



Anti-inflammatory Effects of *Pelargonium endlicherianum* Fenzl. Extracts in Lipopolysaccharide-stimulated Macrophages

Pelargonium endlicherianum Fenzl. Ekstrelerinin Lipopolisakkarit ile Uyarılan Makrofajlarda Anti-enflamatuvar Etkileri

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ABSTRACT

Objectives: This study was designed to investigate the anti-inflammatory effects of *Pelargonium endlicherianum* Fenzl. and *Pelargonium quercetorum* Agnew. root extracts compared with the effects of commercial *Pelargonium sidoides* root extract by production of pro-inflammatory substances and inflammatory signal transduction on LPS-stimulated macrophages.

Materials and Methods: To measure the effects of root extracts on pro-inflammatory mediators, we used the following methods: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (cell viability or cytotoxicity), enzyme-linked immunosorbent assay (cytokine production, prostoglandin E2 production), reverse transcriptase-polymerase chain reaction (COX-2, iNOS mRNA), Western blotting analysis [MAPK activation and NF-κB (p65) traslocation] and the Griess reaction (NO production).

Results: Stimulation of the RAW 264.7 cells with LPS (0.5 µg/mL, 6 hrs treatment) caused an elevated production of pro-inflammatory cytokines (TNF-α and IL-6), increased mRNA expression of COX-2 and inducible NO synthase with release of PGE2 and NO, activated MAPK (phosphorylation of c-Jun N-terminal kinase, extracellular signal-regulated kinase, P38) signalling pathway, and nuclear translocation of NF-κB (p65), which were markedly inhibited by the pre-treatment with 11% ethanol and 70% methanol root extracts of *P. endlicherianum* without causing any cytotoxic effects. *P. quercetorum* root extract only decreased TNF-α production and *P. sidoides* root extract alleviated P38/MAPK activation and COX-2 mRNA expression with PGE2 production.

Conclusion: Our data indicate that especially 11% ethanol root extract of *P. endlicherianum* targets the inflammatory response of macrophages via inhibition of COX-2, IL-6, and TNF-α through inactivation of the NF-κB signalling pathway, supporting the pharmacologic basis of *P. endlicherianum* as a traditional herbal medicine for the treatment of inflammation and its associated disorders.

Key words: Pelargonium species, inflammation, cytokines, cyclooxygenase-2, macrophages

ÖZ

Amaç: Bu çalışma *Pelargonium endlicherianum* Fenzl. ve *Pelargonium quercetorum* Agnew. kök ekstrelerinin anti-enflamatuvar etkilerini, ticari *Pelargonium sidoides* kök ekstresinin etkileri ile kıyaslayarak LPS ile uyarılmış makrofaj hücrelerinde pro-enflamatuvar bileşiklerin üretimi ve enflamatuvar sinyal iletimi yoluyla incelemek üzere dizayn edildi.

Gereç ve Yöntemler: Kök ekstrelerinin pro-enflamatuvar araçlar üzerine etkisini ölçmek için takip eden yöntemler kullanıldı: 3-(4,5-dimetiltiazol-2-il)-difenil tetrazolium bromid deneyi (hücre canlılığı veya sitotoksosite deneyi), enzime bağlı immünosorbent deneyi (sitokin üretimi, prostoglandin E2 üretimi), ters transkriptaz-polimeraz zincir reaksiyonu (COX-2, iNOS mRNA düzeyleri), Western Blot analizi [MAPK aktivasyonu ve NF-κB (p65) translokasyonu] ve Griess reaksiyonu (NO üretimi).

Bulgular: RAW 264.7 hücrelerinde LPS ile uyarım pro-enflamatuvar sitokinlerin üretimini, COX-2 ve iNOS mRNA düzeylerini, PGE2 ve NO üretimini, MAPK sinyali (c-Jun N-terminal kinaz, hücre dışı sinyalle düzenlenen kinaz, P38 fosforilasyonu) yolunda aktivasyonu ve NF-κB (p65) translokasyonunu artırırken tüm bu değerler *P. endlicherianum* %11 etanol ve %70 metanol ekstrelerinin ön-uygulaması ile herhangi bir sitotoksositeye

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Received: 18.04.2017, Accepted: 01.06.2017

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neden olmadan baskılanmıştır. *P. quercetorum* TNF- α düzeylerini azaltırken *P. sidoides* ile ön-uygulama P38/MAPK aktivasyonunu, COX-2 mRNA düzeylerini PGE2 üretimi ile birlikte azaltmıştır.

Sonuç: Sonuçlarımız göstermektedir ki, özellikle *P. endlicherianum* %11 etanol ekstresi COX-2, IL-6, and TNF- α inhibisyonu, NF- κ B ve MAPK sinyal yollarının inaktivasyonu ile enflamatuvar cevabı hedef almıştır, bu durum da enflamasyon ve enflamasyonla ilişkili hastalıkların tedavisinde bitkisel ilaç açısından *P. endlicherianum* ekstresinin farmakolojik esasını destekler.

Anahtar kelimeler: Pelargonium türleri, enflamasyon, sitokinler, siklooksijenaz-2, makrofajlar

INTRODUCTION

Inflammation is a host response to harmful stimuli. This biologic response is a protective mechanism of organisms for the defence against injurious stimuli.¹ Acute inflammation occurs with several typical processes including increased blood flow, increased permeability, and migration of neutrophils and eosinophils. These migrated immune cells are able to neutralize and eliminate potentially injurious stimuli.² If acute inflammation is not resolved, the inflammation may pass to a longer term chronic phase. In chronic inflammation, accumulation of white blood cells also continues, but the composition of the cells changes. The primary cells of chronic inflammation are lymphocytes and macrophages.³ Macrophages play an important role in the initiation and propagation of inflammatory responses by managing inflammation-related signalling pathways such as mitogen-activated protein kinases (MAPKs) signalling cascade and nuclear factor (NF)- κ B signalling⁴ with over-production of pro-inflammatory cytokines and other inflammatory mediators [prostaglandins and nitric oxide (NO)], generated by activated cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS).⁵⁻⁷ A number of inflammatory stimuli such as pro-inflammatory cytokines⁸ and bacterial lipopolysaccharides (LPS)⁹ activate macrophages to up-regulate such inflammatory states. The involvement of macrophages in chronic inflammatory conditions has been the subject of considerable experimental investigation in recent years for developing new anti-inflammatory agents and exploring the molecular anti-inflammatory mechanisms of potential drugs.¹⁰ Over-expression of inflammation-producing enzymes and their inflammatory mediators in macrophages is involved in many inflammation-related diseases such as atherosclerosis,¹¹ rheumatoid arthritis,¹² and cancers.¹³ Additionally, RAW 264.7 murine mouse macrophage cells, which can be stimulated to an inflammatory state by LPS treatment, have been used as an *in vitro* inflammatory cellular model to investigate the effects of anti-inflammatory drugs, herb-derived compounds, and plant extracts.

The *Pelargonium* species, members of the Geraniaceae family, comprise about 750 species and approximately 80% of the genus is indigenous to South Africa. A native South African medicinal plant called *Pelargonium sidoides* DC. has been traditionally used to treat cough, sore throat, congestion, and other respiratory ailments.¹⁴ Pharmacologic studies have demonstrated antibacterial, antituberculosis, antiviral, and immune-modulatory activities of *P. sidoides*.¹⁵⁻¹⁷ Following a number of clinical studies, a medicine with the international name of Umkaloabo, which comes from the plant's local name, has been manufactured using the roots of *P. sidoides*.^{18,19} In Turkey, *Pelargoniums* are represented by two species: *Pelargonium endlicherianum* Fenzl. and *Pelargonium quercetorum* Agnew. *P.*

endlicherianum is known by the common name "solucanotu" (Tansy) and *P. quercetorum* "tolik" in Turkey. The extracts prepared from these species roots and the fresh flowers are used for the treatment of intestinal parasites.²⁰

According to the literature, no scientific study has been reported on the anti-inflammatory activities of *P. endlicherianum* and *P. quercetorum*. Therefore, the aim of this study was to investigate the anti-inflammatory effects of *Pelargonium* species growing in Turkey by measuring MAPK activation, NF- κ B nuclear translocation, pro-inflammatory cytokines, and other inflammatory mediators (prostaglandins and NO), generated by activated COX-2 and iNOS on LPS-stimulated RAW 264.7 macrophages.

MATERIALS AND METHODS

Chemicals

Chromatographic standards, LPS, and all remaining reagents were of the highest purity available and obtained from the Sigma Chemical Company (St. Louis, MO, USA). Cell culture medium and medium supplements were purchased from GIBCO (Invitrogen, USA).

Plant materials and preparation of the extracts

P. endlicherianum was collected from Eskişehir, Dağköprü village in August 2013 and *P. quercetorum* was collected from Hakkari in May 2014. A voucher specimen of *P. endlicherianum* was deposited at the herbarium of the Anadolu University Faculty of Pharmacy, Eskişehir, Turkey (ESSE 14453) and a voucher specimen of *P. quercetorum* was deposited at the herbarium of the Hacettepe University Ankara, Turkey (HUB 30648). The dried *P. endlicherianum* and *P. quercetorum* roots were powdered and extracted with a sufficient amount of 70% methanol and 11% ethanol for 24 h at 40°C in a water bath with shaking. This procedure was repeated three times using the same batch of starting material and the resultant filtrates were combined and the solvent was removed under vacuum (40°C). All extracts were lyophilized and stored at -20°C until required for analysis.

Determination of the total flavonoid and phenolic contents

The total flavonoid content was estimated as catechin equivalents using an aluminium chloride colorimetric assay.²¹ The total phenolic content of the extract was determined using the Folin-Ciocalteu method²² and estimated as gallic acid equivalents (GAE), per gram of extract.

High-performance liquid chromatography determination

Liquid chromatographic equipment (Agilent Technologies 1200 Series) with a photodiode array detector were used. Separations were performed on a 250 x 4.6 mm i.d., 5- μ m

particle size, reverse-phase Mediterranean-C18 analytical column operating at room temperature (22°C) at a flow rate of 1 mL min⁻¹. Detection was performed between the wavelengths of 200 and 550 nm. Elution was conducted using a ternary non-linear gradient of the solvent mixture MeOH/H₂O/CH₃COOH (10:88:2, v/v/v) (solvent A), MeOH/H₂O/CH₃COOH (90:8:2, v/v/v) (solvent B) and MeOH (solvent C). Components were identified by comparison of their retention times to those of standards.

Cell culture

RAW 267.4 murine macrophage cells (a kind gift from Prof. Asuman Sunguroğlu, Department of Medical Biology, University of Ankara, Ankara, Turkey) were grown in DMEM medium containing 2 mM L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin.

Cell viability assay

RAW 267.4 cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described by Janjic and Wollheim.²³ A cell monolayer with cell density of 3×10^4 cells/well was seeded in 96-well plates. After cell attachment, 100 µL of serially diluted extract (concentrations ranging from 400 to 25 µg/mL) in DMEM with 1% FBS was added to the wells. After incubation, 10 µL of MTT solution (5 mg/mL in dH₂O) was pipetted into each well followed by a 3-hour incubation. Violet-coloured formazan crystals were dissolved with 100 µL DMSO and absorbance was determined at 570 nm using a microplate reader (Bio-Tek ELX800, BioTek Instruments Inc., Winooski, VT).

Enzyme-linked immunosorbent assay (ELISA)

Mediums collected after pre-treatment with extracts (20 µg/mL) for 24 h followed by LPS (0.5 µg/mL) treatment (in non-phenol red, serum-free medium) for 6 h and assayed for cytokines [tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6)] and prostaglandin E₂ (PGE₂) production using eBiosciences ELISA kits (San Diego, CA, USA) following the manufacturer's protocol.

Western blotting

RAW 267.4 cells were cultured in 6-well plates pre-treatment with extracts (20 µg/mL) for 24 h, followed by LPS (0.5 µg/mL) treatment (in non-phenol red, serum-free medium) for 6 h. Cells were lysed in 250 µL of RIPA lysis buffer [25 mM Tris (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40] supplemented with a protease inhibitor cocktail (Complete Mini™, Roche, Mannheim, Germany) and 1 mM Na₃VO₄. Protein concentrations were determined using the BCA protein assay (Pierce). Thirty micrograms of protein lysates were heated for 5 min at 94°C in Laemmli sample buffer containing 4% β -mercaptoethanol and loaded on 4–12% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels, then transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with anti-SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), anti-extracellular signal-regulated kinase (ERK), phospho-ERK (Thr202/Tyr204), anti-P38, phospho-P38 (Thr180/Tyr182), and anti-NF- κ B p65

antibodies (Cell Signalling Technology). Protein bands were detected using horseradish peroxidase-conjugated secondary antibodies (Cell Signalling Technology) and visualized using West-Pico enhanced chemiluminescent reagents (Pierce).

Quantitative Real-time polymerase chain reaction (PCR)

RAW 267.4 cells were cultured in 6-well plates pre-treatment with extracts (20 µg/mL) for 24 h followed by LPS (0.5 µg/mL) treatment (in non-phenol red, serum-free medium) for 6 h. Total RNA was isolated using RNeasy lysis reagent (Sigma-Aldrich, St. Louis, MO), in accordance with the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed to cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR was performed using a Light Cycler Nano System (Roche Diagnostics GmbH, Mannheim, Germany). To quantify cDNA, qPCR was performed using a *FastStart Essential DNA probe master mix* (Roche Diagnostics GmbH, Mannheim, Germany) and a catalogue assay kit (kits consist mix of primers and probes for determination of iNOS, COX-2, β -actin). For each sample, the level of target gene transcripts was normalized to β -actin.

Nitric oxide [total nitrite-nitrate (NO_x)] measurement

The mediums were collected after pre-treatment with extracts (20 µg/mL) for 24 h followed by LPS (0.5 µg/mL) treatment (in non-phenol red, serum-free medium) for 6 h and production of NO was assayed using the Griess method with a nitrate-nitrite colorimetric assay kit (Cayman Chemical).

Statistical analysis

Possible associations between groups were analyzed using the SigmaPlot 12. statistical software with Student's t-test. P values <0.05 were considered statistically significant. Fold increase or decrease of mRNA levels was also calculated using the Relative Expression Software Tool software developed for groupwise comparison and statistical analysis of relative expression results.

RESULTS

The total phenol and flavonoid contents of the extracts are shown in Table 1. The total phenolic contents in the obtained extracts ranged from 162.9 to 242.9 mg GAE/g DW. The highest concentration of phenols was measured in the methanol extract of *P. quercetorum*. The total phenolic content was found to be higher in the ethanol extract of *P. endlicherianum* than the ethanol extract of *P. quercetorum*. The total flavonoid contents in the obtained extracts ranged from 36.0 to 64.9 mg RE/g DW. The concentration of flavonoids in the ethanol extract of *P. endlicherianum* was found to be less than the ethanol extract of *P. quercetorum*. The total phenolic and flavonoid compounds were found to be higher in methanol extracts than in the ethanol extracts. The high solubility of phenols and flavonoids in polar solvents provides high concentrations of these compounds in the extracts depending on the polarity of the solvents used in extraction.

The chemical compositions of the extracts were determined using high performance liquid chromatography (HPLC) analyses (Table 2). Apocynin [1-(4-hydroxy-3-methoxyphenyl) ethanone] was identified as the main compound in the *P. endlicherianum* extracts. Based on the results of quantitative HPLC analysis, the highest content of apocynin ($3.51 \pm 0.016\%$ extract) had 70% methanol extract of *P. endlicherianum*. The *P. endlicherianum* 11% ethanol extract had lower ($2.48 \pm 0.002\%$ extract) apocynin levels than the 70% methanol extract of *P. endlicherianum*. The apocynin content of 11% ethanol and 70% methanol extracts of *P. quercetorum* was found as 0.51 ± 0.01 and $0.049 \pm 0.01\%$ extract, respectively. Gallic acid was also identified both in *P. endlicherianum* and *P. quercetorum*. The highest gallic acid content was found in the 11% ethanol extract of *P. endlicherianum* ($1.070 \pm 0.004\%$ extract). The gallic acid content of *P. quercetorum* was found as 0.012 ± 0.001 and $0.098 \pm 0.001\%$ extract, respectively, for 11% ethanol and 70% methanol extracts.

RAW 264.7 macrophage cells were pre-treated with increasing

concentration (10, 20, 25, 50, 100, and 200 $\mu\text{g/mL}$) of *P. sidoides* (EPs® 7630) and each of the other extracts for 24 h. According to these findings (Figure 1), *P. sidoides* (EPs® 7630) was found to be the most cytotoxic towards RAW 264.7 macrophage cells and non-toxic (20 $\mu\text{g/mL}$) concentrations of extracts were selected and used in the subsequent experiments for testing their protective effect on LPS-induced inflammatory response.

The response to LPS (0.5 $\mu\text{g/mL}$, 6 h) treatment, release of pro-inflammatory cytokines TNF- α (Figure 2A) and IL-6 (Figure 2B) were increased approximately 2.0-fold and 38.2-fold, respectively. Pre-treatment with *P. endlicherianum* 11% ethanol extract and *P. quercetorum* 11% ethanol extract caused a reduction in the release of TNF- α and IL-6, but the reduction of IL-6 release after treatment with *P. quercetorum* 11% ethanol extract did not reach statistically significance.

Treatment with LPS resulted in significant upregulation of COX-2 (Figure 3A) and iNOS (Figure 3B) mRNA levels 5.8-fold and 2.9-fold, respectively. However, pre-treatment with *P. sidoides*, *P. endlicherianum* 11% ethanol and *P. endlicherianum* 70% methanol extract displayed a marked decrease in the induction of COX-

Table 1. Total phenol and flavonoid content of ethanolic and methanolic extracts of *P. endlicherianum* and *P. quercetorum* root extracts. Each value is the average of three analysis \pm standard deviation

	Total phenols ^c	Total flavonoids ^d
(<i>P. endlicherianum</i> ^a)	173.93 \pm 7.72	36.03 \pm 0.76
(<i>P. endlicherianum</i> ^b)	201.85 \pm 6.44	41.70 \pm 0.46
(<i>P. quercetorum</i> ^a)	162.90 \pm 6.95	46.63 \pm 1.93
(<i>P. quercetorum</i> ^b)	242.97 \pm 5.52	64.95 \pm 2.95
^a 11% ethanol extract		
^b 70% methanol extract		
^c mg gallic acid equivalent/g dry weight		
^d mg rutin equivalent/g dry weight		

Table 2. Phenolic compound apocynin and gallic acid content of extracts. Each value is average of three analysis \pm standard deviation

	Apocynin ^c	Gallic acid ^c
(<i>P. endlicherianum</i> ^a)	2.486 \pm 0.002	1.070 \pm 0.004
(<i>P. endlicherianum</i> ^b)	3.509 \pm 0.016	0.458 \pm 0.008
(<i>P. quercetorum</i> ^a)	0.510 \pm 0.015	0.012 \pm 0.001
(<i>P. quercetorum</i> ^b)	0.490 \pm 0.012	0.098 \pm 0.001
^a 11% ethanol extract		
^b 70% methanol extract		
^c % extract		

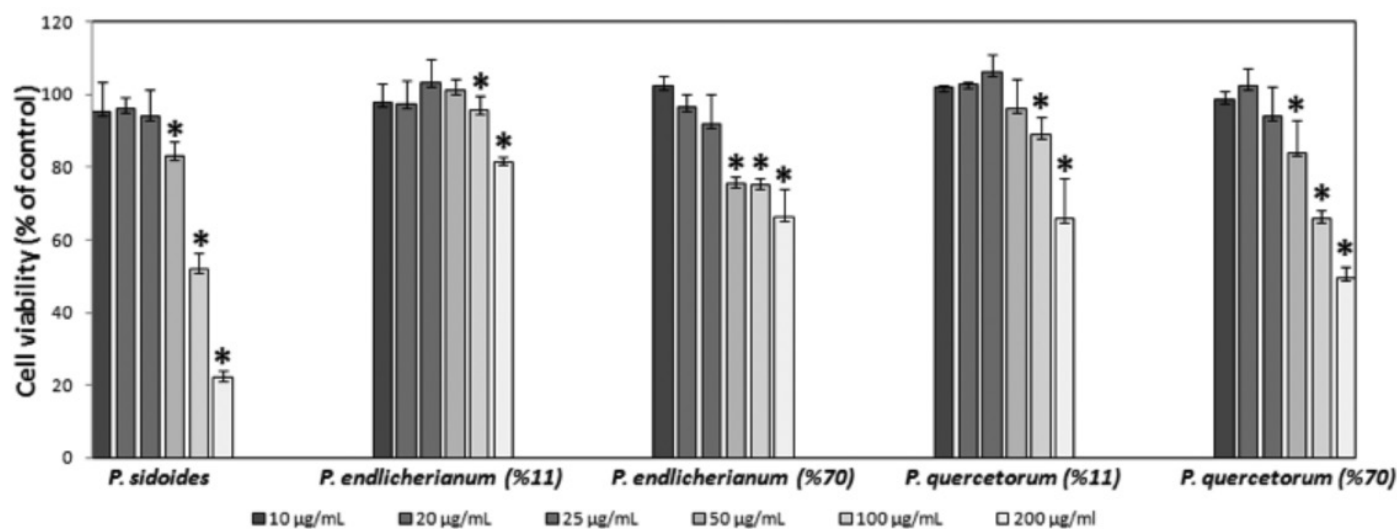


Figure 1. Effects of *Pelargonium* extracts on cell viability
n=4, *p<0.05 vs control

2 mRNA levels. Only pre-treatment with *P. endlicherianum* 11% ethanol extract inhibited the mRNA expression levels of iNOS in LPS-activated RAW 264.7 cells. The increase of COX-2 and iNOS expression in LPS-activated murine macrophage RAW 264.7 was accompanied by the release of large amounts of their products, PGE₂ (Figure 4A) and NO (Figure 4B), respectively. *P. sidoides*, *P. endlicherianum* 11% ethanol and *P. endlicherianum* 70% methanol extract produced also a considerable decrease in the levels of the COX-2 product, PGE₂, which confirms the inhibitory effect of *Pelargonium* extracts toward the COX-2 enzyme. *P. endlicherianum* 11% ethanol extract and *P. quercetorum* 11% ethanol extract completely suppressed the NO production induced by LPS.

In the current study, an increased expression of NF- κ B (p65) protein in the cytoplasm and enhanced p65 nuclear translocation were observed upon LPS activation. All studied extracts excluding *P. sidoides* caused a significant reduction on

cytoplasmic p65 protein expression (Figure 5A). Moreover, *P. endlicherianum* 11% ethanol and 70% methanol extracts had an inhibitory effect on p65 nuclear translocation (Figure 5B).

Finally, we investigated the inhibition of MAPKs activation by *Pelargonium* extracts. Western Blot analysis of RAW 264.7 cells exposed to LPS revealed expression levels of MAPKs, which were partly or markedly downregulated by *Pelargonium* extracts (Figure 6). LPS exposure to RAW 264.7 cells resulted in significant activation in MAPKs including phosphorylated-ERK (p-ERK) (Figure 7A), p-P38 MAPK (p-P38) (Figure 7B), and p-c-Jun N-terminal kinases (p-JNK) (Figure 7C). The LPS-induced activation of ERK was prominently blocked by both *P. endlicherianum* 11% ethanol and 70% methanol extracts. The overactivation of P38 by LPS was suppressed in the presence of *P. sidoides* and *P. endlicherianum* 11% ethanol extract. Activated JNK by LPS exposure was blocked by *P. endlicherianum* 11% ethanol, 70% methanol and *P. quercetorum* 70% methanol extracts.

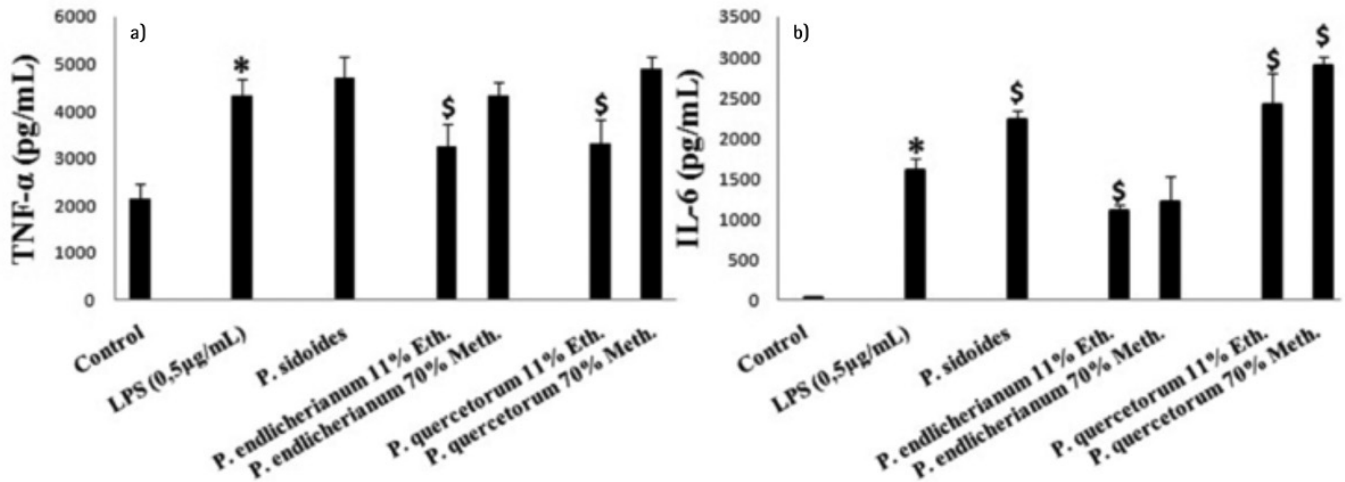


Figure 2. Pre-treatment with extracts modulate cytokine production in LPS treated macrophages

n=4, *p<0.05 vs. control, \$p<0.05 vs. lipopolysaccharide, LPS: Lipopolysaccharide, TNF- α : Tumor necrosis factor- α , IL-6: Interleukin-6

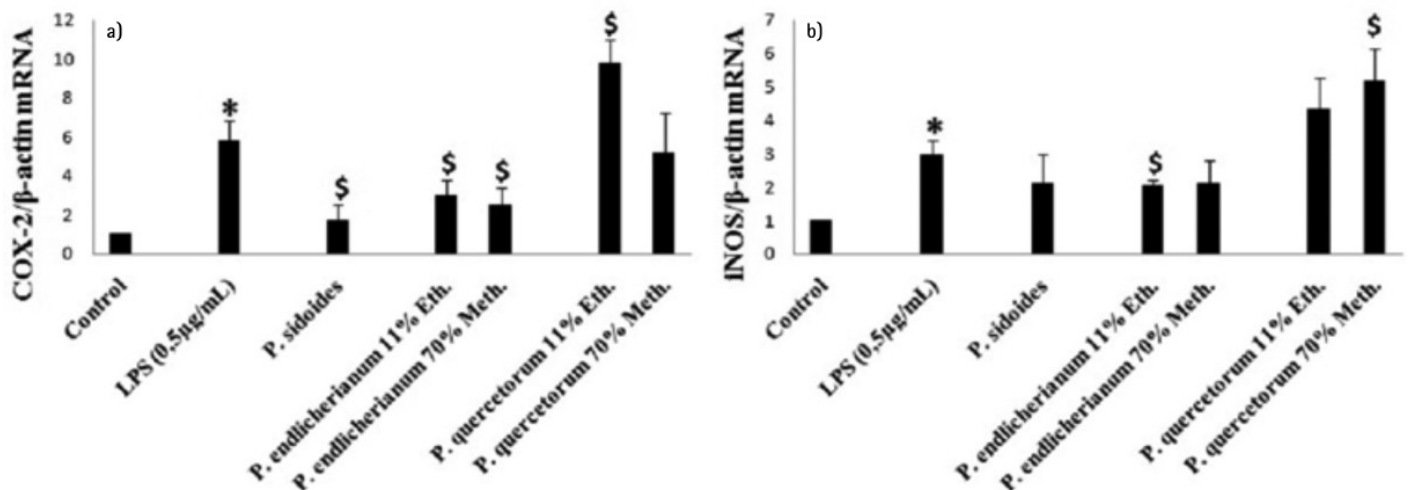


Figure 3. Effect of extracts on inflammation-producing enzymes mRNA levels in LPS treated macrophages

n=3, *p<0.05 vs control, \$p<0.05 vs lipopolysaccharide, LPS: Lipopolysaccharide, COX-2: Cyclooxygenase-2, iNOS: Inducible nitric oxide synthase

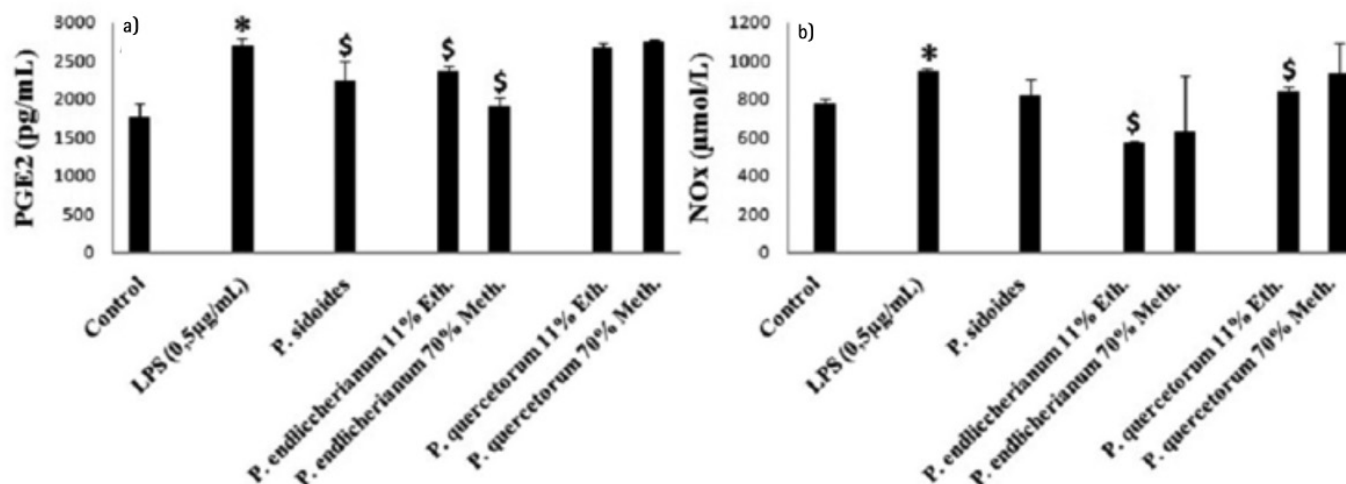


Figure 4. Effect of extracts on the levels of pro-inflammatory mediators; PGE2 and iNOS in LPS treated macrophages
 n=3, *p<0.05 vs control, \$p<0.05 vs lipopolysaccharide, PGE2: Prostaglandin E2, LPS: Lipopolysaccharide, NO: Nitric oxide

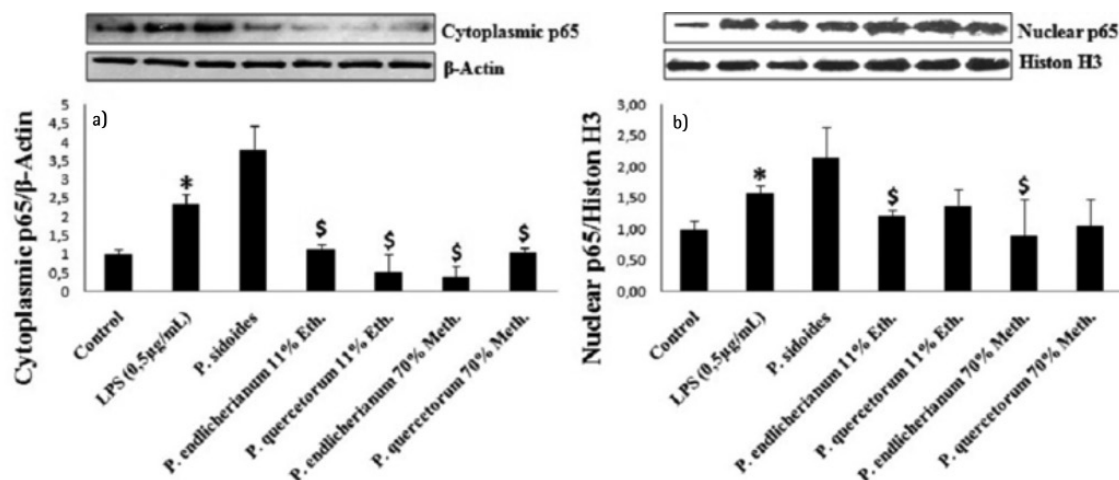


Figure 5. Activation of NF-κB by LPS treatment in macrophage cells and the effects of extracts on cytoplasmic p65 levels and p65 nuclear translocation
 n=3, *p<0.05 vs control, \$p<0.05 vs lipopolysaccharide, LPS: Lipopolysaccharide

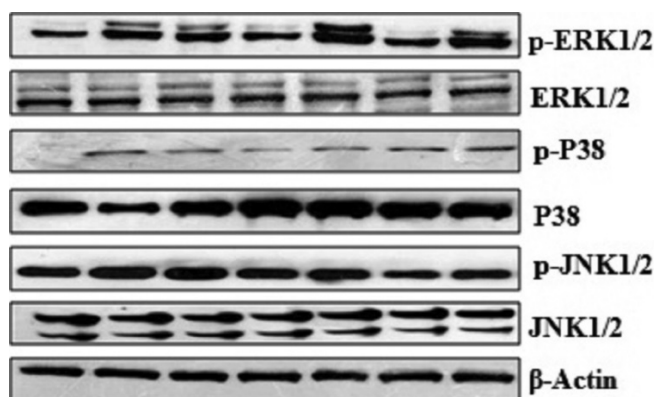


Figure 6. Activation of MAPK pathway by LPS treatment and the inhibitory effects of extracts were shown with Western Blot analysis. Line 1: Control, Line 2: LPS, Line 3: *P. sidoides*+LPS, Line 4: *P. endlicherianum* 11% eth.+LPS, Line 5: *P. endlicherianum* 70% meth.+LPS, Line 6: *P. quercetorum* 11% eth.+LPS, Line 7: *P. quercetorum* 70% meth.+LPS

LPS: Lipopolysaccharide, MAPK: Mitogen-activated protein kinase

DISCUSSION

Medicinal plants continue to be an important source of new chemical substances with potential therapeutic effects. Numerous natural products have been tested in various *in vitro* and *in vivo* models for the development of new anti-inflammatory agents. This study was designed to investigate the anti-inflammatory activities of the root extract (11% ethanol and 70% methanol) of *P. endlicherianum* and *P. quercetorum*, and especially, compare the effects of *P. sidoides* (EPs® 7630) on the induction of inflammatory signalling and production of pro/anti-inflammatory substances in LPS-stimulated RAW 264.7 macrophages.

In South Africa, polyphenol-rich herbal preparations made from roots of *P. sidoides* and *Pelargonium reniforme* are traditionally used to treat respiratory and gastrointestinal infections, dysmenorrhea, and hepatic disorders.¹⁴⁻¹⁶ Inspired by the healing of his tuberculosis, Charles Henry Stevens introduced this phytomedicine drug to England in 1897.²⁴ More

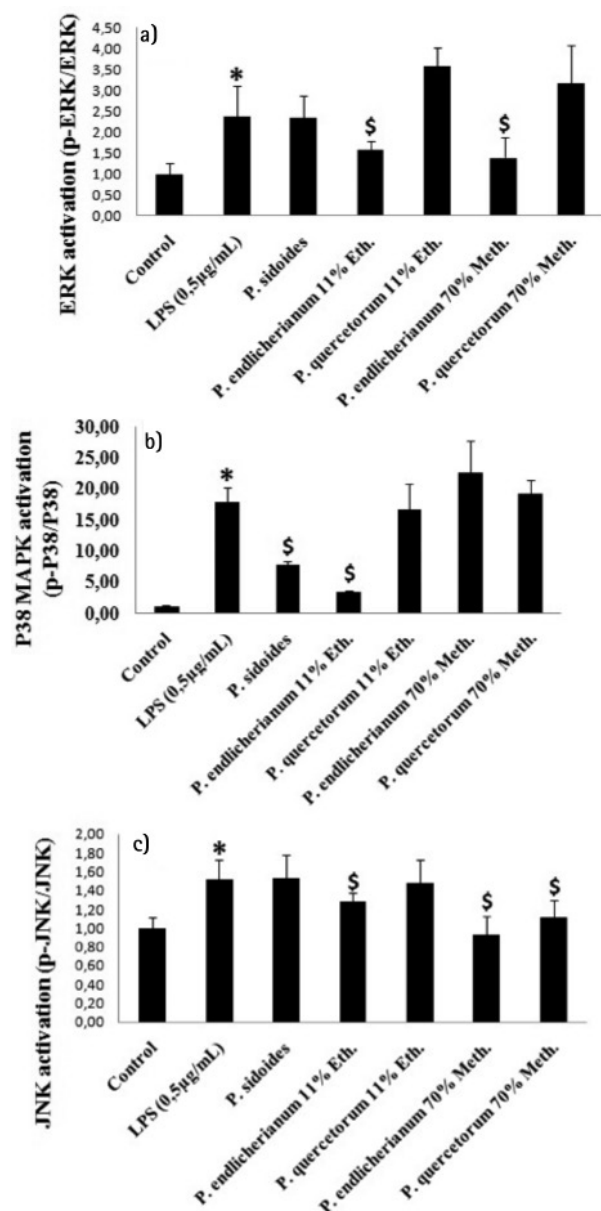


Figure 7. MAPK activation by LPS treatment and the inhibitory effects of extract

A: ERK activation, B: P38 MAPK activation, C: JNK activation. n=3, *p<0.05 vs. control, \$p<0.05 vs. LPS. LPS: Lipopolysaccharide, MAPK: Mitogen-activated protein kinase

than seven decades later, a special ethanol extract of *P. sidoides* roots, was finally developed (EPs® 7630; ISO Arzneimittel, Ettlingen, Germany). In Germany, EPs 7630 is approved today for therapeutic use in patients with acute bronchitis. In addition, EPs 7630 was shown to be effective in clinical trials on patients with tonsillopharyngitis, rhinosinusitis, common cold or chronic obstructive pulmonary disease.²⁵⁻³⁰

According to a meta-analysis about *P. sidoides*, there are several studies exploring the effects of *P. sidoides* in treating acute upper respiratory tract infections; 2,871 patients participated in these studies, an average of 261 in each and the clinical trials were performed in adults, adolescents, and children.²⁶

In Turkey, *Pelargoniums* are represented by two species: *P. endlicherianum* Fenzl. and *P. quercetorum* Agnew. The molecular mechanisms of *P. sidoides* have been partly identified but there are no relevant data revealing the similar effects of *P. endlicherianum* and *P. quercetorum*. Understanding the molecular effects of *P. endlicherianum* and *P. quercetorum* is key to taking advantage of their medical potential such as with *P. sidoides*. To date, data regarding its target cells and effects thereof within the human immune system are lacking. To address this issue, we first investigated how *P. endlicherianum* and *P. quercetorum* influenced the anti/pro-inflammatory responses of LPS-induced RAW 264.7 cells and compared the results with those of *P. sidoides*.

When investigating the signalling cascades induced by *P. endlicherianum* and *P. quercetorum*, we first activated the RAW 264.7 cells with LPS. LPS exposure to RAW 264.7 cells resulted in significant activation in MAPKs including p-ERK, p-P38, and p-JNK, whereas the LPS-induced activation of ERK was prominently blocked by both *P. endlicherianum* 11% ethanol and 70% methanol extracts. The overactivation of P38 by LPS was suppressed in the presence of *P. sidoides* and *P. endlicherianum* 11% ethanol extract. Activated JNK by LPS exposure was blocked by *P. endlicherianum* 11% ethanol, 70% methanol and *P. quercetorum* 70% methanol extracts.

Witte et al.²⁵ treated human peripheral blood mononuclear cells that were isolated from the blood of healthy donors to compare the bacterial and viral stimulation of the immune system and EP7630. The authors found a strong MAPK kinase pathway activation, which included phosphorylation of JNK, ERK1/2, and P38. Furthermore, EPs 7630 slightly provoked NF-κB and phosphoinositide 3-kinase pathway activation. However, pharmacologic blockage of only P38 resulted in a strongly decreased monocyte TNF-α production. The observation that this signalling pattern differed from that induced by TLR3 and TLR4 ligand, inflammatory cytokines, and CD3/CD28 engagement suggests that *Pelargonium* extract affects monocytes via receptors different from those used by the mentioned stimuli.

Differing from the Witte et al.²⁵ study, in our experimental design the cells were stimulated by LPS and the effects of *P. sidoides*, *P. endlicherianum*, *P. quercetorum* were compared in terms of the inflammatory response. Pre-treatment with the extracts decreased the effects of inflammation. Furthermore, stimulation of the RAW 264.7 cells with LPS (0.5 µg/mL, 6 hrs treatment) caused an elevated production of pro-inflammatory cytokines (TNF-α and IL-6), increased mRNA expression of COX-2 and iNOS, with release of prostaglandin E2 and NO, activated MAPK (phosphorylation of JNK, ERK, P38) signalling pathway, and nuclear translocation of NF-κB (p65), which were markedly inhibited by the pre-treatment with 11% ethanol and 70% methanol root extracts of *P. endlicherianum* without causing any cytotoxic effects. *P. quercetorum* root extract only decreased TNF-α production and *P. sidoides* root extract alleviated P38/MAPK activation and COX-2 mRNA expression with PGE2 production.

A study designed to investigate the effects of *P. sidoides* on inflammatory responses was conducted on Leishmania major-infected murine macrophages. In that study, EPs 7630 increased cellular NO production and mRNA levels of iNOS and several cytokines (IL-1 β , IL-10, IL-12, IL-18, TNF- α , IFN- α , IFN- γ).³¹ However, our knowledge regarding the influence of *Pelargonium* extract on human immune cells, in particular on their cytokine production, is still highly restricted. To gain insight into this matter we comprehensively studied the immune-regulatory effects of *P. endlicherianum* and *P. quercetorum* and compared them with the effects of *P. sidoides* on murine macrophage cells. According to our study, TNF- α , IL-6, COX-2, PGE2, iNOS expressions were inhibited by *P. endlicherianum*.

Witte et al.²⁵ revealed that *P. sidoides* (EPs 7630) strongly and dose-dependently induced the production of the pro-inflammatory cytokines TNF- α and IL-6 in human blood immune cells. Moreover, a less prominent induction of the anti-inflammatory acting IL-10 was observed. In line with this study, *P. sidoides* root extract alleviated P38/MAPK activation and COX-2 mRNA expression with PGE2 production. Our data indicate that the 11% ethanol root extract of *P. endlicherianum* in particular targets the inflammatory response of macrophages via inhibition of COX-2, IL-6, and TNF- α through inactivation of the NF- κ B signalling pathway, supporting the pharmacologic basis of *P. endlicherianum* as a traditional herbal medicine for treatment of inflammation and its associated disorders. However, *P. endlicherianum* and *P. quercetorum* pre-treatment inhibited the activation of this pathway revealing that *P. sidoides* has pro-inflammatory effects and can be used more for its protective effect but *P. endlicherianum* can be used as a potential herbal medicine for treatment.

Furthermore, according to our results, apocynin was the major component of the *P. endlicherianum* root extract, which has also been identified by this study for the first time. Apocynin has shown to have strong anti-inflammatory effects in several studies.³²⁻³⁴ In one of the recent studies³⁴ conducted on the RAW 264.7 cells with apocynin, the effects of apocynin on the extracellular release of NO and PGE2 were examined in LPS-stimulated RAW 264.7 macrophages. Cells were incubated with apocynin for 1 h prior to LPS treatment (200 ng/mL). While LPS increased the extracellular release of NO and PGE2 production, approximately 10- and 30-fold, respectively, in RAW 264.7 cells, apocynin (100-500 mM) attenuated the release in a concentration-dependent manner. According to the same study, apocynin inhibited the expression of iNOS and COX-2, reduced NF- α , and inhibited the phosphorylation of MAP kinases JNK, ERK and P38. According to our results, *P. endlicherianum* root extracts, but not the *P. quercetorum* root extracts, were rich in apocynin and the similar strong anti-inflammatory effects that we observed by *P. endlicherianum* might be dependent on the rich apocynin ingredient because *P. quercetorum* did not show such a strong anti-inflammatory effect as *P. endlicherianum*.

CONCLUSION

The present study demonstrated that *P. endlicherianum* suppressed LPS-induced inflammatory responses via the suppression of MAP kinase signalling pathways in LPS-challenged RAW 264.7 macrophages, whereas *P. sidoides* seems to be pro-inflammatory rather than anti-inflammatory. Furthermore, it clearly showed that *P. endlicherianum* is rich in apocynin and probably exerts its anti-inflammatory effects via the suppression of LPS-induced activation MAP kinase signalling pathways over apocynin. In conclusion, all these results suggest that *P. endlicherianum* might be a useful herbal medicine for dissecting inflammation-related pathologies.

ACKNOWLEDGEMENTS

This study was supported by the Scientific and Technical Research Council of Turkey (TUBITAK) (project no. 103S803). *Pelargonium sidoides* (EPs® 7630) crude extract was obtained from Dr. Willmar Schwabe GmbH & Co. KG as a gift.

Conflict of interest: The authors declare that there are no conflicts of interest.

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