Research Article

Evaluation of Methylation and Acetylation Profiles of Dinitroaniline Herbicides and Resveratrol on V79 Cell Line

Dinitroanilin Herbisitlerin ve Resveratrolün Metilasyon ve Asetilasyon Profillerinin V79 Hücre Hattında Değerlendirilmesi

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ABSTRACT
INTRODUCTION: Herbicides are one of the most widely used pesticide compounds for plant growth control worldwide. Risk assessment of dinitroanilin derived herbicides, pendimethalin and trifluralin, is important for foodborne or other means of exposure. In this study, we aimed to evaluate the methylation and acetylation profiles of pendimethalin and trifluralin, which we have high levels of exposure in various ways. Furthermore, it was aimed to determine the protective effect of resveratrol, an antioxidant compound, against the possible toxic effects of these pesticides.
METHODS: The effects of pendimethalin and trifluralin alone (25, 50, 100 µM) and in combination with resveratrol (100 µM) on DNA methyltransferase (DNMT) 1, 3a, 3b; histone deacetylase (HDAC)1 and HDAC3 gene expressions were evaluated by RT-PCR.
RESULTS: : According to the results of the experiment, pendimethalin caused a significant decrease in DNMT and HDAC expressions at all concentrations, whereas HDAC1 and 3 expressions were increased at the concentration of 25 µM, when applied together with resveratrol. Additionally, there is no change in DNMT1 and 3b expression levels. Unlike pendimethalin, trifluralin increased DNMT1 expression in concentration dependent manner. While DNMT3a and DNMT3b expression levels increased significantly, HDAC1 and 3 expression levels did not change significantly. The expression levels of HDAC1 and HDAC3 increased at all concentrations of trifluralin combination with resveratrol. Also, DNMT levels increased at the concentrations of 50 and 100 µM.

DISCUSSION AND CONCLUSION: According to the epigenetic gene expression results, pendimethalin and trifluralin may cause tissue function loss and chromosome damage as a result of direct effects on cell viability by causing expression level changes in all studied genes. Also, it can be concluded that the changes that occur in gene expressions may induce tumor development. Further studies are needed to elucidate the possible toxicity mechanisms of these herbicides considering the relationship between epigenetic changes and various diseases.
Keywords: pendimethalin, trifluralin, epigenetic, DNA methyltransferase, histon deacetylase

ÖZ
GİRİŞ ve AMAÇ: Herbisitler, bitki büyüme kontrolü için dünya genelinde en yaygın kullanılan pestisit bileşiklerindendir. Dinitroanilin türevi herbisitlerden olan pendimetalin ve

trifluralinin risk değerlendirmesinin yapılması, gıda kaynaklı veya diğer yollardan gerçekleşen maruziyetler açısından önemlidir. Bu çalışmada, çeşitli yollarla yüksek düzeylerde maruz kaldığımız pendimetalin ve trifluralinin metilasyon ve asetilasyon profillerini değerlendirmeyi amaçladık. Ayrıca, bir antioksidan bileşik olan resveratrolün, bu pestisitlerin olası toksik etkilerine karşı koruyucu etkisinin belirlenmesi amaçlanmıştır.
YÖNTEM ve GEREÇLER: Pendimetalin ve trifluralinin tek başlarına (25, 50, 100 µM) ve resveratrol (100 µM) ile kombinasyon halinde DNA metiltransferaz (DNMT) 1, 3a, 3b; histon deasetilaz (HDAC)1 ve HDAC3 gen ekspresyonları RT-PCR yöntemiyle değerlendirilmiştir.
BULGULAR: Deney sonuçlarına göre pendimetalin tüm konsantrasyonlarda DNMT ve HDAC ekspresyonlarında anlamlı ölçüde azalmaya neden olurken, resveratrol ile birlikte uygulandığında HDAC1 ve 3 ekspresyonları 25 µM konsantrasyonunda artmıştır. İlave olarak, DNMT1 ve 3b seviyelerinde değişiklik olmamıştır.
Trifluralin pendimetalinden farklı olarak, DNMT1 ekspresyonunu konsantrasyonla orantılı olarak arttırmıştır. DNMT3a ve DNMT3b ekspresyon seviyelerinde de anlamlı artış gözlenirken, HDAC1 ve 3 seviyelerinde anlamlı değişiklik gözlenmemiştir. Resveratrol ile kombinasyon halinde ise, HDAC1 ve HDAC3 tüm konsantrasyonlarda artış göstermiştir. Ayrıca, DNMT seviyeleri 50 ve 100 µM konsantrasyonlarında artmıştır.

TARTIŞMA ve SONUÇ: Epigenetik gen ekspresyonu sonuçlarına göre, pendimetalin ve trifluralin, çalışılan tüm genlerde ekspresyon seviyesi değişikliklerine neden olarak hücre canlılığı üzerindeki doğrudan etkilerinin bir sonucu olarak doku fonksiyon kaybına ve kromozom hasarına neden olabilir. Ayrıca, gen ifadelerinde meydana gelen değişikliklerin tümör gelişimini tetikleyebileceği sonucuna varılabilir. Epigenetik değişikliklerin çeşitli hastalıklarla ilişkisi düşünülerek bu herbisitlerin olası toksisite mekanizmalarının aydınlatılması için ileri çalışmalara ihtiyaç bulunmaktadır.
Anahtar Kelimeler: pendimetalin, trifluralin, epigenetik, DNA metiltransferaz, histon deasetilaz
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1. Introduction

The most important problem of people has been to produce enough nutrients since the establishment of resident life. For this purpose, it is necessary to eliminate insects, fungi, weeds and other harmful organisms that damage the crops in order to increase the quantity and quality of the product. It is also important to combat these pests in terms of health, given the fact that these creatures spread diseases [1]. Although the use of pesticides is necessary, toxic effects can be observed in organisms and in the environment as a result of widespread and uncontrolled use. As a result of incorrect or careless use of pesticides, cases of mass poisoning can occur. Also, long-term pesticide exposure is related to cancer, immune system damage and reproductive toxicity [2].

The herbicides, which are among the most commonly used pesticide compounds in the world, are chemical compounds or cultured biological organisms controlling or suppressing plant growth [3]. Pendimethalin and trifluralin are dinitroaniline herbicides which provide the control of certain broad-leaf and grassy weeds inhibiting mitosis [4-6]. These herbicides have been used

on vegetables, tobacco, oil seed, ornamentals, tomatoes, cotton for a long time [4,5]. For this reason, they can affect the health by environmental pollution or diet [7].

Pendimethalin and trifluralin synthesis can cause the formation of reactive compounds known as nitrosamines. Nitrosamines are alkylating agents and can cause DNA damage by formation of adducts [8]. Additionally, epigenetic changes, which are basically related to DNA methylation and histone acetylation mechanisms, are as important as genetic changes because of 1 genome / n epigenomes relation. The genome-epigenome relationship is thought to play an active role in basic biological functions such as cell viability, cell division, cell differentiation and phenotypic changes [9]. Although epigenetic research has focused on embryonic development, aging and cancer, recent research has been advancing in various areas such as immune system, cardiovascular, neurodegenerative diseases, obesity and diabetes [10,11].

In this study, the epigenetic potential of pendimethalin and trifluralin on Chinese Hamster Lung Fibroblast (V79) cells were investigated. We evaluated the DNA methyl transferase (DNMT) 1, 3a, 3b, and histone deacetylase (HDAC) 1 and 3 levels on V79 cells after 24 h treatment of pendimethalin and trifluralin at the concentrations of 25, 50 and 100 µM, which are determined by the our previous study results of neutral red uptake (NRU) assay and comet assay [12]. Effects of resveratrol, a strong antioxidant compound were also examined at the concentration of 100 µM.

2. Materials and methods

2.1. Pendimethalin, trifluralin and resveratrol solution preparation

Pendimethalin (98.8% purity, CAS NO: 40487-42-1), trifluralin (98.8% purity, CAS NO: 1582-09-8) and resveratrol (99% purity, CAS NO: R5010) were purchased from Sigma – Aldrich. Pendimethalin stock solution (500 mM) was prepared in dimethyl sulfoxide (DMSO) : olive oil (1:3, v/v), trifluralin (500 mM) and resveratrol stock solution (0,5 mM) were prepared in phosphate buffered saline (PBS) containing DMSO (final DMSO concentration was 1% (v/v)).

2.2. Cell culture

The V79 cells obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) were incubated in RPMI 1640 medium supplemented with 1% penicillin-streptomycin solution, 10% heat-inactivated fetal bovine serum (FBS) (Lot:094M3288) and 2 mM L - glutamin at 37°C and 5% CO_2 for 24 h. After 24 h, the cells were harvested and were transferred to 6 well-plates as 30.000 cell/2ml medium of each. Pendimethalin and trifluralin solutions were added to the wells as the last concentrations were 25, 50 and 100 µM after 24 h. Also, 100 µM resveratrol was used as a single concentration and additionally added to the concentrations of pendimethalin and trifluralin. For the negative control, 1% DMSO, 1% DMSO - 3% olive oil were used. The cells were incubated for 24 h and harvested from the wells and centrifugated at 1000 rpm for 5 min.

2.3. Evaluation of gene expression profiles by RT-PCR assay

RNA isolation were performed according to the instructions of RNeasy Mini Kit (QIAGEN). The cell suspensions were filtrated using gDNA eliminator column, after the centrifugation. Then transferred to RNeasy spin column and washed with the solutions as given in the kit procedure.

The measurement of amount and quality of the eliminated RNA samples were performed by Maestrogen Nanodrop. Briefly, 1 µl of the sample was loaded to the base portion fiber terminal. All the samples' OD 260/280 ratio were found in the range of 1,6-1,8.

For the purpose of synthesize cDNA from the RNA samples, RT^2 First Strand Kit (QIAGEN) was used according to instructions. The denaturation of the RNA samples were performed at 42°C for 5 min in qRT-PCR device. To protect the linearity, the samples were placed to cold surface. After that, reverse-transcription enzymes were added to the samples and the cDNA synthesis process was performed at 42°C for 15 minutes and 90°C for 5 minutes. The

synthesized cDNA samples were stored at -20°C. The PCR primers used in this study were listed in Table 1.

For measuring the expression levels of genes, cDNA samples were mixed with RT² SYBR Green qPCR mastermix, RT² qPCR primers (DNMT1, DNMT3a, DNMT3b, HDACI, HDACIII, PPIA) and the expression performed with qRT-PCR device under the conditions of hold 95°C 15 min, cycle 95°C 15 sec and 60°C 30 sec, for 40 cycles. The results were recorded at 60°C. The threshold limit was set to 0,05 and the Ct values of the samples were calculated.

(Table 1. is here)

2.4. Statistical analysis

Statistics of the Ct values were prepared with the on-line based program RT^2 Profiler PCR Data Analysis 3.5. ΔΔCt method was used to interpret the gene expression data [15]. When evaluating the results, the upper limit CT value was taken as 35. Values higher than 35 were also evaluated as 35. All experiments were performed twice.

3. Results

3.1. Effects of trifluralin on gene expression

According to the ΔΔCT values, DNMT1 expression in V79 cells is increased at a higher level and concentration relative to the control group by 24 h incubation of trifluralin. The levels of DNMT3a and 3b only increased significantly at high concentration. There were no significant changes in HDACI and III levels.

When resveratrol was administered alone, the levels of DNMT1, 3a, 3b, HDAC I increased significantly compared to the control, but HDAC III levels remained unchanged.

Furthermore, when trifluralin and resveratrol were co-administered, HDAC I and III expressions were significantly increased at all concentrations. DNMT levels were increased in 25 and 50 μM trifluralin and resveratrol, whereas 100 μM trifluralin and resveratrol were low in expression.

Generally, when the results of fold regulation and biological significance of trifluralin and resveratrol were examined, significant increase in expression was observed in all genes except HDAC III when resveratrol was administered alone. It was observed that trifluralin generally decreased HDAC I and III expressions. When trifluralin combined with resveratrol, it was observed that it caused an increase in HDAC I and III expression, except 100 μM trifluralin and resveratrol administration. Additionally, DNMT1 showed a significant increase in all studied concentrations, whereas DNMT3a and 3b expression levels increased when 100 μM trifluralin was given. DNMT3b decreased at all concentrations when co-administered with resveratrol, while DNMT3a was significantly reduced only when 25 μM trifluralin and 100 μM resveratrol co-administered.

The ΔCT, ΔΔCT, fold change and fold regulation values of genes were given as Table 2-5.

(Table 2-5 are here)

3.2. Effects of pendimethalin on gene expression

When ΔΔCT values were compared, it was observed that DNMT and HDAC expressions were significantly decreased in all concentrations of pendimethalin. Also, DNMT1, HDACI and III expressions were increased significantly at only 25 μM pendimethalin concentration when given together with resveratrol. However, all gene expressions were significantly increased when resveratrol was administered alone.

When pendimethalin and resveratrol fold-regulation and biological significance results were evaluated, it was found that pendimethalin caused a significant decrease in expressions of all genes in all concentrations, whereas resveratrol increased expressions in all genes when administered alone.

Additionally, when biological significance of pendimethalin and resveratrol co-administered concentrations were evaluated, HDACI and III expressions were increased with the effect of resveratrol at a concentration of 25 μM of pendimethalin, but DNMT3a levels were

significantly decreased. There were no changes in DNMT1, 3b and HDACIII levels, while other gene expressions were significantly reduced, when 50 μM pendimethalin and 100 μM resveratrol were co-administered.

The ΔCT, ΔΔCT, fold change and fold regulation values of genes were given as Table 6-9.

(Table 6-9 are here)

4. Discussion

Although genetic material, which is the source of information and life of organisms, is very well protected against degradation by various mechanisms, it may be damaged by exposure to many factors, both internal and external. The DNA repair mechanisms are very active, but they can not be sufficient or repressed in some cases. These damages have temporary or permanent effects and may cause minor or major dysfunctions and diseases in the organism and affect future generations besides the first affected organism.

Within the scope of this study, the possible epigenetic effects of pendimethalin and trifluralin, one of the herbicide compounds that we are frequently exposed to in our country and all over the world, were investigated in V79 cell line. It has been evaluated whether resveratrol, an antioxidant substance, has a protective effect on possible methylation and acetylation profile changes of these herbicides.

DNMT and HDAC expressions were examined to investigate the effects of pendimethalin and trifluralin, one of the dinitroaniline herbicide compounds and whose genotoxicity potentials were determined [12], on epigenetic changes. According to genotoxicity results, the concentrations of pendimethalin and trifluralin as 25, 50 and 100 μM were selected for study.

While pendimethalin results showed a significant decrease in DNMT levels, trifluralin increased DNMT1 expression and increased all of the DNMT genes at a concentration of 100 μM, causing a decrease in all other genes. Embryo death was observed in mice with increased methylation in DNMT1 gene disorder. Changes in DNMT1 expression lead to X chromosome inactivation and imprinting loss [16]. Disorders in DNMT1 gene expression cause proliferation disorders and mitotic defects leading to cell death. These effects in human colorectal cancer cells have been clearly observed [17]. Similarly, it was reported that mouse fibroblast cells with DNMT1 defect were dragged into apoptosis via p-53 pathway after several cell division [18], and apoptosis was observed as a result of decrease in DNMT1 expression in germ cells [19]. Studies have shown that the DNMT1 gene plays a critical role cell proliferation and viability. In addition, DNMT1 function loss was directly associated with tumor formation, demonstrating that tumor growth and chromosome instability in DNMT1 deficient mice [20,21].

Similar to DNMT1, DNMT3a and 3b have also been reported to play a critical role in embryonic development in mice. It was observed that mouse embryos with DNMT3b deficiency died on 9.5 embryonic days and multiple developmental defects occurred, pups without DNMT3a deficiency did not develop and died shortly after birth [22]. Mutations in the DNMT3b gene in humans are cause of a rare autosomal disease ICF-Immune Deficiency Syndrome, centromere stability disorder, facial abnormalities syndrome [23]. Furthermore, mutations in the DNMT3b gene cause a decrease in DNA methylation specific to pericentromeric regions on chromosomes 1, 9 and 16, leading to chromosomal structure and function disorders [24].

CpG methylation levels were found to be increased in lung cancer patients on two genes SFTPA1 and SFTPA2, which encode surfactant protein A associated with lung homeostasis and immunity [25]. In another study, when epigenetic changes were examined in 28 non-smoker lung adenocarcinoma patients, it was found that methylation levels decreased in tumor tissues compared to neighboring non-malignant tissues and methylation increased in tumor tissues in CpG islands [26]. It was observed that these findings were consistent with the results our obtained, and pendimethalin and trifluralin compounds significantly changed methylation levels.

When we evaluated HDAC gene expression levels, it was seen that both herbicidal compounds cause significant decrease in HDACI and III levels. HDACI and III consist of 93% structurally

the same proteins are belong to the class I histone deacetylases group [27,28]. These genes are related to cell cycle control, cell survival and differentiation. For this reason, the use of HDAC inhibitors for the treatment of cancer as an antineoplastic drug is contemplated [29,30]. In a study of non-small lung cancer cells, it was observed that HDAC levels were increased in cancer cells and it was possible to fight cancer cells when using HDAC inhibitors [31]. However, these results are not consistent with our previous study which is about the effects of pendimethalin and trifluralin on apoptosis and anti-apopitosis genes (p53, bax, bcl-2, casp3, casp9, birc). According to our results, trifluralin down regulated all gene expressions (1-500 µM), but pendimethalin up regulated bcl-2 (100 and 500 µg/ml) and birc5 (500 µg/ml) gene expressions and has more effects on anti – apopitosis than trifluralin [32]. These differences of results confirm that in order to reduce the possible carcinogenic effects of pendimethalin and trifluralin on humans, the permissible values and residual limits on foods should not be exceeded.

When the change in epigenetic expressions of resveratrol applied concentrations was examined, the capacity of resveratrol supplementation to reverse the expression changes caused by the studied herbicides was limited. Additionally, normal gene expression levels were not achieved despite resveratrol, especially in HDAC genes. Also, administration of resveratrol alone led to undesirable increases in gene expressions may be the result of pro-oxidant effect of resveratrol [33].

5. Conclusion

Methylation and deacetylation gene expressions are among the main pathways of epigenetic changes and they are the main causes of embryonic development disorders and chronic diseases. According to the epigenetic gene expression results, pendimethalin and trifluralin may cause tissue function loss and chromosome damage as a result of direct effects on cell viability by causing expression level changes in all studied genes. Since the group of cells we studied are healthy lung fibroblast cells, it can be concluded that the changes that occur in gene expressions may induce tumor development. Considering the concentrations exposed, the genotoxic effects appear to be high. However, both herbicidal compounds we investigated are considered by Group C, as a possible human carcinogen by Environmental Protection Agency (EPA).

In addition to the beneficial effects of antioxidants such as resveratrol against oxidative DNA damage, there is also the risk of causing damage by pro-oxidant effects. Therefore, the use of dinitroaniline herbicides with high genotoxicity and epigenotoxicity potentials should be considered carefully and all the effects of antioxidant compounds should be examined in more detail.

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Gene	Forward	Reverse
DNMT1	5'-AAC CTT CAC CTA GCC CCA G-3'	5'-CTC ATC CGA TTT GGC TCT TCA-3'
DNMT3a	5′-CGA CCC ATG CCA AGA CTC ACC TTC CAG-3′	5’- CCT GGT GGA ATG CAC TGC AGA AGG A-3’
DNMT3b	5’-TAC ACA GAC GTG TCC AAC ATG GGC-3’	5’-GGA TGC CTT CAG GAA TCA CAC CTC-3’
HDACI	5′-CTG TCC GGT ATT TGA TGG CT-3′	5′-CAC GAA CTC CAC ACA CTT GG-3′
HDACIII	5′-TCT GAG GAC TAC ATC GAC TCC-3′	5′-GTC GCC ATC ATA GAA CTC AT TG-3′
PPIA	5’-ATG GTC AAC CCC ACC GTG T-3’	5’-TCT GCT GTC TTT GGG ACC TTG TC-3’

Table 1. Gene sequences of primers [13,14]

Table 2. ΔCT values of trifluralin and resveratrol (The values are expressed in mean ± standart deviation format. The control gene PPIA value was taken as 0. The concentrations (25, 50 and 100 µM) of trifluralin were showed as t 25, t 50 and t 100. The concentration (100 µM) of resveratrol was showed as r 100.)

Gene	Control (1% DMSO)	r 100	t 25	t 50	t 100	t 25 + r 100	t 50 + r 100	t 100 + r 100
PPIA	0	0	0	0	0	0	0	0
DNMT1	11,29 ± 2,55	3,465 ± 19,70	6,755 ± 0,346	5,085 ± 2,84	4,67 ± 0,226	3,855 ± 16,94	2,07 ± 14,07	4,785 ± 14,61
DNMT3a	5,68 ± 13,94	2,48 ± 21,10	6,754 ± 0,347	6,375 ± 1,02	4,68 ± 0,225	9,375 ± 22,21	6,4 ± 17,90	6,42 ± 13,50
DNMT3b	7,3 ± 14,96	3,18 ± 20,11	6,756 ± 0,344	6,374 ± 1,02	4,65 ± 0,224	9,135 ± 22,54	9,66 ± 18,76	24,305 ± 3,81
HDACI	5,275 ± 13,88	2,83 ± 20,60	6,753 ± 0,347	6,373 ± 1,01	4,68 ± 0,227	(-) 3,93 ± 5,99	(-) 6,905 ± 5,16	(-) 7,11 ± 21,41
HDACIII	2,54 ± 8,71	2,565 ± 19,93	6,755 ± 0,345	6,376 ± 1,04	4,69 ± 0,223	(-) 6,36 ± 1,61	(-) 7,875 ± 0,86	(-) 6,115 ± 3,58

Gene	Control (1% DMSO)	r 100	t 25	t 50	t 100	t 25 + r 100	t 50 + r 100	t 100 + r 100
PPIA	1	1	1	1	1	1	1	1
DNMT1	0,000399	0,090559	0,009259	0,029462	0,039282	0,069108	0,238159	0,036272
DNMT3a	0,019505	0,179244	0,009258	0,012049	0,039283	0,001506	0,011842	0,011679
DNMT3b	0,006346	0,110338	0,009260	0,012048	0,039280	0,001779	0,001236	0
HDACI	0,025827	0,140632	0,009257	0,012047	0,039283	15,242208	119,842848	70,007239
HDACIII	0,171943	0,168989	0,009259	0,012050	0,039284	82,139257	234,753035	69,310403

Table 3. ΔΔCT values of trifluralin and resveratrol (The values are expressed as ΔΔCT values. The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of trifluralin were showed as t 25, t 50 and t 100. The concentration (100 µM) of resveratrol was showed as r 100.)

Gene	r 100	t 25	t 50	t 100	t 25 + r 100	t 50 + r 100	t 100 + r 100
PPIA	1	1	1	1	1	1	1
DNMT1	226,7565 +	23,1831 +	73,7719 +	98,36 +	173,0446 +	596,3436 +	90,8239 +
DNMT3a	9,1896 +	0,4747 *	0,6177	2,0139 +	0,0772 *	0,6071	0,5987
DNMT3b	17,3878 +	1,459	1,8987	6,1903 +	0,2803 *	0,1948 *	0 *
HDACI	5,4453 +	0,3585 *	0,4665 *	1,521	590,1754 +	4640,2924 +	380,2803 +
HDACIII	0,9828	0,0538 *	0,0701 *	0,2285 *	477,7129 +	1365,2978 +	403,1017 +

Table 4. The fold change values of trifluralin and resveratrol. (The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of trifluralin were showed as t 25, t 50 and t 100. The concentration (100 µM) of resveratrol was showed as r 100. The significant increase in gene expression was showed with +, the decrease in gene expression was showed with * symbol. $p < 0,05$ means significantly different from negative control.)

Gene	r 100	t 25	t 50	t 100	t 25 + r 100	t 50 + r 100	t 100 + r 100
PPIA	1	1	1	1	1	1	1

DNMT1	226,757 +	23,1831 +	73,7719 +	98,36 +	173,0446 +	596,3436 +	90,8239 +
DNMT3a	9,1896 +	-2,1067 *	-1,6189	2,0139 +	-12,9511 *	- 1,6472	-1,6702
DNMT3b	17,3878 +	1,459	1,8987	6,1903 +	- 3,5677 *	- 5,1337 *	-131527,049 *
HDACI	5,4453 +	-2,7895 *	-2,1435 *	1,521	590,1754 +	4640,2924 +	-3,5677 *
HDACIII	-1,0175	-18,571 *	-14,271 *	- 4,3772 *	477,7129 +	1365,2978 +	403,1017 +

Table 5. The fold regulation values and biological significance of trifluralin and resveratrol (The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of trifluralin were showed as t 25, t 50 and t 100. The concentration (100 µM) of resveratrol was showed as r 100. The significant increase in gene expression was showed with +, the decrease in gene expression was showed with * symbol. p <0,05 means significantly different from negative control.)

Gene	**Control (1% DMSO + %3 olive oil)**	**p 25**	**p 50**	**p 100**	**p 25 + r 100**	**p 50 + r 100**	**P 100 + r 100**	**r 100**
PPIA	0	0	0	0	0	0	0	0
DNMT1	1,27 ± 11,07	8,88 ± 2,12	11,13 ± 0,65	7,58 ± 0,52	0,98 ± 13,74	6,98 ± 0,12	7,33 ± 2,03	(-) 10,47 ± 0
DNMT3a	5,545 ± 16,22	10,28 ± 0,14	11, 835 ± 0,34	13,33 ± 0,41	18,09 ± 2,39	14,86 ± 0,18	12,58 ± 1,86	2,11 ± 20,57
DNMT3b	5,36 ± 18,69	10,215 ± 0,049	11, 836 ± 0,33	13,32 ± 0,40	5,43 ± 21,75	15,025 ± 0,049	15,215 ± 0,64	2,81 ± 19,58
HDACI	2,405 ± 15,37	10,27 ± 0,13	11, 834 ± 0,34	13,34 ± 0,41	(-) 8,165 ± 2,05	13,83 ± 1,06	14,26 ± 0,70	(-) 11,74 ± 0
HDACIII	8,485 ± 0,34	9,53 ± 0,91	11,02 ± 0,80	10,765 ± 1,05	(-) 0,545 ± 11,32	9,175 ± 0,17	8,395 ± 1,09	2,21 ± 20,43

Table 6. ΔCT values of pendimethalin and resveratrol (The values are expressed in mean ± standart deviation format. The control gene PPIA value was taken as 0. The concentrations (25, 50 and 100 µM) of pendimethalin were showed as p 25, p 50 and p 100. The concentration (100 µM) of resveratrol was showed as r 100.)

Gene	Control (1% DMSO + %3 olive oil)	p 25	p 50	p 100	p 25 + r 100	p 50 + r 100	P 100 + r 100	r 100
PPIA	1	1	1	1	1	1	1	1
DNMT1	0,41466	0,002123	0,000446	0,005226	0,50698	0,007922	0,006215	1418,352095
DNMT3a	0,021418	0,000804	0,000274	0,000097	0,000004	0,000034	0,000163	0,231647
DNMT3b	0,024349	0,000841	0,000275	0,000096	0,023196	0,00003	0,000026	0,142595
HDACI	0,188809	0,000803	0,000273	0,000098	287,018516	0,000069	0,000051	3420,520118
HDACIII	0,002791	0,001353	0,000482	0,000575	1,45902	0,00173	0,002971	0,216134

Table 7. ΔΔCT values of pendimethalin and resveratrol (The values are expressed as ΔΔCT values. The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of pendimethalin were showed as p 25, p 50 and p 100. The concentration (100 µM) of resveratrol was showed as r 100.)

Gene	p 25	p 50	p 100	p 25 + r 100	p 50 + r 100	p 100 + r 100	r 100
PPIA	1	1	1	1	1	1	1
DNMT1	0,0051 *	0,0011 *	0,0126 *	1,2226	0,0191 *	0,015 *	3420,52 $^{+}$
DNMT3a	0,0376 *	0,0128 *	0,0045 *	0,0002 *	0,0016 *	0,0076 *	10,8153 $^{+}$
DNMT3b	0,0346 *	0,0112 *	0,004 *	0,9526	0,0012 *	0,0011 *	5,8563 $^{+}$
HDACI	0,0043 *	0,0014 *	0,0005 *	1520,1521 $^{+}$	0,0004 *	0,0003 *	18116,3 $^{+}$
HDACIII	0,4846 *	0,1725 *	0,2059 *	522,7582 $^{+}$	0,6199 *	1,0644	77,4396 $^{+}$

Table 8. The fold change values of pendimethalin and resveratrol. (The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of pendimethalin were showed as p 25, p 50 and p 100. The concentration (100 µM) of resveratrol was showed as r 100. The significant increase in gene expression was showed with +, the decrease in gene expression was showed with * symbol. p <0,05 means significantly different from negative control.)

Gene	p 25	p 50	p 100	p 25 + r 100	p 50 + r 100	p 100 + r 100	r 100
PPIA	1	1	1	1	1	1	1
DNMT1	-195,361 *	-929,3 *	-79,3413 *	1,2226	-52,3457 *	-66,7178 *	3420,52 +
DNMT3a	-26,6304 *	-78,249 *	-220,5558 *	-5976,1473 *	-636,934 *	-131,1433 *	10,8153 +
DNMT3b	-28,9401 *	-88,955 *	-250,7316 *	-1,0497	-811,811 *	-926,0845 *	5,8563 +
HDACI	-234,753 *	-689,78 *	-1944,2527 *	1520,1521 +	-2749,5885 *	-3704,3379 *	18116,3 +
HDACIII	-2,0634 *	-5,7958 *	-4,8568 *	522,7582 +	-1,6133	1,0644	77,4396 +

Table 9. The fold regulation values and biological significance of pendimethalin and resveratrol (The control gene PPIA value was taken as 1. The concentrations (25, 50 and 100 µM) of pendimethalin were showed as p 25, p 50 and p 100. The concentration (100 µM) of resveratrol was showed as r 100. The significant increase in gene expression was showed with +, the decrease in gene expression was showed with * symbol. p <0,05 means significantly different from negative control.)