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# Cytotoxic and apoptotic effects of hydroxytyrosol on ovarian cancer cell lines: relationship of cytotoxic effect with ERK 1/2 pathway

## Yumurtalık kanseri hücre hatları üzerinde hidroksitirozolün sitotoksik ve apoptotik etkileri: sitotoksik etkinin ERK 1/2 yolağı ile ilişkisi

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**SUMMARY**

**Objective:** It has been shown that hydroxytryrosol (HT) has antitumoral, anti-proliferative and apoptotic effects on various cancer cell line types. In this study, we investigated cytotoxic and apoptotic effects of HTon human ovarian cancer cell lines OVCAR-3 and MDAH-2774 and relationship of its cytotoxic effects with phosphatidylinositol 3-kinase/Akt (PI3K/Akt) and extracellular signal-regulated kinase 1/2 (ERK 1/2) signaling pathways.

**Method:** XTT cell proliferation kit, Cell Death Detection Elisa Plus Kit and Human Apoptosis Array test were used to determine the cytotoxic and apoptotic effects of HT in OVCAR-3 and MDAH-2774 cell lines at 24, 48, 72 and 96 hours (h). Effect of HT on PI3K/Akt and ERK 1/2 signaling pathways were investigated by using specific inhibitors of these pathways.

**Results:** $IC_{50}$ values of HT were found to be 102.3 µM in MDAH-2774 cells at 72 h and 51.5 µM in OVCAR-3 cells at 96 h. Highest apoptotic effect was seen at 100 µM concentration in OVCAR-3 cells at 96 h and at 50 µM at 72 h in MDAH-2774. HT increased the levels of pro-apoptotic molecules including Bad, Bax, active caspase-3, Htra2/Omi and of proapoptotic death receptors TRAIL R1/DR4, TRAIL R2/DR5, FAS/TNFRSF6 and decreased Survivin levels in MDAH-2774 cells. In OVCAR-3 cells, HT decreased Bcl-2, pro-caspase 3 and CIAP-1, CIAP-2, XIAP, Livin, Survivin levels and increased cytochrome-c level. The cell vitality was increased in both of the cell lines as the result of the treatment with HT and FR180204.

**Conclusions:** These results show that HT has potential cytotoxic and apoptotic effect on OVCAR-3 and MDAH-2774 cells. In both cell lines, the cytotoxic effect of HT could be ocur inhibiting through ERK 1/2 signaling pathway.

**Keywords:** apoptosis; cell line; cytotoxicity; ERK 1/2 pathway; hydroxytyrosol; ovarian cancer

## ÖZET

**Amaç:** Hiroksitirozolün (HT) çeşitli kanser hücre hatları üzerindeki antitümoral, çoğalmayı inhibe etme ve apoptotik etkileri olduğu gösterilmiştir. Bu çalışmada, insan yumurtalık kanser hücre hatları OVCAR-3 ve MDAH-2774 üzerinde HT'nin sitotoksik ve apoptotik etkilerini ve onun sitotoksik etkisinin fosfotidil 3-kinaz/Akt (PI3K/Akt) ve hücre dışı sinyalle düzenlenen kinaz 1/2 (ERK 1/2) ile ilişkisini araştırdık.

**Yöntem:** HT'nin, 24, 48, 72 ve 96 saatlik OVCAR-3 ve MDAH-2774 hücre kültürleri üzerinde sitotoksik ve apoptotik etkilerini belirlemek için, XTT hücre canlılık testi, hücre ölümünü belirleyen ELISA plus ve insan apoptoz array testi kullanıldı. HT'nin sitotoksik etkisinin PI3K/Akt ve ERK 1/2 sinyal yolakları ile ilişkisi, yolakların spesifik inhibitörleri kullanılarak araştırıldı.

**Bulgular:** HT'nin $IC_{50}$ değeri MDAH-2774 hücrelerinde 72. saatte 102.3 µM, OVCAR-3 hücrelerinde 96. saatte 51.5 µM bulundu. En yüksek apoptotik etki OVCAR-3 için 96. saatte 100 µM ve MDAH-2774 için 72. saatte 50 µM'da görüldü. HT, MDAH-2774 hücrelerinde Bad, Bax, aktif kaspaz-3, Htra2/Omi'nin dahil olduğu pro-apoptotik moleküllerin seviyesini ve TRAIL R1/DR4, TRAIL R2/DR5, FAS/TNFRSF6 içeren pro-apoptotik ölüm reseptörlerinin seviyesini artırdı. OVCAR-3 hücrelerinde HT, Bcl-2, pro-caspase 3 and CIAP-1, CIAP-2, XIAP, Livin, Survivin seviyelerini azalttı ve sitokrom-c seviyesini arttırdı. Hücre canlılığı, HT ile FR180204 uygulamasının sonucunda her iki hücre hattında arttı.

**Sonuç:** Bu sonuçlar HT'nin OVCAR-3 ve MDAH-2774 hücreleri üzerinde sitotoksik ve apoptotik etki potansiyeline sahip olduğunu göstermektedir. Her iki kültür hücrelerinde HT'nin sitotoksik etkisi ERK 1/2 sinyal yolağını inhibe ederek gerçekleşiyor olabilir.


**Anahtar sözcükler:** apoptoz; ERK 1/2 yolağı; hidroksitirozol; hücre hattı; sitotoksisite; yumurtalık kanseri

## INTRODUCTION

Ovarian cancer is the most fatal cancer of all the gynaecological cancers seen worldwide in women. The fact that it does not show any symptom in the early stage and that there is no effective diagnostic method today for the early diagnosis as well as the resistance to medication are the factors that complicate the treatment[1,2]. Apart from the chromosomal deletions and mutations, the balance disorder between the vital pathways of the cell and the tumour suppressors is also considered as one of the causes of the development of ovarian cancer[1,3]. The classic treatment approach for the ovarian cancer is the use of post-surgical chemotherapeutic agents, which are platinum derivatives like the cisplatin[1]. However, in less than 40% of the cases are successful results achieved due to the resistance to drugs seen in patients prior to the treatment[4]. On the other hand, the acquired resistance in the patients who had no resistance to drugs at the outset is one of the major problems in the treatment of ovarian cancer[1]. For this reason, there is the need to research into new chemotherapeutic agents for the purpose of preventing the drug resistance in ovarian cancer. The phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathway, which is found in the downstream of the identical tyrosine kinase receptor and plays the key role in the survival of the cell in most of the ovarian cancers, and also the kinase 1/2 (ERK 1/2) signaling pathway, which is regulated by the extracellular signal, are the two major vital pathways. The direct or indirect de-regulation of PI3K/Akt signaling pathway causes the cell cycle to progress abnormally, leading to the development of cancer. Due to the fact that this pathway is hyperactivated in cancer cells, the inhibition of PI3K/Akt pathway is regarded as an important target in the treatment of cancer[3]. ERK, which is the MAPK cascade operated at most in the regulation of cell growth and differentiation, is another pathway that is continuously activated within cancer cells. The dysregulations in both of these pathways contribute to the fact that the ovarian cancer becomes more aggressive and transforms into an invasive/metastatic phenotype[5].

Hydroxytyrosol (HT) is a phenolic component found in olive oil, the antioxidant, anti-proliferative, pro-apoptotic and anti-inflammatory activities of which have been shown[6]. When oleuropein is naturally subjected to hydrolysis in the course of the ripening process of olives, HT is released through the elenolic acid[7]. It has been reported in various *in vitro* and *in vivo* studies that HT has an anti-cancer effect with its cytotoxic and apoptotic characteristics and that it also plays an anti-tumoral role in the prevention of angiogenesis[8-14]. According to the current literature, no study has been found as to whether or not HT has any anti-proliferative effect in any type of ovarian cancer.

In this study, the cytotoxic and apoptotic effects of HT in cell lines of severely serous (OVCAR-3) and endometrioid (MDAH-2774) types of ovarian cancer were investigated. In addition, the roles of PI3K/Akt and ERK 1/2 signaling pathways in the emergence of this cytotoxic effect have been evaluated.

## MATERIALS AND METHODS

### *Chemicals*

Rosewell Park Memorial Institute - 1640 (RPMI-1640), fetal bovine serum (FBS), trypsin/EDTA solution, penicillin/ streptomycin and partricin were purchased from Biochrom Ltd. (Cambridge, UK). Sodium 3'-[1-(phenyl-aminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid hydrate and N-methyl dibenzopyrazine methyl sulphate (XTT) and Cell Death Detection ELISA$^{PLUS}$ Kit were purchased from Roche Diagnostics GmbH (Basel, Switzerland). Human Apoptosis Antibody Array was purchased from R&D Systems (England). Hydroxytyrosol was purchased from PanreacApplichem (Germany) and dissolved in DMSO at the final concentration of 0,16 M. PI3K and ERK inhibitors were purchased from Sigma Aldrich (USA).

### *Cell Line Conditions*

Human ovarian cancer cell lines OVCAR-3 and MDAH-2774 were gift from Ege University Tülay Aktaş Medical Oncology Research Labarotary (İzmir, Turkey) and maintained in RPMI-1640, supplemented with 10% heat inactivated FBS (Biochrom), 1% 5,000 units/mL penicilin, 5,000 µg/mL streptomycin (Biochrom) and partricin (Biochrom). The cells were incubated in 5% $CO_2$ at 37˚C incubator.

### *XTT Viability Assay*

Cell viability was assessed using the XTT assay (Roche, Germany). Cells were harvested by trypsinization, seeded in 96 well-plates at $10^4$ cells per well in 100 µl of medium and allowed to attach for 24 h. Once cells are attached, they were exposed to different concentrations of HT (50 to 400 µM) for 24, 48, 72 and 96 h. Whether or not there was any relationship between PI3K/Akt and/or ERK 1/2 signaling pathways along with the inhibition of cell proliferation observed as the result of HT treatment was investigated. By taking into consideration the $IC_{50}$ values obtained from the cytotoxicity tests performed on two different ovarian cancer cell lines, the experiments related to the specific inhibitors of the pathways were studied with 50 and 100 µM-concentrations of the inhibitors. To test the effect of PI3K/Akt inhibitor (LY294002) and ERK 1/2 inhibitor (FR 180204), cells were treated with inhibitors for 1 h and then treated with HT. XTT solution was prepared by adding N-methyl dibenzopyrazine methyl sulfate (activating agent) to sodyum 3'- [1-(phenyl-aminocarbonyl) -3,4-tetrazolium] -bis (4-methyl-6-nitro) benzene sulfonic acid hydrate in a ratio of 1/50. 100 µl XTT solution was added to each well and incubated at 37°C, 5% $CO_2$ for 4 h. Optical densities (OD) at 450 nm were measured using a plate reader (Tecan Reader, Beckman Coulter, ABD). The mean OD value was calculated for each test sample concentration and cytotoxicity was calculated by using the

following formula: % cytotoxicity = 1 - [($A_{(Treated)}$ /$A_{(Untreated\ control)}$] x 100

### *Histone Associated DNA fragmentation Measurement*

Apoptosis was determined by measuring amount of histone associated DNA fragmentation. For this aim a Cell Death Detection ELISA$^{Plus}$ Kit used according to the kit instruction manual. MDAH-2774 cells were treated with increasing concentrations of HT (50-400 µM) for 72 h and OVCAR-3 cells were treated with HT (50-400 µM) for 96 h. Cells were incubated with a lysis buffer for 30 minutes at room temperature. Then samples were centrifuged at 200xg for 10 min at 4°C. Supernatants of the samples (20 µl) were transferred onto streptavidin-coated plate wells. Then, 80 µl of anti–histone-biotin antibody and anti–DNA-peroxidase antibody mixture was added to coated wells, and incubated for 2 hours at 37°C. The plate was washed and 100 µl of ABTS (2,2′-azino-di[3-ethylbenzthiazalin-sulfonate]) solution was added to each well. At the end of the 15-min incubation, absorbance of the samples was measured at 405 nm with a reference wavelength at 490 nm (Tecan Reader, Beckman Coulter, USA).

### *Human Apoptosis Antibody Array*

The expression profile of apoptosis-related proteins was investigated by a human apoptosis antibody array kit containing duplicate spots of 35 apoptosis-related proteins. The lysis buffer included in kit was used to lyse MDAH-2774 and OVCAR-3 cells treated with 102.3 µM HT ($IC_{50}$ value) and 51.5 µM HT ($IC_{50}$ value) for 72 h and 96 h, respectively. Briefly, the membrane was blocked with blocking buffer for 1 h on a rocking platform at room temperature. The membrane was then incubated with lysates of ovarian cancer cells treated with or without HT overnight at 4°C on a rocking platform. The membrane was washed for 3 times and incubated with streptavidin-horseradish peroxidase conjugate/ biotined antibody cocktail mixture for 1 h. After washing steps, the membranes were exposed to chemiluminescent detection reagent for 10 min. The membranes were scanned and pixel density was presented by quantifying the mean spot densities by Koadarray 2.6 software (UK) from two experiments.

### *Statistical Analysis*

All experiments were run in triplicate and each experiment was repeated for 3 times. Data analysis was done by Graphpad (v5.0) software. The results were expressed as mean ±SD. Significant differences among treatments were assessed using one way ANOVA followed by Dunnett's test. 50 % inhibition concentration ($IC_{50}$) values were calculated with BiosoftCalcuSyn software (Ferguson, MO, USA).

## RESULTS

### *HT inhibits the proliferation of the ovarian cancer cell lines*

According to XTT results, it was seen that depending on the period of time, HT had reduced cell vitality in MDAH-2774 and OVCAR-3 cell lines (Figure 1). While no significant cytotoxic effect was seen on MDAH-2774 cells at the 24$^{th}$ and 48$^{th}$ hours ($p>0.05$), a significant cytotoxic effect was determined at 100 µM concentration and above at the 72$^{nd}$ hour ($p<0.05$). The cytotoxic effect values of HT on MDAH-2774 cells at 100, 200, 300 and 400 µM concentrations at the 72$^{nd}$ hour were specified as 70.5%, 63%, 55.5% and 52%, respectively (Figure 1a), and the $IC_{50}$ value was calculated as 102.3 µM. While HT did not show any significant cytotoxic effect on OVCAR-3 cells at 24$^{th}$ , 48$^{th}$ and 72$^{nd}$ hours ($p>0.05$), it was seen that there was a significant cytotoxic effect at 50 µM concentration and above at 96$^{th}$ hour ($p<0.05$). The cytotoxicity values of HT in OVCAR-3 cells at 50, 100, 200 and 400 µM concentrations at 96$^{th}$ hour were determined as 36%, 72%, 68.5% and 66.5%, respectively (Figure 1b), and the $IC_{50}$ value was calculated as 51.5 µM.

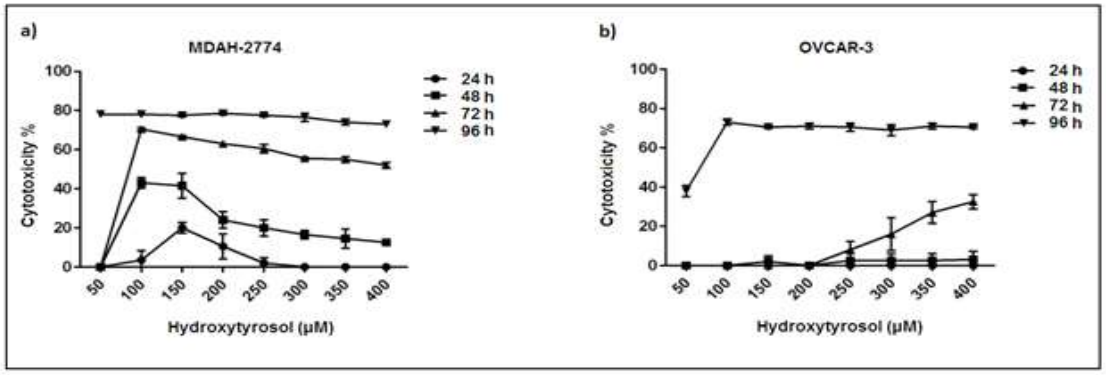



**Figure 1. Effects of HT on cytotoxicity in MDAH-2774 (a) and OVCAR-3 (b) cells at 24, 48, 72 and 96 hours.**

***HT induces apoptosis in ovarian cancer cell lines***

It was determined that the DNA fragmentation had increased by 38% and 21% , respectively, in MDAH-2774 cells treated through HT at 100 and 150 µM for 72 hours ($p<0.05$). At 200 µM concentration and those above, however, no DNA fragmentation was determined to have been observed ($p>0.05$) (Figure 2a). It was ascertained that in the OVCAR-3 cells treated through HT at 50, 100, 150, 200, 250 and 300 µM concentrations for 96 hours, the DNA fragmentation had increased by 39%, 61%, 57%, 42%, 40,5% and 28%, respectively ($p<0.05$). At 350 and 400 µM concentrations, on the other hand, it was determined that no DNA fragmentation was seen ($p>0.05$) (Figure 2b). The significant changes in Human Apoptosis Protein Array results were shown in Table 1 for MDAH-2774 ($p<0.05$) and in Table 2 for OVCAR-3 ($p<0.05$) cells.

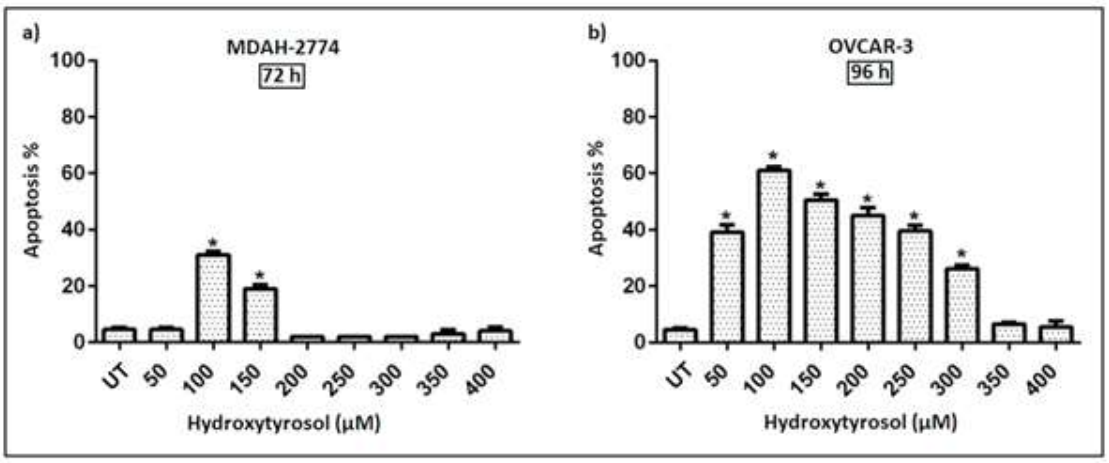



**Figure 2. Apoptotic effects of HT in MDAH-2774 cells at 72 h (a) and OVCAR-3 cells at 96 h (b) (*p< 0.05)**

**Table 1. Fold changes of apoptotic proteins in MDAH-2774 cells exposed to HT ($p<0.05$)**

| | Protein | Fold Changes |
|---|---|---|
| Pro-apoptotic | Bad | + 2.0 |
| | Bax | + 1.4 |
| | Active Caspase-3 | + 1.2 |
| | Htra2/Omi | + 4.2 |
| Death receptors | TRAIL R1/DR4 | + 2.1 |
| | TRAIL R2/DR5 | + 1.7 |
| | FAS/TNFRSF6 | + 1.6 |
| Apoptosis inhibiting proteins | Survivin | - 1.6 |

**Table 2. Fold changes of apoptotic proteins in OVCAR-3 cells exposed to HT ($p<0.05$)**

| | Protein | Fold Changes |
|---|---|---|
| Anti-apoptotic | Bcl-2 | - 3.1 |
| | Pro-caspase-3 | - 8.2 |
| Apoptosis inhibiting proteins | CIAP-1 | - 6.5 |
| | CIAP-2 | - 6.0 |
| | XIAP | - 3.2 |
| | Livin | - 2.2 |
| | Survivin | - 2.7 |
| Pro-apoptotic | cytochrome-c | + 1.2 |

***The Effects of Hydroxytyrosol on PI3K/AKT and ERK 1/2 Signaling Pathways***

For this investigation, an XTT vitality test was performed on MDAH-2774 cells treated with 72-hour-102.3 µM-HT and on OVCAR-3 cells treated with 96-hour-51.5 µM-HT by using the specific inhibitors of PI3K/Akt and ERK 1/2 pathways, which were LY294002 and FR180204, respectively. When compared with the controls, no significant difference was found in neither of the cell lines in terms of cell vitality as the result of the treatment with HT and LY294002 ($p>0.05$) (Figure 3a). Compared to the control groups, the cell vitality was increased in both of the cell lines as the result of the treatment with HT and FR180204 ($p<0.05$) (Figure 3b).

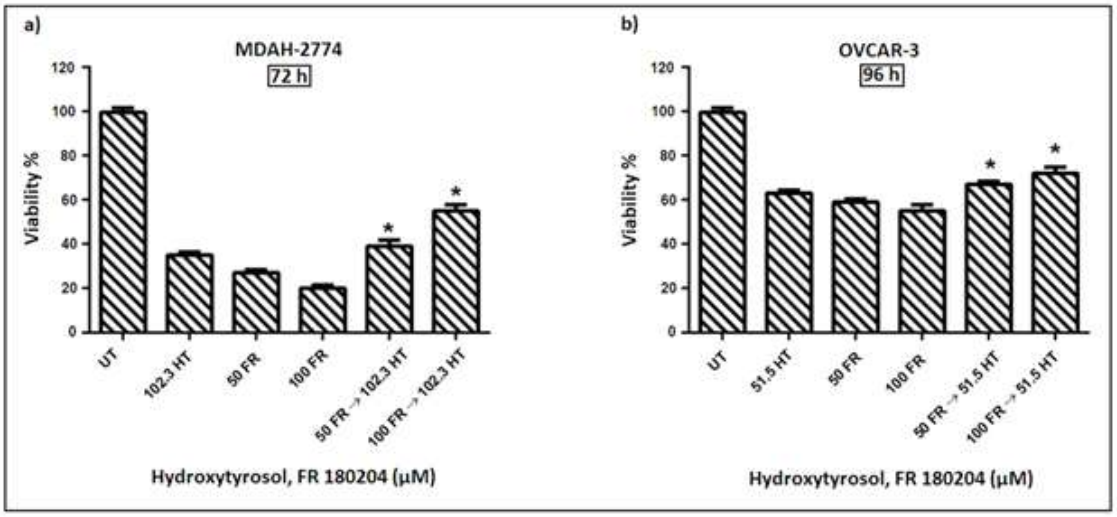



**Figure 3. Effects of FR 180204, a specific inhibitor on the viability of HT treated MDAH-2774 (a) and OVCAR-3 (b) cells. * p < 0.05 was considered significant as compared to any agents alone.**

## DISCUSSION

The ability of cancer cells to develop resistance against chemotherapeutic agents used in the treatment is the greatest obstacle in the course of a successful treatment. Hydroxytyrosol is a potential molecule attracting attention with its antioxidant, anti-inflammatory, anti-proliferative and pro-apoptotic effects for treatment of cancer[15]. The cytotoxic and apoptotic effects of HT in breast, colon, colorectal, melanoma, prostate and human promyelocytic leukemia cell lines and the association of these effects with various signal transduction pathways have been shown in former studies[8,10,11,13,16,17].

In this study, it was determined that HT had showed a cytotoxic effect not by depending on the increasing dose but by depending on the period of time in both of the ovarian cancer cell lines, MDAH-2774 and OVCAR-3.

Notarnicola et al. demonstrated that HT did not pose an anti-proliferative effect at the range of 10-100 µM concentrations at the 24$^{th}$ hour in the human colon cancer cell line called SW620; yet, it showed an anti-proliferative effect at the 72$^{nd}$ hour. Again, in the same study, it was reported that HT in the HT-29 cells had no anti-proliferative effect at the 24$^{th}$ hour; yet, it posed an anti-proliferative effect at 100 µM concentration at the 72$^{nd}$ hour[13].

In the study conducted by Fabiani et al., in which RPMI-1640 medium was used, the anti-proliferative effect of HT on different cancer cell lines was investigated. Two separate cell lines were used for each of the breast, prostate and colon cancer types, and different $IC_{50}$ values were found. The fact that the abilities of different cancer cell lines to remove $H_2O_2$ accumulating in the culture medium are different from one another affects the susceptibility of the cells to the anti-proliferative effect of HT[18]. In our study, two different ovarian cancer cell lines were used. In the dose- and time-dependent applications of HT, the differences in $IC_{50}$ values are thought to be possibly due to the capacities of removing $H_2O_2$ within the medium.

In a study conducted by Corona et al., it was reported that by using sulforhodamine B (SRB) method on the human colon cancer line Caco-2, HT had showed significant cytotoxicity at 5, 50, 100, 130 and 162.5 µM concentrations at 24$^{th}$, 48$^{th}$, 72$^{nd}$ and 96$^{th}$ hours[11].

In Guichard et al.'s study, the $IC_{50}$ value of HT in a different colon cancer cell line,

HT-29, was found to be approximately 200 µM at 24th hour according to the flow cytometric measurements, and it was shown to have ceased the cell cycle at 400 µM concentration at S and G2/M phases[8]. In another study conducted on human promyelocytic leukemia cells, HL60, it was determined that HT had ceased the cell cycle at 100 µM concentration at 25th hour through the use of 3H-thymidine incorporation test[10]. In our study, the $IC_{50}$ values of HT were calculated as 102,3 µM at 72nd hour for MDAH-2774 and as 51,5 µM at 96th hour for OVCAR-3 according to the XTT results. The differences in the $IC_{50}$ values in terms of the investigated molecule and the incubation period are thought to result from the methods preferred for the variety and cytotoxicity measurements over the model cell lines examined.

In our study, it was found that HT had demonstrated the highest value of cytotoxicity at 100 µM at 72nd hour in MDAH-2774 cell cultures, whereas there was a decrease in cytotoxicity in the exceeding doses. In the OVCAR-3 cell lines, however, it was ascertained that the highest cytotoxicity was seen at 100 µM at 96th hour; yet, there was no change in the cytotoxicity values at HT concentrations above this dose. It was shown by Corona et al. that in the colon cancer Caco-2 cell line, there was a dose- and time-dependent increase in the inhibition of cell proliferation as the result of 24th, 48th, 72ndand 96th –hour-incubations of HT at 5, 50, 100, 130, 162,5 µM concentrations. However, it was shown that the proliferation-suppressing effect of the intestinal metabolite of HT, referred to as homovanilil alcohol (HVA), at 5, 50, 100, 150, 200 µM concentrations at 48th and 72nd hours had increased up to 50 µM and continued decreasingly at 100 and 150 µM[11]. The decrease in the cytotoxicity at the concentrations above 100 µM at 72nd hour in MDAH-2774 cell lines examined in our study suggests that HT could be breaking down to its metabolites above the given concentration and time.

In our study, while the 72nd hour-apoptotic effect of HT in MDAH-2774 cell lines was seen at 100 µM at the highest, it was determined that it decreased at 150 µM; yet, no apoptosis was seen at 200 µM and the above concentrations. This result we obtained for MDAH-2774 was found to be concordant not in terms of dose but in terms of the period of time when compared with the study[13] conducted by Notarnicola et al., in which HT concentrations were determined to have showed a pro-apoptotic effect starting from 10 µM at 72nd hour on SW620 cell line and at the dose of 100 µM at 72nd hour on human colon cancer cell line called HT-29. It was shown that apoptosis was induced by HT at 24th hour at 200 µM concentration in human colon cancer cells (HT-29)[8], at the 25th hour, again, at 100 µM concentration in human promyelocytic leukemia cells[10], and at 50 µM concentration at the end of 12 hours in human breast cancer cell line, MCF-7[16]. With these results, the differences in time and concentrations seen within the data regarding the apoptotic effect, may be due to the fact that various cancer cell line lines were studied on.

Also in our study, the highest apoptotic effect of HT in OVCAR-3 cell lines was seen in the 100 µM-concentration at 96th hour, whereas the apoptotic effect was found to have gradually decreased at increasing doses after 100 µM.

The rates in apoptosis, such as 39% and 61% as well as 38% cytotoxicity value at the dose of 50 µM- HT and 73% cytotoxicity value at 100 µM – HT concentration, which were obtained through the XTT test, suggest that the cell death at these concentrations could occur through apoptosis. It was stated that a significant apoptotic effect was seen as the result of a 24-hour-incubation at 50 and 100 µM concentrations during the HT practice on human promyelocytic leukemia cell line, HL-60, whereas at 250 µM, there was a decrease in the rate of apoptosis. In that study conducted by Fabiani et al., it was stated that cell death had occurred at 250 µM concentration

under the effect of necrosis, one of the other mechanisms of death[19]. However, in our study, the decrease in apoptosis at the increasing doses succeeding 100 µM suggests that cell death could occur under the effect of other mechanisms of death. Another reason why there is a decrease in the apoptotic effect in the wake of a given HT- dose in both of the cell lines could be that HT could be breaking down to its metabolites.

In a study conducted on HL-60 cell line, it was found that HT had posed its apoptotic effect by reducing the CDK6 expression, which is one of the important proteins regulating the cell cycle and apoptosis as well as reducing the expressions of $p21^{WAF1/Cip1}$ and $p27^{Kip1}$, which are the CDK inhibitors[10]. We observed a decrease at the level of $p27^{Kip1}$ according to the apoptosis antibody array result obtained from OVCAR-3 cell line. HT may be showing its apoptotic effect by reducing $p27^{Kip1}$ expression.

In our study, it was demonstrated that HT had caused an increase on the level of Bad, Bax, active caspase-3 and Htra2/Omi proteins, which are among the pro-apoptotic molecules, in MDAH-2774 cell lines, and on the level of TRAIL R1/DR4, TRAIL R2/DR5 and FAS/TNFRSF6 molecules, which are the pro-apoptotic death receptors, while giving rise to a decrease in the amount of Survivin, which belongs to the family of apoptosis inhibitor proteins (AIP). The increase on the level of Bad, Bax, active caspase-3 proteins, which we obtained from MDAH-2774 cell line is in concordance with the results of HT regarding the increase in the expressions of Bad, Bak and Bax, the pro-apoptotic proteins, on the human colon cancer HT-29 cell line[8] and the increase in the caspase-3 activity at the doses over 600-800 µM in the human melanoma cells (M14)[20]. In our study, it was determined that HT caused a decrease in the amounts of Bcl-2 and Pro-caspase-3, which are among the anti-apoptotic molecules, in OVCAR-3 cell lines and in the amounts of CIAP-1, CIAP-2, XIAP, Livin and Survivin, which belong to AIP family, while giving rise to an increase in the amount of pro-apoptotic cytochrome-c.

Our findings regarding the fact that HT posed its effect when the expression of Bcl-2 decreased support the finding as to the decrease on human colon cancer HT-29[8] and human cholangiocarcinoma cell lines (TFK-1 and KMCB) and the decrease on the level of Bcl-2 expression obtained from the gall bladder cancer cell line (GBC-SD)[17]. HT could be posing its apoptotic effect by reducing the Bcl-2 level as well as increasing the level of pro-apoptotic proteins. Separately, the fact that it causes a decrease in the amounts of the proteins pertaining to the apoptosis inhibitor family indicates that HT might be posing its effect by reducing or blocking the expressions of the molecules inhibiting apoptosis.

According to the literature, the abnormalities in PI3K signal transduction have been detected in both Type-1 and Type-2 ovarian cancers[5]. Separately, MAPK pathway (ERK 1/2) is known to induce cell proliferation and become up-regulated in several cancer cells[11]. For these reasons, in our study, whether or not the cytotoxic effect of HT occurs over PI3K/Akt and ERK 1/2 pathways on OVCAR-3 and MDAH-2774 cell lines modeling Type-1 and Type-2 ovarian cancers was investigated. In our study, it was found that when each of various concentrations of HT and the PI3K/Akt inhibitor is applied alone and in combination, the difference among them proved to be statistically insignificant for both cell lines. In this case, it can be said that HT on MDAH-2774 and OVCAR-3 cell lines did not pose its cytotoxic effect over PI3K/Akt pathway.

There are studies conducted on different cancer cell lines suggesting that HT may be posing its cytotoxic effect over ERK 1/2 signaling pathway[8,11,17]. It was reported that HT had posed its anti-proliferative effect on the human colon carcinoma cell line (HT-29)[8], (Caco-2)[11], the cholangiocarcinoma cell lines (KMBC and TFK-1)[17], the gall bladder cancer cell line (GBC-SD)[17] and the human breast cancer cell line (MCF-7)[21]

by inhibiting the phosphorylation of ERK 1/2.

In our study, it was determined that when the combination of the specific inhibitor of ERK 1/2 pathway and HT was applied on MDAH-2774 and OVCAR-3 ovarian cancer cell lines, there was a statistically significant decrease when compared with the cytotoxic effect of HT alone, which suggests that HT could be showing its cytotoxic effect through this pathway. There is the need to conduct molecular biological studies to put forward through what protein that plays a role in this pathway.

## CONCLUSIONS

The cytotoxic and apoptotic effects of HT and whether or not its cytotoxic effect occurs on MDAH-2774 and OVCAR-3 ovarian cancer cell lines over PI3K/Akt and ERK 1/2 have been investigated with this study for the first time. It was ascertained that HT, on the endometrioid type of ovarian cancer cell line, MDAH-2774, had posed a significant cytotoxic effect at 100 µM concentration and above at 72$^{nd}$ hour (IC50=102.3 µM). It was also found that it showed a significant cytotoxic effect at 50 µM concentration and above at 96$^{th}$ hour on the severely serous type of ovarian cancer cell line, OVCAR-3 (IC50=51.5 µM). It was determined that the apoptotic effect had occurred at 100 µM at most in the 72$^{nd}$ hour on MDAH-2774 cell line, while it declined at 150 µM, and no apoptosis was seen at 200 µM and the concentrations above that. While its apoptotic effect was observed at 100 µM concentration at 96$^{th}$ hour on OVCAR-3 cell line, this effect was shown to have declined at the concentrations above 100 µM. It was shown to have posed its apoptotic effect on MDAH-2774 cell lines by causing an increase on the level of pro-apoptotic molecular Bad, Bax, active caspase-3, Htra2/Omi and SMAC/DIABLO proteins and on the level of TRAIL R1/DR4, TRAIL R2/DR5 and FAS/TNFRSF6 molecules, which are pro-apoptotic death receptors, as well as leading to a decline in the amount of Survivin, which belongs to apoptosis inhibitor proteins (AIP).

In OVCAR-3 cell lines, on the other hand, HT was determined to have showed its apoptotic effect by causing a decline in Bcl-2 and Pro-caspase-3, the anti-apoptotic molecules, and a decline in the amounts of CIAP-1, CIAP-2, XIAP, Livin and Survivin belonging to AIP family as well as giving rise to an increase in the amount of pro-apoptotic cytochrome-c. It was also found that HT might have been posing its cytotoxic effect on MDAH-2774 and OVCAR-3 cell lines over ERK 1/2 signaling pathway. The data obtained from our research can be the sources of information for further molecular biological studies that can explain what signaling pathway HT poses its anti-cancerous effect through.

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