

***Ferulago pauciradiata* Boiss. & Heldr. Effects of Prantschimgin Compound Isolated from the Root of the Plant on LPS-Induced Inflammation Model in the L929 Cell Line**

Zerrin KUTLU^{1*}, Sevgi KARABULUT UZUNCAKMAK², Songul KARAKAYA³,
Fatmagül DELİMUSTAFAOĞLU⁴

¹Department of Biochemistry, Faculty of Pharmacy, Atatürk University, Erzurum 25240, Turkey

²Health Services Vocational School, Bayburt University, Bayburt, 69000, Turkey

³Department of Pharmacognosy, Faculty of Pharmacy, Ataturk University, Erzurum, 25240, Turkey

⁴Department of Pharmaceutical Botany, Faculty of Pharmacy, Ankara University, Ankara, 06000, Turkey

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Abstract

Investigation of the effects of prantschimgin (Prn) compound isolated from the root part of the *Ferulago pauciradiata* (Apiaceae) plant in the lipopolysaccharide (LPS) cytotoxicity model created in the L929 cell line. L929 cell lines were applied in a 5% CO₂ incubator at 37 °C, using standard cell culture procedures, LPS was applied with Prn pure substance with a final concentration of 2 µL and 1 µL after 1 hour. Following the LPS application, the MTT protocol, a colorimetric method, was applied to determine cell viability at the end of the required 24th, 48th, and 72nd hours incubation times. IC₅₀ values were calculated as 0.28 µg/mL for Prn and 1 µg/mL for LPS. Application of LPS to L929 cell lines caused a significant decrease in cellular index depending on time. Also, in the Prn + LPS groups, it was found that the decreased cell index significantly increased even closer to the control compared to the LPS applied group. It was found that cytotoxicity and cell damage caused by LPS applied to the L929 cell line improved after Prn application.

Keywords: *Ferulago pauciradiata*, cytotoxicity, L929, LPS, MTT, prantschimgin

L929 Hücre Hattında Lipopolisakkaritle İndüklenen Sitotoksosite Modeli Üzerine *Ferulago pauciradiata* Boiss. & Heldr. Bitkisinin Kökünden İzole Edilen Prantschimgin Bileşiğinin Etkisinin İncelenmesi

Öz

L929 hücre hattında oluşturulan lipopolisakkarit (LPS) kaynaklı sitotoksosite modelinde *Ferulago pauciradiata* (Apiaceae) bitkisinin kök kısmından izole edilen prantschimgin (Prn) bileşiğinin etkisinin incelenmesi. L929 hücre hatları, 37°C'de %5 CO₂'li inkübatörde standart hücre kültürü prosedürleri kullanılarak son konsantrasyonu 2 µL olacak şekilde Prn saf maddesi ve 1 saat sonra son konsantrasyonu 1 µL olacak şekilde LPS uygulandı. LPS uygulamasının ardından, gerekli olan 24, 48 ve 72 saat inkübasyon süreleri sonunda sitotoksik etkilerin belirlenmesi amacıyla kolorimetrik bir metot olan MTT protokolü uygulandı. IC₅₀ değerleri, Prn için 0.28 µg/mL ve LPS için ise 1 µg/mL olarak hesaplandı. L929 hücre hatlarına LPS uygulanmasının zamana bağlı olarak hücresel indekste anlamlı derecede azalmaya neden olmuştur. Ayrıca Prn+LPS gruplarında ise sadece LPS uygulanan grubuna göre azalmış hücre indeksini, anlamlı derecede artırdığı hatta kontrole yaklaştırdığı tespit edilmiştir. L929 hücre hattına uygulana LPS ile oluşturulan sitotoksosite ve hücre hasarını Prn uygulaması sonucunda düzeldiği tespit edildi.

*Corresponding Author: kutluzerrin@atauni.edu.tr

Anahtar kelimeler: Apiaceae, *Ferulago pauciradiata*, sitotoksisite, L929, LPS, MTT, prantşimgin

1. Introduction

Our country has a very wide flora in terms of plant diversity. Many of the drugs used today are directly or indirectly obtained from plants. (Tokur and Aksoy, 2017) Some plants have various activities due to the compounds such as coumarins, phenolic compounds and flavonoids they contain. Because of these properties of plants, researchers have recently preferred plants as therapeutic agents. (Zhang et al., 2017; Kutlu et al., 2020)

Medicinal plants used for various reasons since ancient times have made many contributions to human health. (Sheba ve Anuradha, 2019) *Ferulago pauciradiata* Boiss. & Heldr. (Apiaceae) is a perennial species. *Ferulago* species are used in folk medicine in ulcers and dermatological diseases. (Karakaya et al., 2018). Studies have found that coumarins have many pharmacological properties such as antibacterial, anticoagulant, antipyretic, vasodilator, and anticarcinogen (Srikrishna et al., 2018). Fibroblasts are a type of cell found in all tissues of the body (Thomas 2019). In the normal process, fibroblasts act as sentinel-like cells in the tissue and are the first to respond to damage signals (Millar et al., 2012).

The lipopolysaccharide (LPS) induced cytotoxicity model, which is a component of the cell wall of Gram-negative bacteria, is the most preferred model in in vitro cytotoxicity studies. We aim to investigate the effects of the prantschimgin (Prn) compound isolated from the root part of the *Ferulago pauciradiata* (Apiaceae) plant in the lipopolysaccharide (LPS) induced cytotoxicity model created in the L929 cell line.

2. Materials and Methods

2.1. Materials

2.1.1. Plant Material

Ferulago pauciradiata specimens were collected in Nevşehir in 2013 and identified by XXXX (Department XXXX). The voucher specimens were kept at the herbarium of Ankara University, Faculty of Pharmacy, with the number of AEF 26360.

2.1.2. Cell Lines

In this study, the L929 cell line was used to evaluate the effects of Prn compound isolated from the root part of *Ferulago pauciradiata* on LPS-induced cell damage in the L929 (ATCC® CRL-2091) cell line. The L929 cell line was purchased from the American Type Culture Collection (ATCC, USA) and used by reproducing from existing stocks available in the Cell Culture Laboratory, affiliated to the Department of Medical Pharmacology, Atatürk University School of Medicine.

2.2. Methods

2.2.1. Extraction and Isolation

Prantchimgin was obtained using the method described by Karakaya et al. (Karakaya et al. 2017 and Karakaya et al. 2018). 125 g dried crushed plant material was utilized and known compound prantchimgin was obtained. *Ferulago pauciradiata* specimens were collected in Nevşehir in 2013 and identified by Prof. Dr. Hayri DUMAN. The voucher specimens were kept at the herbarium of Ankara University, Faculty of Pharmacy, with the number of AEF 26360.

2.2.2. Experimental Protocol and Details

This study was carried out in the Cell Culture Laboratory of the Department of Medical Pharmacology, Faculty of Medicine, Atatürk University. First, different concentrations of doses were determined to calculate the IC₅₀ value of the Prn compound and LPS (Table 1).

Table 1. Doses used to calculate IC₅₀ values of LPS and Prn

Concentrations	
LPS	Prn
20 µg/mL	8 µg/mL
15 µg/mL	4 µg/mL
10 µg/mL	2 µg/mL
7.5 µg/mL	1 µg/mL
5 µg/mL	0.5 µg/mL
2.5 µg/mL	
1 µg/mL	

LPS; lipopolysaccharide, Prn; prantchimgin

After the cells preserved in Cryotube in the liquid nitrogen tank were dissolved, they were planted in flasks of T75 cm² (Gibco®, UK) with filtered cap. After the cells were determined with the help of Inverted (Leica®, Germany) light microscope (20X-40X), where 85-90% of them became confluent (20X-40X), 5x10³ cells were sown in each well of 96-well microplates. After that, Prn and LPS applications were performed and a colorimetric method (3- [4,5- dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT) protocol was applied to determine cytotoxic effects.

2.2.2.1. Preparation of Prantchimgin and LPS

Since the Prn compound is insoluble in water, 1% dimethyl sulfoxide was added to dissolve the substance after vortexing. Different concentrations (8 µg / mL; 4 µg / mL; 2 µg / mL; 1 µg / mL; 0.5 µg / mL) after passing through the sterile filter (Catalog no: 1520012, GyroDisc CA-PA) with sol pore diameter of 0.2 µm It was prepared. LPS (*E. Coli* O55: B5, Sigma-Aldrich®, St. Louis, USA) in lyophilized form was dissolved in pure water to prepare different concentrations.

2.2.2.2. Cell Culture Studies

Different cell lines grow in different environments. The L929 cell line is 89% DMEM (Gibco®, New York, USA), 10% FBS (Gibco®, New York, USA), 1% PSA (Gibco®, New York, USA) and 2% L-Glutamine (It is grown in medium containing CAS No: 56-85-9, Sigma-Aldrich®, St. Louis, USA). Cells were proliferated using standard cell culture procedures in a 5% CO₂ incubator (NuAire®, America) at 37 °C. With the help of the Inverted light microscope (Leica®, Germany), after determining that the cells are in a confluent state with 85-90% density, after mixing 100 µL trypan blue, 90 µL PBS, 10 µL medial cell in the micro eppendorf tube, 10 µL of this mixture was taken to determine the number of live cells in the cell counting device (INNOVATIS®, Virginia). After making the necessary calculations, sowing was performed with 5x10³ cells (number of groups x 5x10³) in each well.

2.2.2.3. Evaluation of Cell Viability with the MTT Method

L929 cell lines cultivated in three microplates (for 24th, 48th, and 72nd hours) with 5x10³ cells (ie 5000 cells at 150 µL), the standard cell in a 5% CO₂ incubator at 37 °C. The cells were allowed to incubate for 24th hours using culture procedures. At the end of the incubation period, the Prn compound was applied with a final concentration of 2 µL at the doses determined first and LPS was applied after 1 hour with a final concentration of 1 µL. Following the LPS application, a colorimetric method MTT (Cat. No. 11 465 007 001, Roche) protocol was applied to determine cytotoxic effects at the end of the required 24th, 48th, and 72nd hours incubation times. The % viable cell ratio was calculated using the following equation.

$$\% \text{ Percentage viability} = \frac{(\text{Compound-treated cell absorbance}) - (\text{absorbance of the empty well})}{(\text{control cell absorbance} - \text{absorbance of the empty well})} \times 100$$

Statistical Analysis

The data were evaluated statistically using IBM 22.00 SPSS software. To test the compliance of the data with a normal distribution (Gaussian), a Shapiro-Wilk W-test was applied to each group. Data with a p-value greater than 0.05 was considered to be normally distributed, and a skewness test was used to test its normal distribution. Levene's test was used to test the homogeneity of the variances. The variance of data with a p-value greater than 0.05 was considered homogeneous. The statistical analysis data that did not exhibit homogeneous variance was conducted using a Games-Howell test, which involved a one-way ANOVA and post-hoc multiple comparison tests; p<0.05 was considered significant. The statistical analysis of data that did not exhibit homogeneous variance were conducted using a Tukey and Duncan tests, which consisted of a one-way ANOVA and post-hoc multiple comparative tests.

3. Research Findings

3.1. LPS and IC₅₀ Values of Prantchimgin

To determine the most effective dose on the L929 cell line, Prn pure substance and LPS obtained from the root part of *Ferulago pauciradiata* at different concentrations were applied. IC₅₀ values that inhibit the proliferation of 50% of different concentrations of Prn and LPS on the L929 cell line *in vitro* were determined (Table 1).

IC₅₀ values were calculated as 0.28 µg/mL for Prn (Figure 1) and IC₅₀ for LPS (Figure 2) as 1 µg/mL using the logarithmic transformation of Prn and LPS concentration and nonlinear regression sigmoidal dose-response analysis (GraphPad 8.0 Prism, La Jolla, CA).

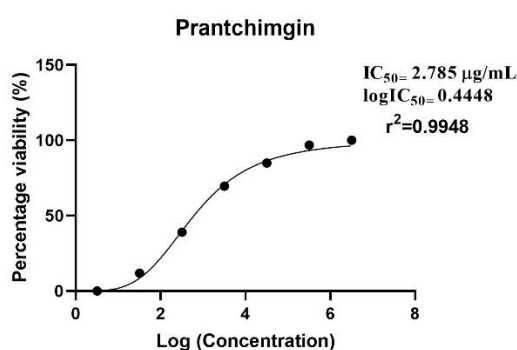


Figure 1. IC₅₀ value of prantchimgin

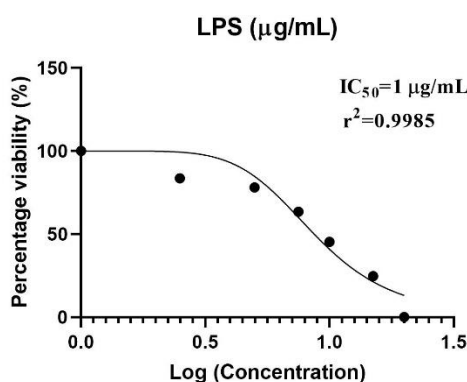


Figure 2. IC₅₀ value of LPS

3.2. Results of Cell Viability

The cell viability of the control group was considered as 100%. After applying LPS to the L929 cell line, when we compared the live-cell ratio in the LPS group with the live-cell ratio of the control group, a statistically significant difference was found ($p < 0.0001$). When LPS is applied 1 hour after applying different doses of Prn pure substance (8 µg / mL; 4 µg / mL; 2 µg / mL; 1 µg / mL; 0.5 µg / mL) obtained from the root part of the *Ferulago pauciradiata*, the concentration of Prn pure substance increases. It was determined that it increased cell proliferation and the highest live cell ratio was observed at a dose of 8 µg / mL (Figure 3).

According to the MTT protocol, the proportion of viable cells in the LPS group was 64.39% at 24th hours. When the proliferative effect of Prn pure substance on L929 cells was compared with the control group, it was determined that there were 91.78% live cells, and in the group that received Prn + LPS, 85.12% were live cells (Figure 4).

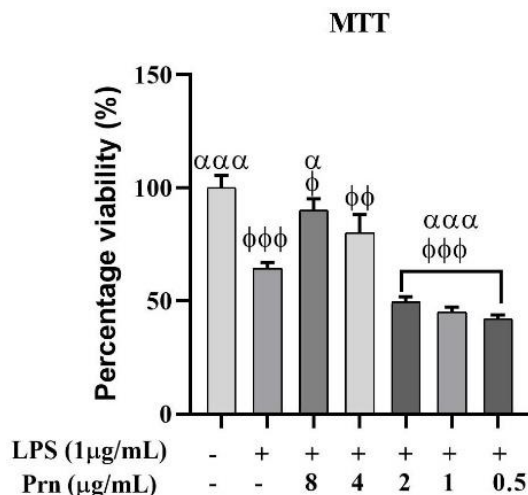


Figure 3. The effect of LPS and Prn agents applied on the L929 cell line on cell viability. (Φ; according to control group, $\Phi p < 0.05$, $\Phi \Phi p < 0.01$, $\Phi \Phi \Phi p < 0.001$; α: according to LPS group $\alpha p < 0.05$, $\alpha \alpha p < 0.01$, $\alpha \alpha \alpha p < 0.001$)

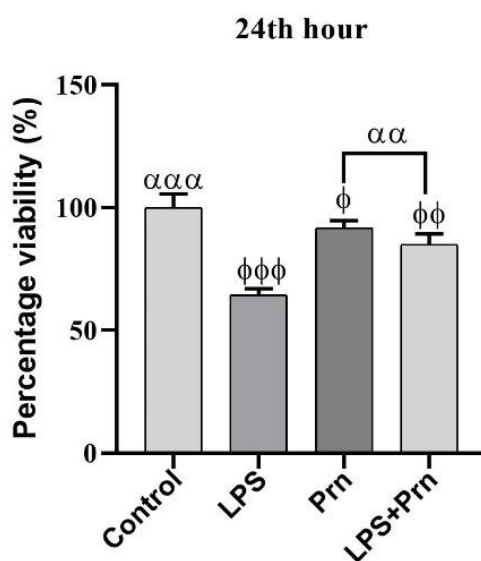


Figure 4. The effects of Prn and LPS on L929 cell line in 24th hours. (Φ; according to control group, $\Phi p < 0.05$, $\Phi \Phi p < 0.01$, $\Phi \Phi \Phi p < 0.001$; α: according to LPS group $\alpha p < 0.05$, $\alpha \alpha p < 0.01$, $\alpha \alpha \alpha p < 0.001$)

The live-cell ratio was 48.4% 48th hours after the Prn compound was applied on the L929 cell line. 48th hours after the LPS application, the live cell ratio in the LPS group was 38.79% and 87.33% in the Prn + LPS group (Figure 5).

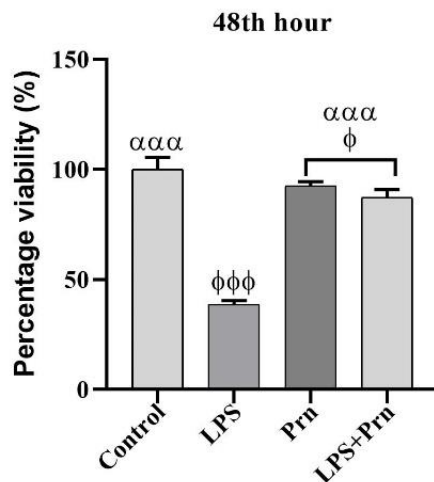


Figure 5. The effects of Prn and LPS on L929 cell line in 48th hours. (Φ ; according to control group, $\Phi p < 0.05$, $\Phi\Phi p < 0.01$, $\Phi\Phi\Phi p < 0.001$; α : according to LPS group $\alpha p < 0.05$, $\alpha\alpha p < 0.01$, $\alpha\alpha\alpha p < 0.001$)

When we look at the effects of the Prn compound on L929 cells after 72nd hours, the live cell ratio was found to be 93.78% in the Prn group and 89.18% in the group receiving Prn + LPS. In the LPS group, it was determined that 19.92% live cells (Figure 6).

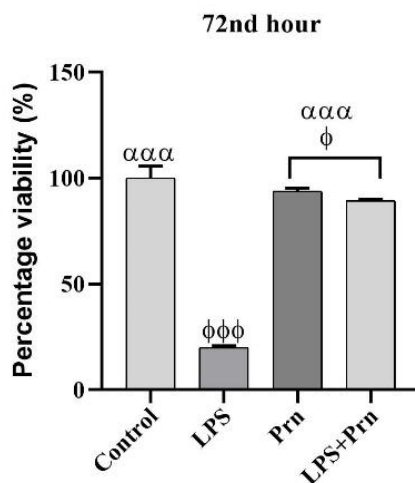


Figure 6. The effects of Prn and LPS on L929 cell line in 72nd hours. (Φ ; according to control group, $\Phi p < 0.05$, $\Phi\Phi p < 0.01$, $\Phi\Phi\Phi p < 0.001$; α : according to LPS group $\alpha p < 0.05$, $\alpha\alpha p < 0.01$, $\alpha\alpha\alpha p < 0.001$)

According to our results, Prn compound was determined to prevent cytotoxicity-related cell damage in the L929 cell line created with LPS. It was determined that L929 cells reduce cell damage caused by cytotoxicity caused by LPS depending on time. It was determined that the cell viability rate in the Prn + LPS group increased the cell proliferation at 24th hours ($p < 0.01$), 48th hours ($p < 0.001$), and more 72nd hours ($p < 0.001$) and was statistically significant compared to the LPS group. When we look at our results as a whole, the cell viability rate in all periods (24th hour, 48th hour, and 72nd hour) is above 85% and well

above the 70% rate recommended by ISO 10993-5: 2009 for cytotoxicity (DIN EN ISO, 2009) found to be.

4. Discussion

In this study, the effects of Prn compound isolated from the root of *Ferulago pauciradiata* on LPS-induced cytotoxicity cell injury model in L929 cell line were investigated. In this study, it was determined that Prn compound prevented cytotoxicity on L929 cell line at 24th hours, 48th hours and more 72nd hours, and increased cell proliferation and brought it closer to cell viability in the control group. When the results of our study were analyzed as a whole, results consistent with our hypothesis that the Prn compound reduced cytotoxicity cell damage was obtained.

One of the most common coumarin compounds found in the Apiaceae family, and especially in the genus *Ferulago*, is Prn. In studies conducted (Srikrishna et al., 2018; Hoang et al., 2017; Venugopala et al., 2013). Coumarins have been reported to have various pharmacological properties such as anti-inflammatory, antibacterial, antioxidant, antifungal, and anticoagulant. In accordance with the results of the studies conducted, while the effects of Prn, which is a coumarin derivative, on L929 cells at 24th, 48th and 72nd hours did not show any toxic effect compared to the control group, it was determined that Prn application increased the amount of decreased cell index in LPS groups.

As a result, studies (Mustafa et al., 2018; Kasumbwe et al., 2017) found that coumarins have a proliferative effect. When LPS was applied 1 hour after applying different doses of the Prn compound obtained from the root part of the *F. pauciradiata*, it was found that as the concentration of the Prn pure substance increased, the cell proliferation increased and the highest viable cell ratio was observed at a dose of 8 µg / mL.

5. Conclusion

When we look at the results of our study as a whole, it was determined that the viability rates of the cells were above 85% as a result of the Prn application and it was significantly above the 70% viable cell ratio recommended by ISO 10993-5: 2009 Din en ISO (2009), for cytotoxicity. These results showed us that Prn does not have a cytotoxic effect.

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Competing interests

The authors declare that they have no competing interests.

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