, bitkisel antioksidanların çeşitli kanser türlerinin tedavisinde yardımcı madde olarak kullanılabileceği öngörülmektedir. Bu çalışmada, güçlü bir antioksidan olan resveratrolün sağlıklı ve kanserli karaciğer hücrelerinde antioksidan enzimlerin gen ekspresyonu ve aktiviteleri üzerindeki etkileri araştırılmıştır. Karaciğer kanseri hücreleri (HepG2) ve sağlıklı hepatositler (THLE2) resveratrol ile farklı dozlarda (25-, 50-, 75- µM) 48 saat süreyle muamele edildikten sonra antioksidan enzimlerin gen ekspresyon seviyeleri qRT-PCR ile, aktiviteleri ise spektrofotometrik olarak ölçülmüştür. Sonuçlar, THLE2 hücrelerindeki tüm antioksidan enzimlerin resveratrolün tüm dozlarında baskılandığını, ancak bunun tersine HepG2 hücrelerinde antioksidan enzimlerin yukarı regülasyonunu göstermiştir. Sağlıklı hücrelerde görülen gen ekspresyonundaki azalma, katalaz ve glutatyon S-transferazın enzimatik aktivitelerinin baskılanmasıyla paralel değiştiği, ancak kanser hücresindeki aktivite değişikliklerinin anlamlı olmadığı belirlenmiştir. Sağlıklı ve kanserli hepatositlerde antioksidan enzim sistemlerinin farklı olarak düzenlemesi, kanser hücrelerinde antioksidan enzimlerin oksidatif hasara karşı koruyucu bir işlev olarak uyarılmasını gösterirken, sağlıklı hücrelerde azalmış ekspresyon, tersine antioksidan ihtiyacının azalmasına işaret etmektedir. Bu nedenle resveratrol, kanser hücrelerinde antioksidan enzimleri aktive ederek hücresel stresi azaltabilecek moleküler mekanizmaları etkileyerek kanser tedavisinde etkili olabilir.

Anahtar Kelimeler: Resveratrol, antioksidan enzimler, gen ekspresyonu, oksidatif stres

Citation: Bostancı A, Şencan EN, Kütük A, Sadi G (2022). Differential regulation of antioxidant enzymes by resveratrol in healthy and cancerous hepatocytes. Anatolian Journal of Botany 6(2): 62-68.

1. Introduction

Free radicals, due to the unpaired electrons in their structure, are unstable and more energetic than non-radical molecules and so they deteriorate the structures of biomolecules such as lipids, carbohydrates, proteins, and nucleic acids (Studer and Curran, 2016). An imbalance between the accumulation of reactive species and their rate of removal is defined as oxidative stress which is considered as the main contributor to cellular pathologies (Sadi and Sadi, 2010). In the cells, several enzymatic and non-enzymatic antioxidant systems reduce or eliminate the stress conditions caused by elevated oxidative stress. The body's principal enzymatic defense mechanisms are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) (Sadi and Sadi, 2010). Besides, there are also molecular antioxidants such as glutathione, vitamin C, carotenoids, and vitamin E that form non-enzymatic defense systems. Free radicals and oxidative stress play effective roles in many disease conditions such as aging, atherosclerosis, ischemia-reperfusion, inflammation, rheumatoid arthritis, neurodegenerative diseases, cancer, and liver diseases (Khan et al., 2010; Abdul-Rahman et al., 2012; Klaunig, 2018). The development of hepatocellular carcinoma which

is the most common form of liver cancer cases has also been associated with the free radicals and oxidative stress (Fitzmaurice et al., 2017; Tarocchi and Galli, 2017).

Resveratrol (3, 5, 4′-trihydroxystilbene) is a natural antioxidant found in grapes, blueberries, raspberries, mulberries, and peanuts (Kataria and Khatkar, 2019). It reduces cellular stress, microbial infections, or the chemical effects of pesticide treatments in plants (Akbel et al., 2018). Besides, the potential effects of the resveratrol obtained from plant sources on human health are also being studied by many researchers. For example, antidiabetic (Zhang et al., 2018; Dai et al., 2019), anti-aging, anti-inflammatory, antioxidant, anti-cardiovascular disease, antimicrobial (Vestergaard and Ingmer, 2019), anti-estrogenic (Stivala et al., 2001; Berman et al., 2017), anti-carcinogenic (Athar et al., 2007; Bertelli, 2007), and vascular occlusion preventing roles (Catalgol et al., 2012) are well described. Many target molecules have been identified that mediate the protective effects of resveratrol such as endothelial nitric oxide synthetase, sirtuin 1, nuclear factor E2-related factor-2, and nuclear factor-kappa (Liu et al., 2015). In addition, resveratrol was also found to regulate antioxidant enzyme systems in several tissues (Karabekir and Özgörgülü, 2020). Recent studies have demonstrated either antioxidant or pro-oxidant effect of resveratrol depending on the specific cells, different tissues, or disease types. The exact mechanisms that make resveratrol a protective agent for normal cells as well as a radical generator with cytotoxicity against cancer cells are currently being debated (Khan et al., 2013). Besides, the effects of resveratrol on antioxidant enzymes' expressions and activities in various cancers are contradictive.

Therefore, in this study we evaluated the possible regulatory roles of resveratrol over main antioxidant enzymes in both healthy and cancerous hepatic cells. Differential expression patterns of antioxidant genes together with enzyme activities were assessed under different resveratrol concentrations the results of which might provide a better understanding of pharmacological uses of resveratrol in cancer prevention or treatment.

2. Material and Method

2.1. Cell culture and resveratrol treatment

Human healthy liver (THLE2) and human liver hepatocarcinoma (HepG2) cell lines were cultured using a Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (fetal bovine serum), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37ºC in the presence of 5% CO_2 and providing 95% humidity until they reached 90% confluency. Getting 90% confluency, cells were removed with trypsin/EDTA with phenol red solution and subcultured to the new growth media. After examining the cytotoxic properties of resveratrol over these cells, resveratrol in different concentrations (25 µM, 50 µM, and 75 µM) was applied to the cells for 48 hours. These doses of resveratrol are selected according to IC_{50} values for cellular cytotoxicity results (data not shown).

2.2. Total RNA isolation and cDNA library synthesis

The RNesy total RNA isolation kit (Qiagen, Hilden, Germany) was used to isolate total RNA from THLE2 and HepG2 cells according to the manufacturer's procedure. After isolation, spectrophotometry (Multiskan Go, Thermo Scientific, Waltham, MA, USA) at 260/280nm was used to assess the quantity and quality of total RNAs. Complementary DNA (cDNA) libraries were prepared using RNA samples using the First-Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). Accordingly, 1 µg total RNA, 1 µl oligo$(dT)_{18}$ primer (100 µM), 4 µl 5X-M-MuLV reaction buffer, 1 µl RiboLock™ (20 U/µl) (Thermo Scientific, USA), 2 µl dNTP (10 mM) was mixed with 1 µl of M-MuLV Reverse Transcriptase (200 U/µl) and the total volume was made up to 20 µl. cDNA synthesis was carried out at 42ºC for 1 hour in a thermal cycler (Bioneer, South Korea).

2.3. Determination of antioxidant enzymes' genes expression with quantitative real-time polymerase chain reaction (qRT-qPCR)

A real-time quantitative polymerase chain reaction (qRT-PCR, LightCycler480 II, Roche, Basel, Switzerland) was used to assess the expression levels of *sod1*, *sod2*, *cat*, *gpx*, and *gstm*. Accordingly, 5 µl of SYBR Green Master Mix (Roche, Germany) was mixed with 2 µl forward and 2 µl reverse primers (2 µM each), and then 1 µl of cDNA was added. After initial denaturation at 95 °C for 10 minutes, denaturation at 94 °C for 15 seconds, annealing at 58 °C for 15 seconds, and extension at 72°C for 30 seconds were repeated 40 times, the resulting fluorescence after the extension step of each cycle was determined with the LightCycler 480-II (Roche, Germany) instrument. Primer pairs (Table 1) that were designed with the NCBI Primer Blast tool were also validated before the experiments. Measurements were performed in triplicate for each sample, and threshold cycle count (C_T) values were calculated for each amplification curve. The internal standard *gapdh* gene was used for normalization. The relative expressions of genes to *gapdh* were determined by the activity-corrected gene quantification tool of LightCycler™ 480 SW 1.5.1 software (Roche, Germany). Melt analysis was performed on the products to control the purity of the qPCR products and genomic DNA contamination was also tested using blank reaction tubes that did not contain any cDNA or DNA during the reaction.

Table 1. Primer sequences of *cat*, *sod1*, *sod2*, *gstµ*, *gpx*, and internal standard *gapdh* used for the mRNA expression determination.

Gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
cat	GAACAGATAGCCTTCGACCC	AGTAATTTGGAGCACCACCC
gpx	CAGTCGGTGTATGCCTTCTC	TTCTTGGCGTTCTCCTGATG
sod1	AGATGACTTGGGCAAAGGTG	TTGGGCGATCCCAATTACAC
sod2	GCACATTAACGCGCAGATCA	AGCCTCCAGCAACTCTCCTT
gstmu	AGAAGCAGAAGCCAGAGTTC	GGGGTGAGGTTGAGGAGATG
gapdh	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTGGGCCAT

2.4. Protein isolation

Resveratrol treated THLE2 and HepG2 cells were scraped from the cell surface with a cell scraper (Sarstedt, USA) in 100 µl homogenization medium containing 50 mM Tris, 150 mM sodium chloride, 1 mM EDTA, 1% (w/w) NP-40, 0.25% (w/v) sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM PMSF (Phenylmethylsulfonyl fluoride), pH:7.4. After cell harvesting, they were incubated on ice for 45 minutes and then homogenized with an ultra sonicator (Sonopuls, Bandelin, Germany). Cell homogenates were centrifuged at 1.200*g* for 10 minutes at +4ºC and supernatants were removed. Total protein concentrations of the supernatants were determined by the Lowry method (Lowry et al., 1951).

2.5. Determination of Catalase and Glutathione S-transferase atıvities

Catalase enzyme activity was measured according to the protocol described by Aebi et al. (1974) with slight modifications. In this method, spectrophotometric measurement of the reduction in H_2O_2 to water through catalase activity was followed. Accordingly, 240 µl phosphate buffer (50 mM, pH: 7.0) and 10 µl cell homogenate were mixed thoroughly in a UV-permeable microtiter plate and the enzymatic reaction was initiated with the addition of 50 µl H_2O_2 (200 mM), and the absorbance changes were monitored for 2 min at 240 nm. Specific activity was calculated as the amount of decomposed hydrogen peroxide by 1 mg total protein in 1 minute and the extinction coefficient of hydrogen peroxide was 0.00364 ($L.mmol^{-1}.mm^{-1}$).

Total GST enzyme activity was determined according to the procedure developed previously (Habig et al., 1974). Accordingly, 15 µl cell homogenate was mixed with 20 µl GSH (50 mM), 15 µl CDNB (50 mM, dissolved in 2/3 ethanol), and 250 µl phosphate buffer (50 mM; pH 7.0) in a UV-permeable microtiter plate. The changes in absorbance were followed for 2 minutes at 340 nm. Total GST activity in cells was calculated as the amount of chromogenic product formed in one minute by the homogenate containing one mg of protein. The extinction coefficient was 9.6 $M^{-1}.cm^{-1}$ in specific activity calculations.

2.6. Statistical analysis

The GraphPad Prism 5.02 software was used to analyze the data. All data were given as mean ± standard error of the mean (SEM) values of three biological replicates with three technical replicate measurements.The corresponding *gapdh* levels were used to normalize the gene expression data. One-way ANOVA followed by a post-hoc test (Tukey's Honestly Significant Difference) was utilized for statistical evaluations. The statistical significance of comparisons with *P* values less than 0.05 was accepted.

3. Results

3.1. The changes in *cat, sod1* and *sod2* gene expressions with resveratrol in healthy and cancerous hepatocytes

Superoxide dismutase (SOD) isozymes; SOD1 and SOD2, which neutralize superoxide radicals in the cytoplasm and mitochondria, respectively, are the most important antioxidant enzymes in cells. The resultant hydrogen peroxide is then converted into non-toxic compounds with catalase (CAT) to eliminate the radical-induced stress conditions in hepatocytes. In this study, gene expressions of main antioxidant enzymes; *sod1, sod2,* and *cat* were determined by qRT-PCR for deep scanning of the exact modulation mechanisms over those enzymes with resveratrol in both cancerous and healthy hepatocytes. Results demonstrated that the gene expression levels of *cat*, *sod1,* and *sod2* were significantly repressed with resveratrol (Figure 1A, 1C, and 1E) in THLE2 cells. This reduction compromises the 80% of control expressions in all three doses and was found to be statistically significant ($P<0.05$). On the contrary to healthy cells, in HepG2 cells, gene expression of *sod1, sod2,* and *cat* were significantly induced in all doses of resveratrol in a dose-dependent manner (Figure 1B, 1D, and 1F). Gene expression of *cat* increased about two-fold in 25 µM, and about four-fold in 50- and 75 µM resveratrol treated HepG2 cells.

3.2. The changes in *gpx* and *gstm* gene expressions with resveratrol in healthy and cancerous hepatocytes

Glutathione-dependent antioxidant enzymes, the most important of which is glutathione peroxidase (GPx), catalyze the breakdown of hydrogen peroxide to water in the presence of glutathione, are another protective mechanism against oxidative stress. Some glutathione S-transferases (GSTs), which catalyze the conjugation of glutathione to a wide spectrum of electrophiles, share this function. The main isoform of this enzyme family having peroxidase-like antioxidant activity is the mu isoform (GST-mu). Herein, the expression levels of both *gpx* and *gstm* were also determined by qRT-PCR in both resveratrol treated THLE2 and HepG2 cells and the results are summarized in Figure 2.

Similar to *cat,* and *sod* expressions, *gpx,* and *gstm* levels were also suppressed in healthy THLE2 cells with all doses of resveratrol. Especially 50- and 75 µM resveratrol reduced the *gstm* expressions further ($p<0.05$) (Figure 2C). The behavior of these enzymes in cancerous hepatic cells (HepG2) is again contradictory in such a way that both *gpx* and *gstm* were induced tremendously with resveratrol. An approximately two-fold increase in *gpx* expression and a four-fold increase in *gstm* levels were observed at high doses of resveratrol in HepG2 cells and these changes were statistically significant ($p<0.05$).

3.3. The changes in CAT and total GST activities in THLE2 and HepG2 cells

Antioxidant enzyme systems catalyze processes that counteract free radicals and radical-induced byproducts, contributing the protection of cellular damage caused by free radicals. In this study, enzymatic activities of CAT and GST enzymes were determined, and the results are summarized in Figure 3. In line with the gene expression data, CAT and total GST activities were repressed with resveratrol in THLE2 cells (Figure 3A, 3C) showing that the suppressive effect starts at the gene expression level and continues up to the activity level. On the other hand, while resveratrol augmented gene expression levels of antioxidant enzymes in HepG2 cells, there was not any significant modulation in their activities (Figure 3B, 3D). This result indicated only gene expression regulatory function of resveratrol in cancerous hepatocytes and other

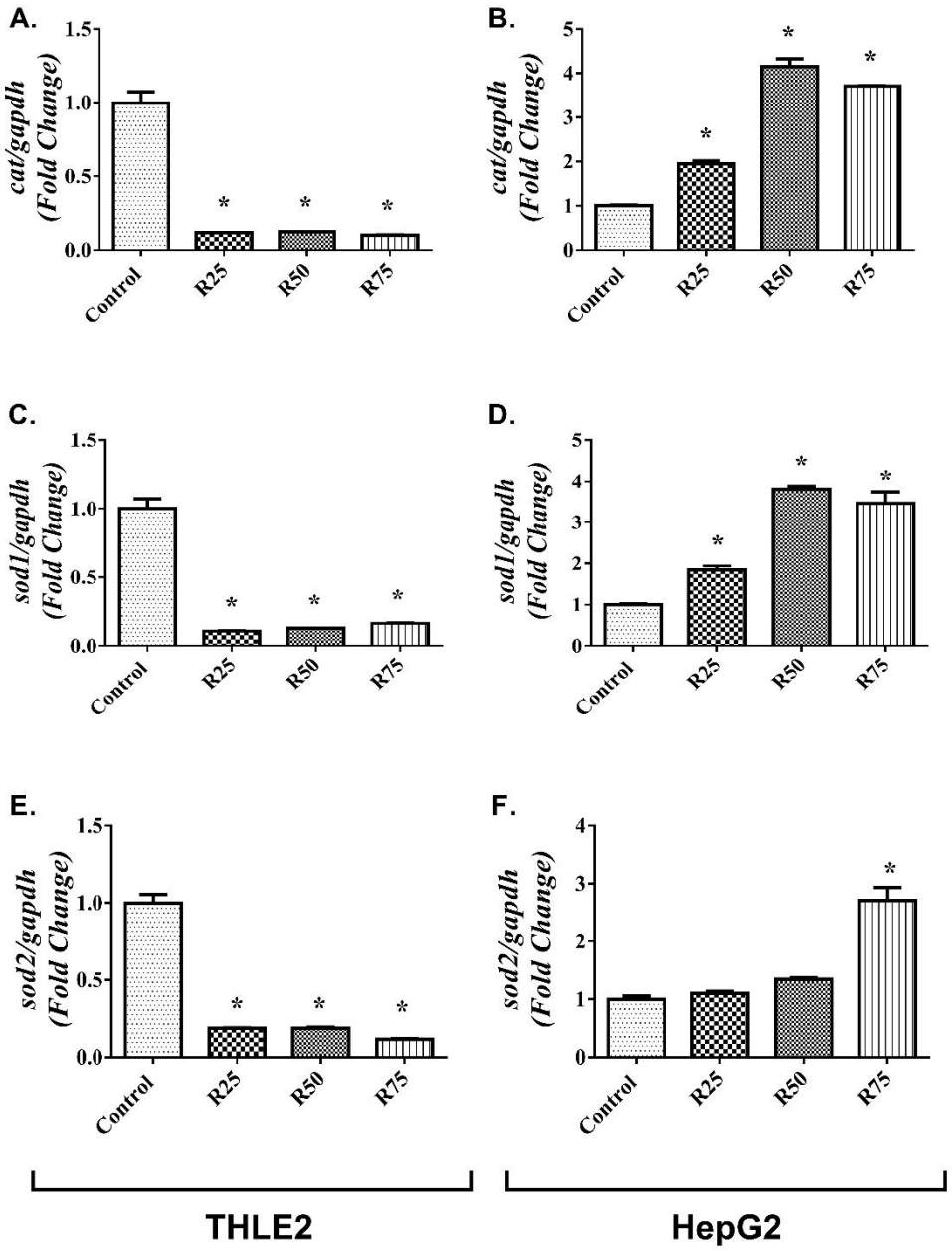

Figure 1. Genes expression levels of *cat* (A,B*), sod1* (C,D) and *sod2* (E,F) in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. The results are normalized to internal control *gapdh* gene and expressed as a fold change values over control cells. *Represents significance at $P < 0.05$ as compared with control group. All data were given as mean ± standard error of the mean (SEM) values of three biological replicates with three technical replicate measurements.

post-translational mechanisms that might stabilize the activities of CAT and GST in HepG2 cells.

4. Discussions

Cancer is a disease that spreads throughout the body from the organs and tissues in which cells divide uncontrollably. The causes of cancer have many genetic and environmental factors one of which is free radicals leading to damage to the nucleic acids, proteins, and lipids (Liou and Storz, 2010). Recent studies postulated that oxidative stress is a major contributor to carcinogenesis since it disrupts several intracellular signal transduction pathways (Alves et al., 2021) and it promotes carcinogenesis by providing sufficient components for cancer cell survival. Thus, cancer cells, in turn, might benefit from the advantages of elevated levels of free radicals to keep their survival, by adapting the content and regulation of their antioxidant machinery (Moloney and Cotter, 2018). Any pharmacological agent that prevents the build-up of radicals might sequentially increase the antioxidant capacity of cancer cells which could prevent the progression and induce apoptotic processes (Moloney and Cotter, 2018).

A plant-derived polyphenol, trans-resveratrol, exhibits strong antioxidant properties and has been previously shown in the prevention and treatment of cancer complications (Ko et al., 2017). It acts on cancer cells by

activating or deactivating molecular pathways and modulating the antioxidant enzymes' expression in diseases conditions such as cancer and diabetes (Khan et al., 2013; Sadi et al., 2013). Thus, inducing the expression of enzymatic antioxidants or enhancing their activities might be beneficial cancer prevention and treatment strategy. Despite the promising value of resveratrol to suppress tumor growth in various cancer research, the precise mechanism of anti-proliferative effects of resveratrol needs to be inspected in detail. Therefore, this study is conducted to reveal the modulatory effects of resveratrol over main antioxidant enzymes in both healthy and cancerous liver cells to enlighten the exact molecular mechanisms for antiproliferative action over cancer cells.

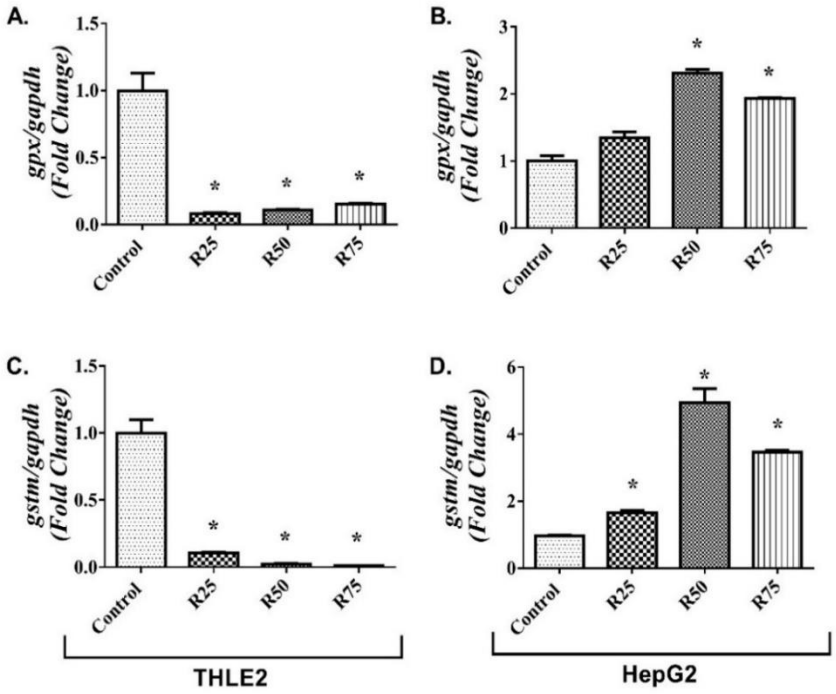

Figure 2. Genes expression levels of *gpx* (A,B*)* and *gstm* (C,D) in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. The results are normalized to the internal control *gapdh* gene and expressed as a fold change values over control cells. *Represents significance at $P < 0.05$ as compared with the control group. All data were given as mean ± standard error of the mean (SEM) values.

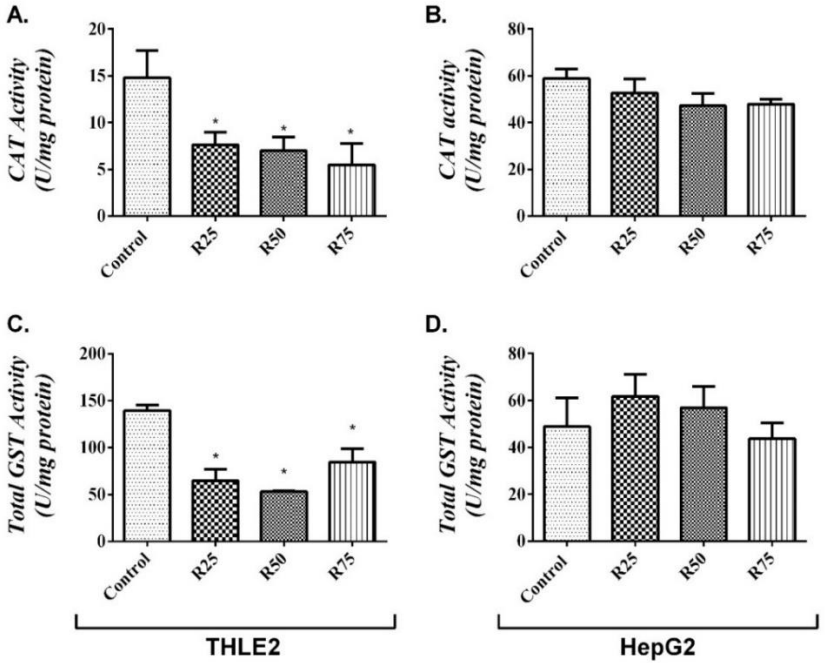

Figure 3. Specific activities of CAT (A,B) and GST (C,D) enzymes in resveratrol treated THLE2 and HepG2 cells. R25: cells treated with 25 µM resveratrol; R50: cells treated with 50 µM resveratrol; R75: cells treated with 75 µM resveratrol. *Represents significance at $P < 0.05$ as compared with the control group. All data were given as mean ± standard error of the mean (SEM) values.

Herein, we used THLE2 cells as a control cell line to distinguish the effects of resveratrol on cancer cells (HepG2) from that on non-cancerous cells. The results demonstrated significant up-regulation of main antioxidant enzymes with resveratrol in cancerous HepG2 cells at gene expression levels in a dose-dependent manner due to a compansatory mechanism for increased oxidative stress levels. On the contrary to this result, gene expressions of antioxidant enzymes were down-regulated significantly in THLE2 cells with all used doses of resveratrol. We also noticed that the reduction in gene expression in control cells was in line with the enzymatic activities of CAT and GST enzymes, however, enzyme activity in HepG2 cells did not change significantly.

Simultaneous up-regulation of *cat*, *sod1*, *sod2*, and *gstm* in cancer cells, and their disproportional suppression in healthy cells would be valuable for cancer pharmacotherapy. Since free radicals keep the cancer cells malignant and provide resistance to normal cell cycle arrest mechanisms. Low radical content would lead to lower levels of DNA damage and genetic instability in cancer cells which might in turn decrease cell survival and proliferation. Teoh-Fitzgerald and coworkers have demonstrated that overexpression of SOD3 decreased breast cancer metastasis in vivo (Teoh-Fitzgerald et al., 2012). Besides, inhibition of cell growth with SOD2 overexpression in human pancreatic carcinoma (Ough et al., 2004), and suppression of the malignant phenotype in pancreatic cancer by overexpression of GPx glutathione peroxidase (Graves et al., 2006) supports our hypothesis that reducing radicals in cancer cells by up-regulating antioxidant enzymes might inhibit pro-tumorigenic signaling pathways in the treatment of cancer. We can better understand the significance of enhanced free radical generation in cancer and identify particular target pathways to more effectively treat cancer using this information.

In conclusion, the prevention of oxidative stress, inflammation, and cancer-cell proliferation, as well as the activation of tightly regulated cell-death processes, are all roles of resveratrol in preventing carcinogenesis. Because of the intricacy and number of biological systems involved, further research is needed to completely understand how resveratrol can be used to prevent the development and progression of cancer.

Conflict of Interest

Authors have declared no conflict of interest.

Authors' Contributions

AB: performed qPCR experiments, ENŞ: conducted cell culture studies, AK: measured the enzyme activities, GS: designed and supervised the study, made data analysis and wrote the manuscript.

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