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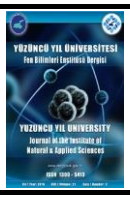

Research Article

Investigation of the Effects of Piperlongumine and Doxorubicin Combined Treatment on Cell Death via PTEN in HeLa Cells

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Abstract: Doxorubicin (Dox), which is used in treating many types of cancer including cervix cancer nevertheless, its effect alone is low especially in recurrent cases. Therefore, investigating of agents that can increase the impact of Dox continues. The aim of the present study is to answer the question: Can Piperlongumine (PL) a natural alkaloid cause an increase in the efficacy of Dox in the HeLa cell line? In this study, the effects of Dox and PL on cell viability by MTT and Acridine orange/propidium iodide staining, and expression levels of the PTEN (Phosphatase and tensin homolog 10) gene by Real-Time PCR and Western-Blot were evaluated in HeLa cells. It was determined that PL combined with Dox increased cell death and suppressed cell proliferation. The PTEN gene expression was decreased in all experimental groups, but the PTEN protein phosphorylation increased in cultures treated with PL and when Dox/PL was combined. The fact that PL application increases the activation of PTEN, which is a tumor suppressor. This indicates that it can be used to increase the effectiveness of Dox in the treatment.

HeLa Hücrelerinde Piperlongumin ve Doksorubisin Kombine Tedavisinin PTEN Yoluyla Hücre Ölümü Üzerine Etkilerinin Araştırılması

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Anahtar Kelimeler
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Öz: Doksorubisin (Dox) birçok kanser türünün yanı sıra serviks kanseri tedavisinde de kullanılmaktadır ancak özellikle tekrarlayan vakalarda tek başına etkisi düşüktür. Bu nedenle Dox etkisini artırabilecek ajanların araştırılması devam etmektedir. Bu çalışmada amaç: Doğal bir alkaloid olan Piperlongumine (PL), HeLa hücre hattında Dox etkinliğini artırabilir mi? sorusuna cevap vermektir. Çalışmamızda, HeLa hücrelerinde MTT ve Akridin oranj/propidyum iyodür boyama ile Dox ve PL'nin hücre canlılığı, Real-Time PCR ve Western-Blot ile PTEN (Fostataz and tensin homolog 10) gen/proteininin ekspresyonu üzerindeki etkileri değerlendirilmiştir. Dox ile kombine edilen PL'nin hücre ölümünü arttırmış ve proliferasyonunu baskılamıştır. Tüm deney gruplarında PTEN gen ekspresyonu azalmıştır ancak PL ve Dox/PL kombine uygulanan kültürlerde PTEN protein fosforilasyonu artmıştır. PL uygulamasının bir tümör baskılayıcı olan PTEN aktivasyonunu arttırması tedavide Dox etkinliğini arttırmak amacı ile kullanılabileceğini göstermektedir.

1. Introduction

Cancer is one of the most important health problems of our age and new treatment methods or chemotherapeutics are still among the most researched subjects. Piperlongumin (PL) is also called piplartin, is an herb used in Indian alternative medicine to treat cough, respiratory infections, stomachache, and other ailments. It is an active alkaloid isolated from the *Piper longum* and the chemical structure of PL is well characterized. Although it's newly discovered its pharmacological properties have been revealed in recent years (Piska et al., 2018). Many studies have shown that piperlongumine (PL) can selectively kill cancer cell lines, and subsequent studies have confirmed the anticancer activities of piperlongumine (Kumar & Agnihotri, 2019; Seber et al., 2020). Piperlongumin has been found to induce cell death both by caspase-dependent apoptosis and via necrosis and autophagy (Guamán-Ortiz et al., 2017).

Doxorubicin, is a chemotherapeutic that inhibits topoisomerase II during DNA replication and then prevents nucleotide chain attachment after double strand breakage (Momparler et al., 1976). Doxorubicin (DOX) is one of the abundant and effective chemotherapeutic agent used for cancer therapy (Xia et al., 2018). However, like other chemotherapeutic agents, the efficacy of doxorubicin is severely limited due to chemoresistance (Zhou et al., 2013).

The PTEN gene encodes a phosphatase enzyme expressed in many tissues. PTEN functions as a tumor suppressor in regulating cell growth and apoptosis and arresting cell migration by interacting with the extracellular matrix. The function of PTEN prevents cells from uncontrolled cell proliferation and regulate cell division (Moon et al., 2004). Moreover, AKT1/NF-kappaB/Notch1/PTEN axis has an important role in the development of chemoresistance, downregulation of PI3K/AKT activities through overexpression of PTEN gene sensitizes gastric cancer cells to chemotherapeutic agents (Zhou et al., 2013).

In this study we conducted with the cervical cancer cell line HeLa, the effects of doxorubicin (Dox) and PL on PTEN (Phosphatase and tensin homolog 10) expression and cell proliferation/migration were investigated. Actin, a cytoskeletal component expressed in all cell types, was used as an internal control gene in the study. Changes in PTEN gene and protein expression were evaluated by comparing them with β-Actin expression used as an internal control.

In our study, Dox and PL were applied to the HeLa cell line for 24 hours, and the effects of drugs on PTEN and β-Actin genes were investigated. RNA isolations were performed, and the Real Time PCR method was applied to determine the expression amount of the determined genes. At the same time, protein analysis was performed after drug administration at the end of 24 hours and western-blot method was used for protein expression of PTEN. Furthermore, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] analysis and acridine orange/propidium iodide (AO/PI) staining were performed to determine the viability of HeLa cells. In this manner, the expression level of the PTEN gene associated with metastasis and tumorigenesis were compared and the possible role of piperlongumine, a natural product, in the treatment of cervical cancer was examined (Moon et al., 2004; Seber et al., 2020).

2. Material and Methods

2.1. Cell culture

HeLa cells were grown in DMEM medium containing 10% FBS (Fetal Bovine Serum), 1% Penicillin streptomycin, and L glutamine, and placed in 96-well culture dishes at 3.2 x 10^4 cells/well for MTT analyses and AO/PI staining, 9.5x10^5 cells/well into 6-well culture dishes for RNA and protein isolations. IC50 doses were determined by applying increasing doses of PL and Dox to cells incubated at 37 ^{0}C for 24 hours. 50, 75, 100, 125, 150, and 175 µM PL and 500, 750, 1000, 1500, and 2000 nM Dox were applied to determine the IC50. The applications were carried out in three repetitions. Then, the combined doses of 100 µM PL, 1000 nM Dox, and 100 µM PL + 1000 nM Dox were determined for Real time PCR and western blot analyzes were applied to the control and experimental groups. Experimental groups, application time, application, and amount of PL and Dox applied are summarized in Table 1.

Table 1. Experimental groups, application time, application, and amount of PL and Dox applied

Group No	Application	Application Period	Application Amount
1	Control	24 h	-
2	PL	24 h	100 μM
3	Dox	24 h	1000 nM
4	PL + Dox	24 h	100 μM + 1000 nM

2.2. MTT cell viability analysis

In our study, cell viability analysis was performed in cultures where Dox and PL were applied for 24 hours using the Vybrant® MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Cell Proliferation Assay Kit (V-13154, Thermo Fisher Scientific, USA) by the company's instructions. The optical density (OD) value obtained from the control group samples was accepted as 100% viable, and the % viability of experimental groups was calculated according to the formula % viability = (OD test groupX100)/(OD control group).

2.3. AO (Acridine Orange) /PI (Propidium Iodide) staining

HeLa cells cultured in 96-well plates were stained with AO/PI simultaneously with MTT analysis and visualized under a fluorescent microscope (Leica DM2500, DH Life Sciences, USA). AO/PI staining was used to confirm MTT data and evaluation of cell viability.

2.4. RNA isolation, cDNA extraction, and quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was isolated by the manufacturer's instructions (Pure Link, Ambion Kit). Using 100 ng RNA obtained from the experimental and control groups, the cDNA reaction was established in the presence of 10x Buffer, 25 dNTP mix (100 mM), Random Primer, Multiscribe Reverse Transcriptase, and dH_2O, which are included in the kit (Applied Biosystems, USA). The reaction protocol was as follows: incubation at 25^0C for10 minutes, at 37^0C for 120 minutes, and at 85^0C for 5 minutes.

Gene-specific assays; PTEN (catalog #: 433118003, PTEN: 433118003, PTEN) 1) and β-Actin (catalog # 4331182, Actb. Rn00667869_m1, RefSeq NM_031144.3, Thermo Fisher Scientific, Waltham, MA USA), were used to identify gene expression using Applied Biosystems 7300/7500 Real-time PCR system.

2.5. Western blot analysis

The western blot method was used to determine PTEN protein expression. Total proteins were obtained using Triton X-100 (Sigma-Aldrich, Cat # 10789704001, Germany) and protease inhibitor cocktail (Roche, Cat # 04693159001, Germany).

The total protein amount was determined by using Bradford protein assay, and 100 ng protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then transferred to the PVDF membrane (Thermo Fisher Scientific, Cat # IB401001, Waltham, MA, USA) for immunoblotting. Membranes were incubated with phospho-PTEN (Ser380) polyclonal antibody (Thermo Fisher Scientific, Cat # PA5-17826, Waltham, MA, USA) diluted 1:1000 and monoclonal β-Actin antibody diluted 1:5000 (Thermo Fisher Scientific, Waltham, Cat # MA1-140) O/N at room temperature. Secondary antibody labeling was performed using the Western Breeze chemiluminescence kit (Thermo Fisher Scientific, Cat # WB7104, Waltham, MA, USA) and protein bands were visualized using X-ray film (Thermo Fisher Scientific, Cat # 34090, Waltham, MA, USA). Band intensity was then calculated using ImageJ software to examine fold changes in protein expression.

2.6. Statistical analysis

The parametric or nonparametric nature of the data was determined by applying Shapiro Wilk (for normality) and Levene (homogeneity of variance) tests. Spearman correlation coefficients were calculated for linear relationships between the control and the variables (Piperlongumine and Doxorubicin applications) (Steel & Torrie, 1960). A value of $p<0.05$ was accepted as significant. Shapiro Wilk, Levene and Spearman tests were performed using SPSS version 15.0.

3. Results

3.1. Cell viability analysis

The % viability determined as a result of PL administration were as follows: PL50 81.9 %, PL75 73.0 %, PL100 66.9 %, PL125 67.1 %, PL150 67.2 %, and PL175 59.9 % (Figure 1).

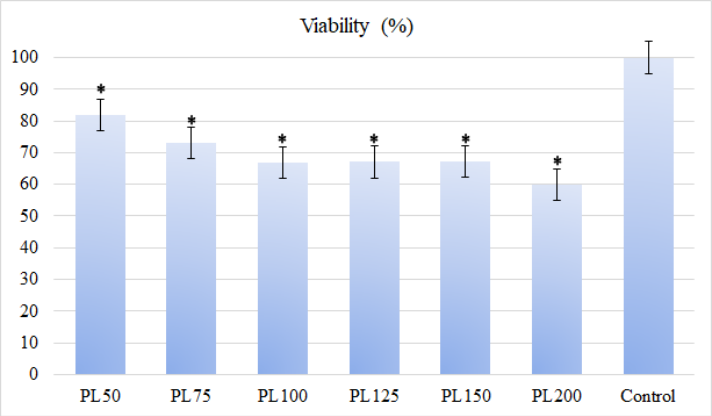

Figure 1. Graph of % viability in PL administrated cultures. (50, 75, 100, 125, 150, and 175 µM PL). * Negative correlation of cell viability with the increasing doses of piperlongumine were statistically significant ($p = 0.002$).

The % viability levels obtained as a result of Dox application were determined as D500 78.9 %, D750 70.7 %, D1000 69.4 %, D1500 62.4 %, D1750 61.4 %, and D2000 58.4 %. The graph of these data is given in Figure 2.

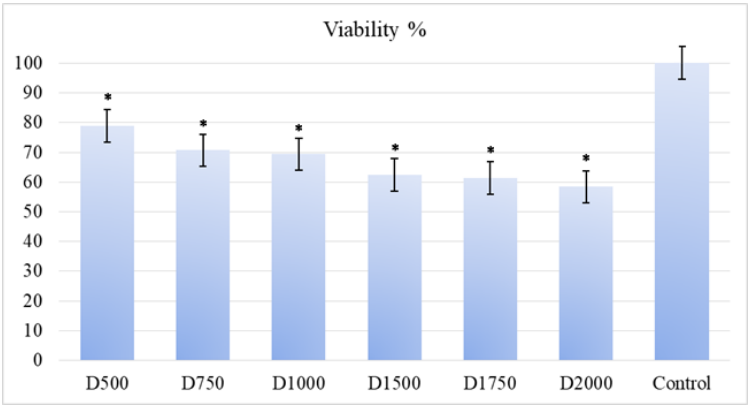

Figure 2. Graph of % viability in Dox administrated cultures. (500, 750, 1000, 1500, 1750, and 2000 nM Dox). * Negative correlation of cell viability with the increasing doses of Dox were statistically significant ($p < 0.001$).

To confirm the results of the MTT analysis and to determine whether the detected cell deaths were apoptotic, AO/PI staining was performed in all samples treated with PL and Dox. The microphotographs of the AO/PI staining can be seen in Figure 3. It was determined that as the application dose increased, cell death increased in the samples treated with both PL and Dox. In cultures treated with 100 μM PL and 1000nM Dox, cell viability was found to be 66.9% and 69.4%, respectively, after MTT analysis. However, in AO/PI staining, it was determined that cell proliferation was also suppressed above these doses and thus the number of cells decreased. In the next stages of the study, 100 μM PL, 1000nM Dox, and 100 μM PL + 1000nM Dox were applied in combination. In Figure 4, graph of % viability in PL100, D1000 and PL100+D1000 (viability 39.1%) experimental groups are given. In the Figure 5, AO/PI staining of the control group 100 μM PL, 1000nM Dox and 100 μM PL + 1000nM Dox combined applications are given.

3.2. Statistical analysis

In the cell culture experiments, viability was negatively correlated with the increasing doses of piperlongumine ($r = -0.745$, $p = 0.002$), also negative in the increasing doses of doxorubicin applications ($r = -0.816$, $p < 0.001$). But, maximal in cell viability occurred at PL100+D1000 combination (Figure 4).

3.3. Quantitative Real-Time PCR analysis

qRT-PCR experiments were performed on a total of 4 groups; PTEN gene expressions were normalized to β-Actin expression used as an internal control gene from the same sample.

After the Rn values were determined in all samples, the level of gene expression in all samples was calculated concerning the control group (Group 1), and the relative quantity (RQ) value was given in the table (Table 1). In the reference sample, RQ=1, that is, the gene expression level was accepted as 100%, and the change in gene expression in the samples belonging to the experimental groups was determined as fold change.

As a result of the analyses, in group 2, where only PL was administered for 24 hours, PTEN gene expression (RQ=0.38) decreased by 62%. In group 3, where only Dox was administered for 24 hours, PTEN gene expression (RQ=0.42) decreased by 58%. In group 4, where piperlongumine and Dox were administered combined for 24 hours PTEN gene expression decreased by 53% (RQ=0.47) (Table 2).

Table 2. RQ values of β-Actin, and PTEN gene expressions were obtained as a result of qRT-PCR

Group No	β-Actin	PTEN
1	1	1
2	1	0.38
3	1	0.42
4	1	0.47

3.4. Western-Blot analysis

Western-blot experiments were performed on a total of 4 samples in all experimental groups; Expressions of PTEN protein were normalized to the expression of β-Actin used as an internal control gene from the same sample. The demonstrative picture of the protein bands obtained by western blot is given in Figure 6. Pictures of WB were analyzed using the ImageJ program and the change in protein expression was determined in folds (Table 3).

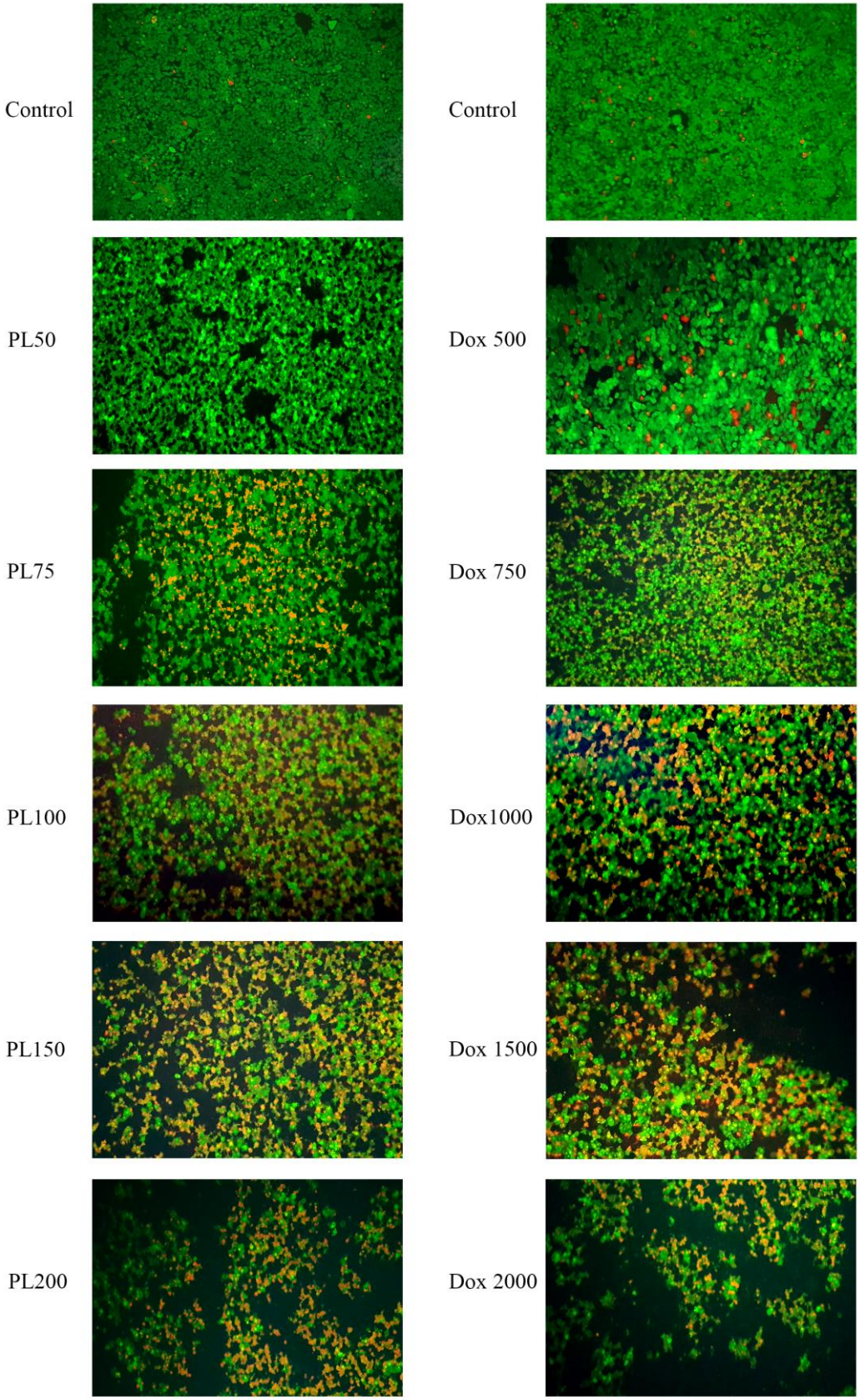

Figure 3. The microphotographs of the AO/PI staining of 50, 75, 100, 125, 150, and 175 µM PL and 500, 750, 1000, 1500, 1750, and 2000 nM Dox application. Dead cells are stained red and live proliferating cells are stained green.

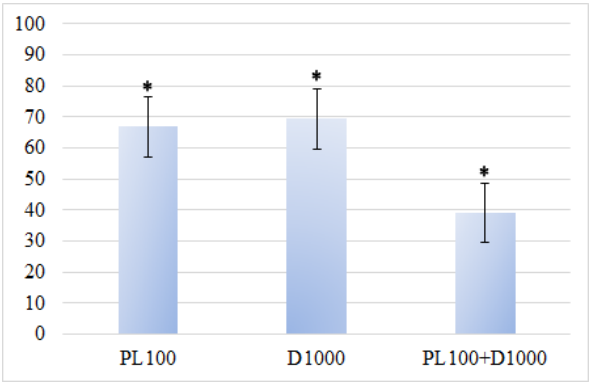

Figure 4. Graph of % viability in PL100, D1000 and PL100+D1000 experimental groups. *Negative correlation of cell viability with in PL100, D1000 and PL100+D1000 experimental groups were statistically significant ($p < 0.001$) and maximal in the PL100+D1000 combination group.

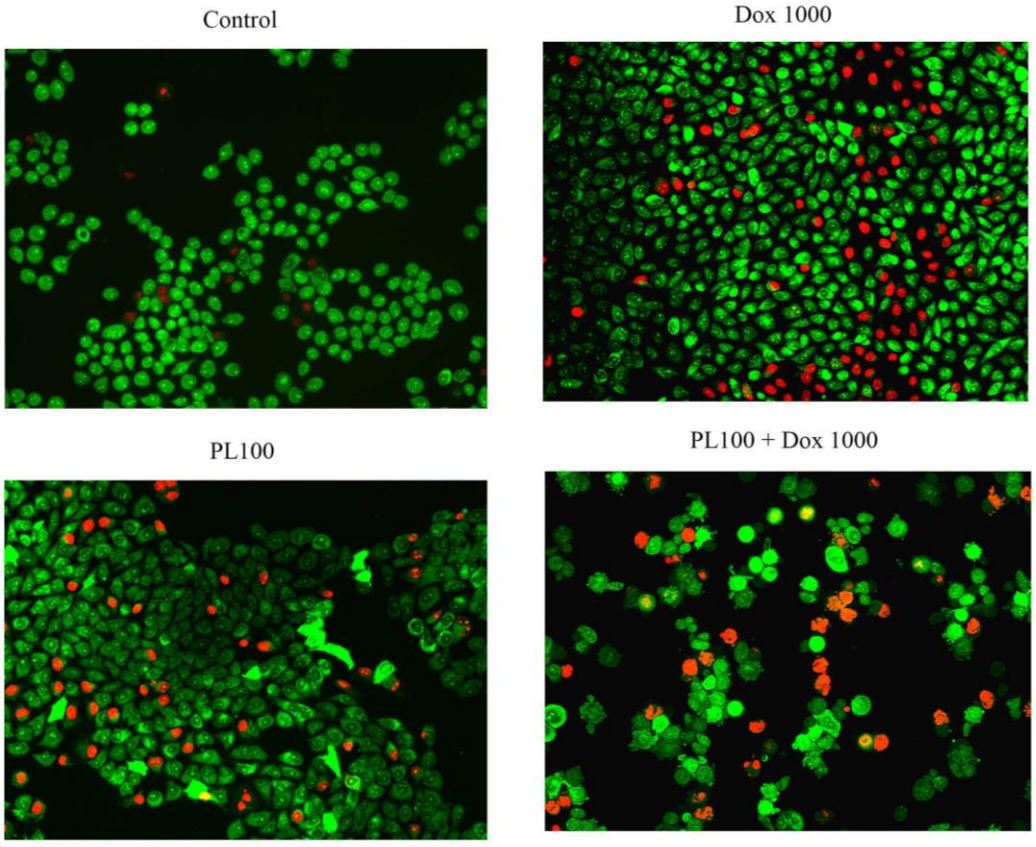

Figure 5. AO/PI staining of the control group 100 µM PL, 1000nM DOX, and 100 µM PL + 1000 nM Dox combined applications. Dead cells are stained red and live proliferating cells are stained green.

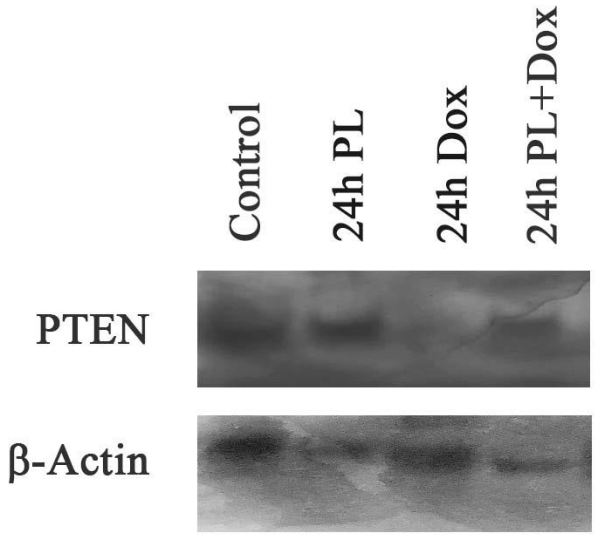

Figure 6. The PTEN and β-Actin protein bands obtained by western blot

Table 3. Fold changes of β-Actin and PTEN expressions

Group No	β-Actin	PTEN
1	1	1
2	1	1.33
3	1	0.77
4	1	3.16

4. Discussion and Conclusion

PL is a natural product in alkaloid structure found in *Piperlongum linn* plant species. Numerous studies in cancer cell lines and animal models suggest that PL exhibits anti-tumorigenic activity (Chen et al., 2018). In the study of Gong et al. (2014); it has been shown that PL causes the cells to go to apoptosis by stopping the cell cycle in the G2/M phase and also provides the accumulation of intracellular reactive oxygen species (ROS) in a dose and time-dependent manner. The same study suggested that low-dose PL combined therapy with cisplatin or paclitaxel has a growth-inhibiting synergistic effect in ovarian cancer cells (Gong et al., 2014). Induction of oxidative stress by exogenous ROS production therapy can now be considered an effective cancer therapy. That's because this approach selectively kills cancer cells without affecting normal cells. Piperlongumine (PL) functions as an inducer of ROS in anticancer therapy. Piperlungumine has been shown to increase ROS levels and induce selective cell death in a few cancer cells but not in normal cells (Raj et al., 2011). Accumulation of PL-activated ROS leads to apoptosis of the cancerous cell through activation of the JNK/ERK pathway (Kumar & Agnihotri, 2019). Niu et al. (2015) reported that piperlongumin inhibits interactions with chromosomal maintenance 1 (CRM1), also known as export 1 (XPO1), in HeLa cells. They showed that tumor suppressor proteins cause accumulation in the nucleus and that piperlongumin itself directly interacts with Cys528, which is conserved in CRM1. The authors examined the effect on CRM1 mutant HeLa cells. They suggested that CRM1 mutant HeLa cells are resistant to piperlongumin and thus piperlongumin-mediated inhibition of nuclear export of tumor suppressor proteins could be used to develop strategies to treat cancers including cervical cancer (Niu et al., 2015). Similarly, in a study evaluating tumor formation, cell migration, invasion, proliferation ability, and sensitivity to chemotherapy and radiotherapy in oral cancer cells, the anti-tumor effect of PL is emphasized (Karki et al., 2017).

Doxorubicin, is a chemotherapeutic that is frequently used in the treatment of many cancers, including cervix cancer. It inhibits replication and prevents cell division by stabilizing topoisomerase II and DNA complex during DNA replication (Momparler et al., 1976). The antitumor effects of doxorubicin are mechanisms including micromolecule synthesis, generation of reactive oxygen species (ROS), DNA binding, cross-linking, suppression of topoisomerase IIb (TOPIIb) and its intercalation to DNA resulting in the induction of apoptosis and prevention of DNA damage. TOPIIb cleaves DNA strands during replication, transcription or recombination. Doxorubicin is known as an inhibitor that prevents DNA synthesis by interfering with DNA strands. Although doxorubicin is toxic to both cancer and normal cells, the mechanism of cell death in both cells may not be similar (Takemura & Fujiwara, 2007; Wang et al., 2019). At the same time, DOX binds to the cell membrane and changes the physical properties of the membrane, thus disrupting the membrane function of the cell (Alves et al., 2017).

In the present study, the anti-tumorigenic effect of PL and also combined therapy with Dox on HeLa cells was investigated. In our study, MTT analysis was performed in all samples treated with PL and Dox to determine cell viability and proliferation. Furthermore, AO/PI staining was performed to confirm the results of the MTT analysis. In MTT analysis and AO/PI staining, it was determined that as the application dose increased, cell death increased in both PL and Dox-applied samples ($p<0.001$). In cultures treated with 100 µM PL and 1000 nM Dox combined, cell viability was found to be 66.9% and 69.4%, respectively, after MTT analysis. AO/PI stains confirm the MTT data. These data demonstrated the anti-tumor effect of PL in HeLa cells, in agreement with the literature (Seber et al., 2020). Although the antitumor activity of PL is known, the underlying molecular mechanisms have not been fully elucidated yet. Therefore, in the continuation of our study, the expression levels of genes associated with metastasis and tumorigenesis such as PTEN were evaluated.

The phosphatidylinositol 3-kinase/protein kinase B signaling pathway (PI3K/Akt signaling pathway) is one of the key regulators of several signaling pathways including cell survival, proliferation, differentiation, endocytosis and vesicle trafficking, metabolism, and host inflammatory responses. Active Akt plays a central role in cell signaling downstream of PI3K in a variety of cellular stimuli. Controls its substrates, such as Bad, caspase-9, glycogen synthase kinase 3β (GSK3β), mTOR, and many other transcription factors. Disruption of Akt activity is among the pathophysiological causes of various diseases, including type 2 diabetes and cancer (Nakayama et al., 2015). In parallel with research on cancer, research in other fields has revealed the catalytic and regulatory roles of the PI3K signaling pathway in normal cell function (Fruman et al., 2017). PI3K is negatively controlled by PTEN. Although PTEN is a bidirectional phosphatase enzyme that has effects on many proteins, its main function for the cell is to dephosphorylate phosphatidylinositol 3,4,5-triphosphate (PIP3) to phosphatidylinositol 4,5-biphosphate (PIP2) by working as a negative regulator of PI3K (Myers & Tonks, 1997; Tamura et al., 1998). It is well known that loss of PTEN gene expression is directly related to increasing Akt activity (Stambolic et al., 1998).

As a result of qRT-PCR analyses, in group 2, where only piperlongumine was administered PTEN gene expression (RQ=0.38) decreased by 62%. In group 3, where only Dox was administered for 24 hours, PTEN gene expression (RQ=0.42) decreased by 58%. In group 4, where piperlongumine and Dox were administered combined for 24 hours, PTEN gene expression decreased by 53% (RQ=0.47). However, western blot analyzes showed that PTEN protein expression persisted despite decreased gene expression. In group 2, where only piperlongumine was administered PTEN expressed 1.33 fold higher compared to control and in group 4, where piperlongumine and Dox were administered combined for 24 hours, PTEN was expressed 3.16 fold higher. Only in group 3, where only Dox was administered for 24 hours, PTEN expression (0.77 fold) decreased by 23%.

In this study, the effect of PL on cell proliferation and viability was evaluated when used alone or in combination with Dox in HeLa cells, a cervical cancer cell line. In our literature review, we did not find any study reporting the effect of PL on PTEN gene/protein expression when used alone or in combination with Dox in HeLa cells. Our study made a valuable contribution to the literature as the first study in which the anti-tumor effect of PL on cell viability was evaluated by determining the PTEN gene and protein expression.

While it was determined that PTEN gene expressions were decreased in all groups, it was observed that protein expression continued as a result of the western-blot study. This suggests that PTEN protein phosphorylation and activation are enhanced when piperlongumine is administrated alone or together with doxorubicin. Increasing the activation of PTEN, a tumor suppressor, in the cancer cell is

a desirable outcome. However, it should be considered that the PTEN gene expression is decreased. Also, of course, it is not enough to specify the PTEN expression alone. Other signaling molecules that regulate the expression of this gene or are affected after its activation need to be investigated. And, whether it causes PTEN change in normal cells should be confirmed.

4.1. Conclusion

It was determined that cell death was increased and cell proliferation was suppressed in the samples treated with both PL and Dox. These data showed the anti-tumor effect of PL in agreement with the literature. In addition, PTEN protein phosphorylation and activation were enhanced when piperlongumine was administrated alone or together with doxorubicin. This shows that despite anti-cancer effect of PL, this compound can be considered for therapeutic use to increase drug efficacy in drug-resistant cancer cells.

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