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Oleandrin, İnsan Endometriyal Karsinom Hücrelerinde Toll Benzeri Reseptör Yolağı Genlerinin İfadesini Baskılar

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ÖZET

Endometrial kanser, endometriyal dokularda kötü huylu hücrelerin gelişiminden kaynaklanır ve dünya genelinde kadınlar arasında altıncı en yaygın kanser türüdür. Doğuştan gelen bağışıklık sisteminin bir bileşeni olan Toll benzeri reseptörler (TLR'ler), patojenlerin tanınmasında kritik bir işlev üstlenir. Önceki araştırmalar, TLR'ler ile farklı kanser türleri arasında bir ilişki olduğunu ve TLR ekspresyonunun kanser hücrelerinde progresyon, apoptoz ve hayatta kalma ile ilişkili olduğunu göstermiştir. Apocynaceae familyasından olan *Nerium oleander* L., geleneksel olarak çeşitli hastalıkların tedavisinde kullanılmıştır. *N. oleander*'in yaprakları ve tohumlarından elde edilen oleandrin bileşiği, çeşitli insan kanser hücrelerinin büyümesini baskıladığı gösterilmiştir. Bu çalışma, oleandrinin Ishikawa insan endometrial karsinom hücrelerinde TLR yolağı genleri üzerine etkisini incelemeyi amaçlamıştır. Oleandrin uygulamasının ardından, XTT proliferasyon testi 24., 48. ve 72. saatlerde gerçekleştirilmiştir. IC_{50} değeri 48. saat için 75,3 nM olarak belirlenmiştir. Oleandrinin TLR'ler (1-10) ve MYD88 genlerinin ekspresyon seviyeleri üzerindeki etkisi qPCR ile analiz edilmiştir. Oleandrin uygulaması sonucunda TLR1, TLR6 ve MYD88 genlerinin ekspresyon seviyelerinde belirgin bir azalma ile sonuçlanırken, diğer TLR'lerin ifadesi nispeten stabil kalmıştır. Sonuç olarak, oleandrin, Ishikawa kanser hücrelerinin canlılığını önemli ölçüde azaltarak, TLR1, TLR6 ve MYD88 genlerinin seviyelerinin modülasyonu yoluyla TLR sinyal yolaklarını etkileyen sitotoksik etkiler göstermiştir.

Oleandrin Downregulates Toll-Like Receptor Pathway Genes in Human Endometrial Carcinoma Cells

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ABSTRACT

Endometrial cancer arises from the development of malignant cells in endometrial tissues and ranks as the sixth most prevalent neoplasm among women globally. Toll-like receptors (TLRs), as part of the innate immune system, have a key function in the detection of pathogens. Previous research has previously indicated an association between TLRs and different types of cancers, highlighting the role of TLR expression in cancer cells in relation to progression, apoptosis, and survival. *Nerium oleander* L., a member of the Apocynaceae family, has been traditionally employed in addressing various diseases. Oleandrin, a compound obtained from the leaves and seeds of *N. oleander*, has been shown to suppress the growth of various human cancer cells. This study aimed to examine the impact of oleandrin on TLR pathway genes in Ishikawa human endometrial carcinoma cells. Following oleandrin treatment, XTT proliferation assay was conducted at the 24., 48. and 72. hours. The IC_{50} value was found to be 75.3 nM at the 48 hour. The impact of oleandrin on the expression levels of TLRs (1-10) and MYD88 genes in Ishikawa cells was analyzed using qPCR. Oleandrin administration caused a notable decrease in the expression levels of TLR1, TLR6 and MYD88, whereas the expression of other TLRs remained relatively stable. In conclusion, oleandrin exhibited cytotoxic effects, significantly decreasing the viability of Ishikawa cancer cells and influencing the TLR signaling cascade through modulation of the levels of TLR1, TLR6, and MYD88 genes.

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INTRODUCTION

Endometrial cancer (EC) is a cancer that develops in the inner lining of the uterus [1]. It is the most common cancer of the female reproductive system [2]. The prognosis of endometrial cancer varies significantly based on its histological type. Type 1 tumors are the most prevalent, hormone-responsive, and typically at a lower stage, with a highly favorable outcome. In contrast, type 2 tumors have high malignancy and poorer prognosis and prone to recurrence even in early stages [3]. The primary treatment for EC is surgery and supplementation by adjuvant radiation or chemotherapy depending on the tumor's grade and stage [4]. Research continue for the investigation of novel agents for EC treatment and the development of new treatment strategies.

Nerium oleander L. is a highly toxic ornamental shrub that is commonly cultivated worldwide. *N. oleander* contain several toxic compounds, such as oleandrin, oleandrigenin and other cardiac glycosides [5]. Oleandrin is a compound, in monomeric form, extracted from the leaves and seeds of the plant. It is widely utilized in managing various diseases such as congestive heart failure. Studies have shown that oleandrin may be a potential antitumor agent, and it has the ability to effectively restrain the expansion of various cancer cells and promote apoptosis. Oleandrin has recently attracted considerable attention because of its remarkable anti-cancer and anti-viral effects [6,7].

Toll-like receptors (TLRs) are recognized as key factors in tumor pathogenesis, influencing both tumor cells and immune cells, including those involved in innate and acquired immunity [8]. In humans, ten distinct types of TLRs have been identified [9]. TLR1, 2, 4, 5, 6 and 10 present on the cell surface, while TLR3, 7, 8 and 9 are found in endosomes [10]. MyD88 serves as a fundamental adaptor protein in TLR pathways and holds a crucial function in innate immune responses. MyD88 stimulation results in the generation of inflammatory cytokines, which facilitate immune cell infiltration, polarization and immune escape within the tumor microenvironment [11,12]. TLR expression and its role in cancer cells and its relation with tumor formation and tumor progression are areas that need to be investigated [13].

While previous studies have demonstrated the anticarcinogenic effects of oleandrin in various cancer cell types, the underlying mechanism responsible for these effects remains unclear. In this study, the impact of oleandrin on Ishikawa human endometrial carcinoma cells was investigated, focusing on cell proliferation and TLR signaling pathways. For this purpose, XTT assay was used to assess cytotoxicity, and qPCR was employed to analyze the expression levels of genes encoding key proteins involved in the TLR pathway (TLR1–TLR10 and MyD88).

MATERIALS AND METHODS

Chemicals

Oleandrin (Cat#06069) was supplied by Sigma-Aldrich Chemical Company, USA, while the XTT kit (Cat#20-300-1000), FBS, PBS, RPMI-1640, and pen-strep were supplied by Biological Industries. QIAzol was purchased from Qiagen, USA. The cDNA synthesis kit and SYBR green mix were obtained from Bio-Rad.

Cell culture and culture conditions

The human endometrial adenocarcinoma Ishikawa (ECACC 99040201) cell line was obtained from ATCC and cultured in RPMI-1640 medium supplemented with 10% FBS, 1% pen-strep and 2 mM L-glutamine. The cells were maintained in a humidified incubator at 37°C with 5% CO_2 and 95% air to ensure optimal growth conditions.

Cytotoxicity assay

The effect of oleandrin on the viability of Ishikawa cells was assessed using the XTT assay. In summary, Ishikawa cells were plated at a density of 10^3 cells per well in a 96-well plate and treated with seven varying oleandrin concentrations (0–150 nM) for 24, 48, and 72 hours. Following treatment, a microplate reader was used to assess cell viability at 450 nm, with 630 nm as the reference wavelength.

The formula used to calculate cell viability was: Viability (%) = (Optical density of treatment well / Optical density of control well) × 100.

The half-maximal inhibitory concentration (IC_{50}) of oleandrin was determined using CompuSyn Version 1.0 software.

RNA isolation, cDNA synthesis and quantitative real time PCR (qPCR)

Total RNA was extracted using QIAzol Reagent to evaluate the expression of TLR pathway genes. The cDNA was synthesized following the manufacturer's guidelines using the cDNA Synthesis Kit (Bio-Rad iScriptTM cDNA synthesis kit, Cat#170–8891, USA). The primer sequences used in the qPCR analyses were given in Table 1 [14]. The qPCR reaction for each gene was assembled in a total volume of 10 µl, comprising 5 µl of 2× SYBR Green Supermix, 5 pMol of both forward and reverse primers and 2 µl of cDNA. The thermal cycling conditions included an initial denaturation at 95°C for 10 minutes, followed by 40 amplification cycles: denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and strand extension at 72°C for 30 s. ACTB was used as the reference gene to standardize qPCR data and to normalise the data.

Table 1
The primer sequences used for gene expression analysis in qPCR [14].

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	PCR product size (bp)
TLR1	CAGCGATGTGTTCGGTTTTCCG	GATGGGCAAAGCATGTGGACCA	111
TLR2	TTATCCAGCACACGAATACACAG	AGGCATCTGGTAGAGTCATCAA	160
TLR3	GGCTAGCAGTCATCCAACAGAA	GCAGTCAGCAACTTCATGGC	135
TLR4	CCCTGAGGCATTTAGGCAGCTA	AGGTAGAGAGGTGGCTTAGGCT	126
TLR5	CCTTACAGCGAACCTCATCCAC	TCCACTACAGGAGGAGAAGCGA	130
TLR6	TTCTCCGACGGAAATGAATTTGC	CAGCGGTAGGTCTTTTGGAAC	75
TLR7	CTTTGGACCTCAGCCACAACCA	CGCAACTGGAAGGCATCTTGTAG	142
TLR8	ACTCCAGCAGTTTCCTCGTCTC	AAAGCCAGAGGGTAGGTGGGAA	145
TLR9	TGAGCCACAACTGCATCTCGCA	CAGTCGTGGTAGCTCCGTGAAT	134
TLR10	GGTTAAAAGACGTTCATCTCCACG	CCTAGCATCCTGAGATACCAGG	145
MyD88	GAGGCTGAGAAGCCTTTACAGG	GCAGATGAAGGCATCGAAACGC	129
ACTB	AGCACGGCATCGTCACCAACT	TGGCTGGGGTGTTGAAGGTCT	179

Statistical Analyses

All experimental procedures were performed in triplicate. The qPCR analysis was conducted using the $2^{(-\Delta\Delta Ct)}$ method, while comparisons between the control and dose groups were performed through "Volcano Plot" analysis in "RT2 Profiles™ PCR Array Data Analysis," followed by statistical evaluation using the Student's *t*-test. Statistical significance was considered when $p \leq 0.05$ in all experiments.

RESULTS

Oleandrin has anti-proliferative effects on Ishikawa cells

Cytotoxic effect of oleandrin in Ishikawa cells was evaluated through the XTT assay. Oleandrin inhibited the cell proliferation of human endometrial adenocarcinoma cells. The inhibition occurred in a time- and dose-dependent manner. IC_{50} of oleandrin in Ishikawa cells was determined to be 75.3 nM at 48. hours, using CompuSyn version 1.0 software (Figure 1) [15]. This dose was used in subsequent gene expression analysis using qPCR.

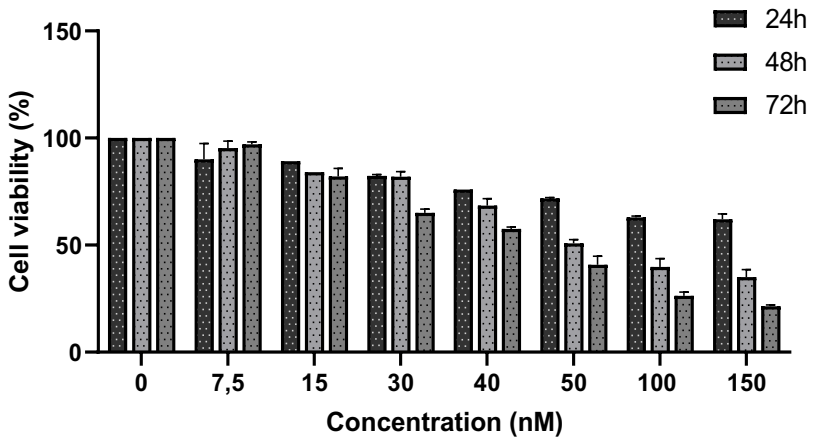

Figure 1
Effects of oleandrin on cell survival in Ishikawa cells.

Oleandrin effects TLR pathway genes

After isolating total RNA and synthesizing cDNA from both control and oleandrin-treated groups, gene expression levels were assessed using qPCR. The impact of oleandrin on gene expression in the TLR pathways in Ishikawa cell lines were illustrated in Figure 2. Oleandrin treatment in the Ishikawa cells resulted in a notable decrease in the expression of TLR1, TLR6 and MYD88. An elevated but insignificant expression was observed for TLR2, 3, 4, 7, 8, 9 and 10. The expression of other TLRs did not show any significant differences.

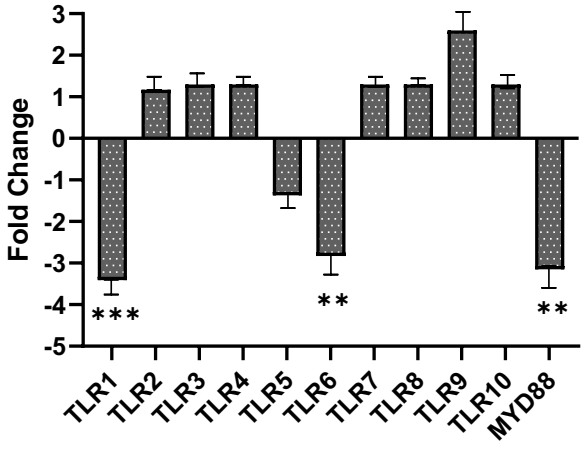

Figure 2
*Effects of oleandrin on TLR pathway genes in Ishikawa cells (**p<0,01, ***p<0,001).*

DISCUSSION

Cancer is a severe genetic disorder resulting from a series of abnormalities in the genome [16]. EC is the fourth leading cancer in women, following breast, lung and colorectal cancers, with a rising incidence and mortality rate [2,17,18]. The need for new treatments continues. This research investigates the possible anticancer effect of oleandrin on human endometrial adenocarcinoma Ishikawa cells by examining cell proliferation and expression levels of genes having critical roles in TLR pathways.

Oleandrin is a cardenolide that contributes to the potent heart-related effects and is present in the aerial parts of *Nerium oleander*. A study demonstrated that oleandrin displayed notable cytotoxic effects on four colon, one gastric and one cervical cancer cell lines in vitro. These findings suggested that cardiac glycosides could specifically inhibit the growth of tumor cell lines [19]. It was shown that oleandrin, ouabain and digoxin induce cell death in androgen-resistant human prostate cancer cells by promoting sustained increases in intracellular Ca^{++} levels [20]. Gunes et al. demonstrated that oleandrin treatment reduced the level of proinflammatory cytokine IFN-γ and thereby apoptosis in melanoma cells. Additionally, oleandrin demonstrated anticarcinogenic effects by influencing TLR pathway genes and associated miRNAs [14]. Pan et al. reported that oleandrin notably decreased cell viability in SW480, HCT116 and RKO cells. The IC_{50} values for SW480 and NCM460 cells were calculated as 0.02 μM and 0.56 μM, respectively. Oleandrin suppressed pro-caspase-3 and pro-caspase-9 expressions while enhancing the activation of caspase-3 and caspase-9, suggesting its role in inducing mitochondrial-mediated apoptosis in human colorectal cancer cells [21]. Ko et al. [22] reported that oleandrin and odoroside A suppress invasion in both MDA-MB-231 and radiotherapy-resistant variants by inhibiting phospho-STAT3, which consequently downregulates invasion-related molecules such as OCT3/4, β-catenin, and MMP-9. Gunes et al. [23] proposed that oleandrin could serve as an anti-cancer agent, displaying anti-proliferative, apoptotic, anti-metastatic and anti-invasive effects in human melanoma cells. Oleandrin also displays a strong cytotoxic impact on breast cancer cells without affecting the growth of MCF-10A cells. It was also indicated that oleandrin suppresses breast cancer cells derived from patients in 3D culture and induces mitochondrial-mediated apoptosis by triggering ER-stress [24]. Our present study demonstrated that lower oleandrin doses suppressed cell proliferation of Ishikawa cells.

It has been estimated that >15% of all malignancies are initiated by inflammation. It was observed that prolonged administration of nonsteroidal anti-inflammatory drugs lowers cancer risk and

strengthens the proposed link between inflammation and cancer [25]. Recent findings suggest that TLRs are involved in the onset and advancement of cancer as well as autoimmune diseases [26]. Droemann et al. [27] identified TLR9 expression across different human lung cancer tissues and several tumor cell lines. Various TLR4 sequence variants have been linked to an increased risk of developing prostate cancer. This underscores the distinct function of TLRs in the body's first line of defense and their impact on prostate cancer susceptibility [28]. Previous research have reported an increased TLR expression in gastric carcinoma cells and their precursor lesions. Since *H. pylori* stimulates epithelial TLR expression to drive gastric carcinoma-associated factors like IL-8, the existence of TLRs in gastric cancer cells could amplify tumor-promoting inflammatory responses, leading to detrimental effects [29]. A study found that TLR2 expression was significantly increasedin MDA-MB-231 cells and was crucial for cell invasion. Compared to the less invasive MCF-7 cells, MDA-MB-231 cells showed a 10.5-fold higher expression of TLR2. The study suggested that TLR2 is a crucial receptor involved in NF-kappaB activation and the increased invasive capacity of MDA-MB-231 cells [30]. One study indicated that TLR2-5 are prominently found on the epithelial surface of normal ovaries, and these receptors are also present in non-cancerous conditions, tumor tissues, and ovarian cancer cell cultures. These findings highlight the existence of multiple TLRs in both normal ovarian tissue and ovarian cancer cells, suggesting a mechanism in which epithelial tumors activate inflammatory pathways that promote tumor progression [31]. TLR5 may be crucial in the progression of cervical neoplasia. It could also act as a key indicator for the transformation of cervical squamous cells into cancerous ones [32]. Bakbak et al. reported that TLR2 and TLR6 were significantly upregulated in cases of endometrial cancer and endometrial hyperplasia. It also proposed that the presence of TLR6 might be associated with late-stage endometrial cancer [33]. Altered MyD88 signaling drives tumor cell growth and metastasis, which are significantly associated with a poor prognosis. Thus, MyD88 could serve as both a tumor biomarker and a valuable target for cancer therapy [12]. A study also indicated that the MyD88 gene plays a significant role in promoting colorectal cancer [34].

In this study, qPCR results revealed that oleandrin treatment led to a significant decrease in the expression of TLR1, TLR6, and MyD88 genes, which are essential components of the TLR signaling pathway. This downregulation suggests that oleandrin may inhibit the TLR-mediated signaling pathway, which is crucial for the inflammatory response and immune system activation in cancer cells. Consequently, oleandrin displayed an anticarcinogenic effect by targeting TLR pathway genes in Ishikawa cells.

These findings propose that oleandrin could exert its anti-cancer effects by modulating key components of the innate immune response, potentially offering a novel anticancer approach for endometrial carcinoma. The limitation of this study is that a control (non-cancerous) cell line was not used for comparison of the findings. The inclusion of a healthy control would have provided valuable insights into the specificity of oleandrin’s effects, helping to determine whether these effects are cancer-specific or if they also impact normal cells. Further research is needed to explore its impact on other pathways in more detail.

CONCLUSION

This study demonstrates that oleandrin exerts significant anti-proliferative and cytotoxic effects on Ishikawa human endometrial carcinoma cells. The XTT assay revealed a dose-and time-dependent inhibition of cell proliferation. Moreover, gene expression analysis through qPCR showed a notable downregulation of TLR1, TLR6 and MYD88, key components of the TLR signaling pathway, suggesting that oleandrin may modulate immune responses by targeting these receptors. These findings indicate that oleandrin has the potential to inhibit cancer cell growth through modulation of TLR-

mediated inflammatory pathways, offering a promising avenue for further research into its application as an anticancer agent, particularly for endometrial carcinoma. However, additional studies are necessary to fully understand its mechanisms and explore its effects on other signaling pathways.

Ethical Statement

This article is the revised and developed version of the unpublished conference presentation entitled "A Cardiac Glycoside of *Nerium oleander* L. Downregulates TLR1, TLR6 and MYD88 Genes in Human Endometrial Carcinoma Cells", orally presented at the Second International Congress on Biological and Health Sciences-2022".

Author Contributions

Research Design (CRediT 1) S.A. (%30)- C.E.G. (%30)- F.S.Ç. (%20)- E.K. (%20)
Data Collection (CRediT 2) S.A. (%30)- C.E.G. (%20)- F.S.Ç. (%30)- E.K. (%20)
Research - Data Analysis – Validation (CRediT 3-4-6-11) S.A. (%40)- C.E.G. (%20)- F.S.Ç. (%20)- E.K. (%20)
Writing the Article (CRediT 12-13) S.A. (%30)- C.E.G. (%20)- F.S.Ç. (%20)- E.K. (%30)
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Sustainable Development Goals (SDG)

Sustainable Development Goals: 3 Healthy and quality life.

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