Abstract. – OBJECTIVE: Fusaric acid is a derivative of picolinic acid produced by some Fusarium species. In this study, we aimed to determine the mRNA expression and antiproliferative effects of fusaric acid in Ishikawa endometrium cancer cells in signal pathway genes associated with Toll-like receptors (TLRs). The effect of fusaric acid on the viability of Ishikawa cells was evaluated using XTT.

PATIENTS AND METHODS: After total RNA was isolated from control and dose group cells, cDNA synthesis was performed, and mRNA expression changes of genes involved in the Toll-like signaling pathway were evaluated by real-time reverse-transcription polymerase chain reaction (RT-PCR).

RESULTS: The decrease in viability of Ishikawa cells was observed in a time- and dose-dependent manner. The inhibitory concentration (IC50) dose of fusaric acid at the 72nd hour in the Ishikawa cell line was 142.81 µM. When the dose group treated with 125 µM fusaric acid at the 72nd hour was compared with the control group, significantly decreased toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, and Myeloid differentiation primary response protein 88 (MYD88) gene expressions were observed.

CONCLUSIONS: Fusaric acid inhibits cell proliferation and downregulates Toll-like receptors pathway gene expression in Ishikawa endometrial cancer cells.

Key Words: Cell proliferation, Endometrial cancer, Fusaric acid, Ishikawa cell line, Toll-like signaling pathway.

Introduction

Endophytic fungi produce different mycotoxins, which are toxic to humans and animals. Fusaric acid (FA, 5-butylicolinic acid) is a derivative of picolinic acid produced by some Fusarium species. These fungal species are ubiquitous in soil and are known to parasitize many agricultural foods. FA has phytotoxic effects on plants, such as necrosis and wilt disease. Neurochemical effects have been studied in mice and rats. FA is also toxic to humans. By increasing oxidative stress in the cell, it induces mitochondrial energy dysfunctions, DNA damage, and apoptotic cell death.

Inflammation is an important factor in carcinogenesis. Some signaling pathways are activated in response to inflammation. One of these signaling pathways is the Toll-like signaling pathway. Toll-like receptors (TLRs) can trigger an inflammatory response and cell survival in the tumor microenvironment. It has been reported that the Toll-like signaling pathway is associated with many disease groups, such as cancer, autoimmune diseases, diabetes, and dermatological diseases. TLRs are involved in important processes such as the regulation of apoptosis, DNA repair, autophagy, and angiogenesis. TLRs are localized in cell components such as cell surface, endosome, endolysosome, and lysosome. Ten TLRs (TLR1-TLR10) have been identified in humans.

Endometrial cancer (EC) is a type of cancer that is most common in women and has a high mortality rate. Classical antineoplastic drugs negatively affect the quality of life of patients due to their different side effects. For this reason, herbal studies have been carried out for many years to find new and more effective treatments for malignant cells with minimal risk of toxicity to healthy cells.

As an experimental model, we used the Ishikawa cell line. Ishikawa cells are one of the best-charac-
terized human endometrial cell lines\(^1\). These cells are derived from a well-differentiated adenocarcinoma of the human endometrial epithelium and express estrogen and progesterone receptors\(^1\,\^2\).

In this study, we aimed to determine mRNA expression and antiproliferative effects of FA in signal pathway genes associated with Toll-like receptors in Ishikawa cells in order to shed light on endometrial cancer, which is commonly seen in women.

**Patients and Methods**

**Cell Culture**

Ishikawa endometrial cancer cells were grown up in RPMI-1640 medium (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Capricorn Scientific GmbH, Ebsdorfergrund, Germany), 20 µg/ml streptomycin and 20 units/ml penicillin, 1 mM sodium pyruvate (Biological Industries, Beit Haemek, Israel) and 0.1 mM amino acid solution (Biological Industries, Israel) and cultured at 37°C in 5% CO\(_2\) in this study. Fusaric acid was treated in the Ishikawa cells with different concentrations, including 12.5 µM, 25 µM, 50 µM, 100 µM, 125 µM, 250 µM, and 500 µM to evaluate the antiproliferative activity at 24, 48, and 72 hours according to time and dose-dependent manner.

**XTT Assay**

The antiproliferative effects of fusaric acid, on Ishikawa endometrial cancer cells were determined using XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay at a concentration of 1×10\(^4\) cells per well in 96-well plates according to the kit’s instructions (Cell Proliferation Kit; Biological Industries, Israel). The XTT combination was administered in line with the dose and time prescribed by the manufacturer after the dosing intervals were completed. Formazan formation was measured spectrophotometrically with a microplate reader (Multiskan GO microplate spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) at 450 wavelengths (reference wavelength 630 nm) and colorimetrically. The stated formula was used to calculate cell viability (percentage) using absorbance measurements.

\[
\text{Viability} (\%) = \frac{\text{Absorbance of experiment well}}{\text{Absorbance of control well}} \times 100
\]

The AAT Bioquest online tool (available at: https://www.aatbio.com/tools/ic50-calculator) was used to assess the inhibitory concentration (IC\(_{50}\)) dosage of fusaric acid on Ishikawa cells. The IC\(_{50}\) dosage was used as dose group in the other molecular studies of this study.

**Real-Time PCR Assay**

Total RNA isolation from cells was carried out using Trizol (Invitrogen, Waltham, MA, USA) in accordance with the manufacturer’s instructions. The A.B.T. synthesis kit with RNase Inhibitor was used for cDNA synthesis (Atlas Biotechnology, Çankaya/Ankara, Turkey). Real-Time polymerase chain reaction (RT-PCR, Applied Biosystem, Step One Plus, Foster City, CA, USA) was used to assess changes in mRNA expression of toll-like receptor 1 (TLR1), TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, Myeloid differentiation primary response protein 88 (MYD88). Normalization was accomplished through the use of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences

| Table I. Primer sequences of the genes used in the study. |
|-----------------|-----------------|
| **Forward**     | **Reverse**     |
| TLR1            | CAGCGATGTGTTGCGTTTCCG |
| TLR2            | TTACGCACACGACAGAATACAG |
| TLR3            | GGCTAGCAGCTCATCCAACAGA |
| TLR4            | CCTGAGGCTATTTAGGCGATCA |
| TLR5            | CTTACAGCAGAACCTCACTCA |
| TLR6            | TTTCGTGACGGAAATGTAATTG |
| TLR7            | CTTTGACCTCCAACCAACCA |
| TLR8            | AACTCCGACTTTCCTCTGTCTC |
| TLR9            | TGAGCCACAAGCTGATCTGCA |
| TLR10           | GGTTAAGAGACGCCATCTCAG |
| MYD88           | GAGGCATTGAAGGCCCTTTACAG |
| GAPDH           | GCACCGCTCAGGGCTGAGAAC |

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used in this study are given in Table I. RT-PCR was used to perform real-time PCR tests using the ABT™ 2X qPCR SYBR-Green Master Mix (Atlas Biotechnology, Cankaya/Ankara, Turkey) protocol.

**Statistical Analysis**

The parametric and nonparametric analysis of the dosage and control groups was performed using the IBM SPSS Version 23 (IBM Corp., Armonk, NY, USA) analytical tool. In all statistical analyses, a p-value lower than 0.05 was accepted as statistically significant. In the analysis of RT-PCR data, quantitation was performed using the 2^ΔΔCT method via RT-PCR analysis RT² Profiler™ PCR Array Data Analysis program.

**Results**

**XTT Assay**

Upon treatment with FA, the viability of Ishikawa cells was assessed by XTT assay. A decrease in viability Ishikawa cells was observed in a time- and dose-dependent manner. Although the cell viability in the cells treated with fusaric acid decreased partially due to the increasing dose at the 24th and 48th hours, a more effective effect was observed at the 72nd hour. IC_{50} dose of FA was found as 142.81 µM at 72nd hour in Ishikawa cell line (Figure 1).

**Real Time-PCR**

After total RNA was isolated from control and dose group cells, the cDNA synthesis was performed. The expression analysis of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, MYD88 were performed by RT-PCR according to the SYBR Green qPCR Master Mix protocol. As a result of RT-PCR, mRNA expression changes of genes involved in the Toll-like signaling pathway were evaluated. All gene fold changes, and p-values are summarized in Table II.

When the dose group treated with 142.81 µM FA at 72nd hour was compared with the control group, significantly decreased TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, and MYD88 gene expressions were observed (Table II). The decrease in TLR3 (-2.77, p=0.020180), TLR4 (-2.38, p=0.018438), TLR5 (-2.42, p=0.010049), TLR6 (-2.34, p=0.006882), TLR7 (-4.06, p=0.009164), TLR8 (-5.71, p=0.009339), TLR9 (-5.93, p=0.010035), TLR10 (-6.23, p=0.003637) and MYD88 (-2.53, p=0.039905) gene expression levels were statistically significant. TLR1 (-7.22, p=0.353098) and TLR2 (-1.20, p=0.436636) expressions were also decreased, but not statistically significant.

**Discussion**

EC is a gynecological malignancy common in women and has a high incidence and mortality rate. Although more molecular-targeted drugs are used in the treatment of EC and other cancers, the side effects of chemotherapy drugs make the treatment process of the disease dif-
in many types of cancer, and its adverse effect on endometrial tissue was first reported by Modugno et al.\textsuperscript{27} in 2005. Under physiological conditions, endometrial tissue is exposed to cyclic inflammation. Therefore, endometrial tissue is different from other tissues. Because of this feature of the endometrial tissue, TLRs can be used as diagnostic and prognostic markers for endometrial cancer\textsuperscript{28}. TLRs regulate both oncogene and tumor suppressors at the posttranscriptional level\textsuperscript{16}. Although the expression of TLRs has been studied in breast\textsuperscript{29}, pancreatic\textsuperscript{30}, stomach\textsuperscript{31}, and colon\textsuperscript{32} cancers, studies on gynecological malignancies are limited. Ng et al.\textsuperscript{33} investigated the relationship between TLR2 and oral squamous cell cancer and reported that TLR2 expression was significantly higher in inflammatory cells close to cancer cells. Pandey et al.\textsuperscript{34} found a significant relationship between cervical cancer and TLR2 and TLR4 expressions. Allhorn et al.\textsuperscript{35} showed that TLR3 and TLR4 expression play a role in pathological changes in the endometrium. Bakbak et al.\textsuperscript{28} showed that TLR2 and TLR6 were significantly more expressed in cases with endometrial cancer. In the same study, it was concluded that the presence of endometrial hyperplasia and TLR6 may indicate the presence of late-stage endometrial cancer\textsuperscript{28}. Young et al.\textsuperscript{36} detected and reported TLR1-6 and TLR9 mRNA species both in the whole endometrium and in separated endometrial epithelial cells. In our study, a significant decrease was observed in TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, and MYD88 gene expressions in the dose group treated with 142.81 µM fusaric acid at 72nd hours compared to the control group. Expression of TLRs has been shown to be increased in various cancer types. However, there are almost no studies in the literature investigating the effect of FA on the expression of TLRs.

In this study, using the XTT assay, the viability of Ishikawa cells was determined as 142.81 µM at the 72nd-hour IC\textsubscript{50} dose. FA was shown to be more cytotoxic in Ishikawa endometrial cancer cells than in normal cells. Mamur et al.\textsuperscript{37} investigated the antiproliferative activities of FA in human cervical carcinoma (HeLa) cell lines with the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide test. Accordingly, they reported that a dose of 12.50 µg/mL FA produced a significant decrease in the mitotic index. Devnarain et al.\textsuperscript{38} showed that FA was cytotoxic and induced increased apoptosis in human esophageal carcinoma cell line (SNO) cells. Abdul et

<table>
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<th>Genes</th>
<th>Fold regulation</th>
<th>(p)-value</th>
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<tr>
<td>TLR1</td>
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<tr>
<td>TLR2</td>
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<td>TLR3</td>
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<td>TLR7</td>
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<tr>
<td>MYD88</td>
<td>-2.53</td>
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also showed that in the hepatocellular carcinoma (HepG2) cell line, FA caused a dose-dependent decrease in metabolic activity with significant depletion of intracellular ATP.

Conclusions

This study showed for the first time that FA downregulates TLR pathway genes mRNA expression and inhibits antiproliferative activity in Ishikawa endometrial cancer cells. Our results suggest that FA may be an effective and safe natural compound in the treatment of endometrial cancer. Multiple, larger, and multicenter joint clinical studies are needed for clinical evaluation of FA.

Conflict of Interest

The authors declare no potential conflicts of interest with respect to the research, authorship and publication of this article.

Authors' Contribution

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Ethics Approval

Since it is a cell culture study, it does not require any Ethics Committee approval.

Availability of Data and Materials

Not applicable.

Informed Consent

No investigation was conducted on human participants in this study.

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References

2) Singh VK, Upadhayay RS. Fusaric acid induced cell death and changes in oxidative metabolism of Solanum lycopersicum L Bot Stud 2014; 55: 66
18) Mena J, Elgueta E, Gonzales FE, Cardenas H, Orihuela PA. Hydroethanolic Extracts of the Ar-


