Biochemical Genetics (2021) 59:315–334
https://doi.org/10.1007/s10528-020-10005-y

ORIGINAL ARTICLE

Biochemical Research of the Effects of Essential Oil Obtained from the Fruit of *Myrtus communis* L. on Cell Damage Associated with Lipopolysaccharide-Induced Endotoxemia in a Human Umbilical Cord Vein Endothelial Cells

Zerrin Kutlu[1] · Mine Gulaboglu[1] · Zekai Halıcı[2] · İrfan Cınar[2] · Busra Dıyarbakır[2]

Received: 10 September 2018 / Accepted: 4 December 2018 / Published online: 12 October 2020
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Abstract

The aim of this study to investigate the potential effects of essential oils and compounds obtained from MC fruit on sepsis induced endothelial cell damage in human umbilical cord vein endothelial cells (HUVECs) at molecular and cellular levels on in vitro sepsis model. A sepsis model was induced by the application of LPS. The HUVEC treatment groups were as follows: control, LPS, MC, MC plus LPS, 1.8 cineole, 1.8 cineole plus LPS, α-pinene, α-pinene plus LPS, α-terpineol, and α-terpineol plus LPS. Following the treatments, cell proliferation was analyzed using the xCELLigence® system. The mRNA expression of various cytokines [tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and IL-6] and endothelial nitric oxide (eNOS) were determined by quantitative polymerase chain reaction (qPCR) analysis. The 1.8 cineole and α-pinene treatments at specific doses showed toxic effects on α-terpineine, although it did not result in a change in the cellular index as compared with that of the control group. The application of LPS to HUVECs led to a significant decrease in the cellular index, depending on the treatment time. It did not correct the decreased cell index of MC plus LPS and α-terpineol plus LPS groups as compared with that of the LPS-only group. The 1.8 cineole plus LPS treatment and α-pinene plus LPS treatment significantly increased the cell index as compared with that of the LPS-only treatment, and the cell index in these groups was closer to that of the control. According to the results of the qPCR analysis, neither the MC-only treatment nor the α-terpineol-only treatment significantly reduced cellular damage caused by LPS-induced increases in TNF-α, IL-1β, IL-6, and eNOS mRNA expression. However, both the 1.8 cineole treatment and α-pinene treatments significantly decreased TNF-α, IL-1β, IL-6, and eNOS mRNA expression induced by LPS. Volatile oil obtained from MC fruit and the MC compound α-terpineol had no effect on the decreased cell index and increased cytokine response due to LPS-induced endothelial cell damage. However, 1.8 cineole and α-pinene, other major

Extended author information available on the last page of the article

components of MC fruit, ameliorated LPS-induced damage in HUVECs at cellular and biomolecular levels (TNF-α, IL-1β, IL-6, and eNOS).

Keywords HUVEC · LPS · *Myrtus communis* L. · 1.8 Cineole · α-Pinene · α-Terpineole

Introduction

Many medicines used today are derived from natural products or from compounds produced from natural products. In both developed and developing countries, the prevalence of traditional medicine use is increasing. According to data from the World Health Organization, 80% of the world's population favors traditional medicine, and 50% of medicines today are derived from natural sources, especially plants. Fruit produced by the plant *Myrtus communis* L. (MC) has a long history of use as food and as medicine. In Turkey, the fruit is generally known as blueberries. MC fruit has many biological properties, such as antioxidant, anti-inflammatory, and antibacterial activities (Akin et al. 2010). Different parts of the plant (e.g., fruit, leaves, and foliage) are widely used in traditional (popular) medicine as food additives and in the perfume industry (Alipour et al. 2014; Bouzabata et al. 2016; Kafkas et al. 2013; Sümbül et al. 2011).

A number of studies confirmed that MC is rich in essential oils, with monoterpenes constituting 90% of the plant's essential oil content (Aleksic and Knezevic 2014; Asgarpanah and Ariamnesh 2015; Bouzabata et al. 2016). Today, more than 3000 chemically defined essential oils are known, of which about 300 are used in medical, cosmetics, healthcare, dental, and food (preservatives) industries. Various studies have described the use of essential oils in the treatment of organ dysfunction and systemic irregularities and their antibacterial, antiviral, antifungal, antioxidant, antiseptic, diuretic, carminative, and anticancer effects (Belmimoun et al. 2016; Hennia et al. 2015). Researchers also reported that the essential oils 1.8 cineole, α-pinene, and α-terpineol were the major monoterpenes in MC fruit (Asgarpanah and Ariamnesh 2015; Belmimoun et al. 2016). The monoterpene 1.8 cineole, also called cineole, has been found in the essential oils of many plants (Touaibia 2015). It has antioxidant and anti-inflammatory properties and is used in the treatment of respiratory system diseases, asthma, bronchitis, rheumatism, and coughs. It is also used in nasal sprays and disinfectants. Due to its antiatherosclerotic, anticancer, and antimicrobial properties, 1.8 cineole is employed in pharmaceutical preparations for septic shock, where it targets related pathogens (Adriana et al. 2013; Touaibia 2015; Zhao et al. 2014). α-pinene is a bicyclic monoterpene found in essential oils of many plant species (Cho 2012; Jiao et al. 2014). α-pinene is used in the dye processing, paper, pharmaceutical, and odor and taste industries (Hennia et al. 2015). Furthermore, α-pinene has pharmacological properties (e.g., antioxidant and anti-inflammatory activities), (Hennia et al. 2015; Cho 2012; Ana et al. 2012) as well as anticancer, antinociceptive, analgesic, and antimicrobial effects (Cho 2012; Türkez and Aydın 2016). α-terpineol is a major volatile monoterpene alkaloid found in many plant

species (Rita et al. 2013). α-terpineol is an isomer of terpinen-4-ol (Aydin et al. 2013). Among monoterpenoids, α-terpineol is a unique molecule, which exhibits strong therapeutic and pharmacological properties (Silva et al. 2016). Thus, it is used as an anticonvulsant, antinociceptive (Aydin et al. 2013; Silva et al. 2016), antibacterial, antifungal, anti-inflammatory, and antioxidant agent, as well as a sedative and hypertensive agent (Parvardeh et al. 2016; Souza et al. 2011).

Given the anti-inflammatory, antibacterial, and antioxidant effects of MC, the essential oil content of MC may have uses in the treatment of sepsis. Thus far, no studies have investigated such effects. One study reported that 1.8 cineole ameliorated pathological effects of lipopolysaccharide (LPS)-induced liver damage (Santos et al. 2001). Sepsis is a systemic inflammatory disease, which causes shock, organ failure, and even death. Despite the development of various treatment methods, sepsis-related mortality remains relatively high (Alberti et al. 2002; Angus et al. 2001). Factors associated with elevated mortality rates are insufficiencies that occur in vital organs during sepsis (Russell 2006). Worldwide, 19 million cases of sepsis are reported each year, and 1400 deaths per day are attributed to sepsis (Chikkamenahalli et al. 2016). In terms of the pathophysiology of sepsis or septic shock, it results in hypotension, systemic inflammatory response syndrome, tissue damage, multiple organ failure, and even death (Ozdulger et al. 2003). The pathogenesis of sepsis includes inflammation, impaired fibrinolysis, and coagulation, and microbiological agents and inflammatory response in physiopathology. Any traumatic damage or infection that occurs in tissues leads to the activation of the humoral system and the release of various cytokines, thereby resulting in a severe inflammatory response, which is accompanied by hemostatic changes and tissue damage (Bone 1991; Richard and Irene 2003). This inflammatory response to sepsis can be studied by determining the levels of various molecules, mediators, and cytokines. Endotoxin-stimulated monocytes release tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, IL-8, and thrombocyte activating factor. A wide range of activation, including cellular and humoral immunity, in the first encounter of the microbial agent and host starts. During this phase, mononuclear cells play a key role in this activation by releasing proinflammatory cytokines IL-1, IL-6, and TNF. TNF and IL-1 are released during the first phase of sepsis, and these result in the release of other mediators (Yorganci 2005).

Sepsis is very common in Turkey and worldwide and is a major cause of death. Thus, sepsis is no longer only a health problem and poses problems for society in social and economic terms. Finding a solution to this crucial health problem is both a primary goal for many researchers, and it is important for patients who have lost their health or are getting worse. For these reasons, many studies have investigated the effects of different compounds on sepsis. A number of studies found that essential oil and its components from MC fruit exhibited very high anti-inflammatory effects and that they were effective against cytokines (Bouzabata et al. 2016; Juergens 2014; Zhao et al. 2014). Thus, essential oil from MC may represent a new source of study that will remove the strong inflammatory response and the resulting damage in the sepsis process. In vivo and in vitro studies have shown that sepsis plays a key role in host cell and tissue damage, and more importantly, in the irreversible stage of the clinic, where endothelial cell damage plays a key role. For this

reason, much of the work on sepsis in recent years has focused on endothelial injury and prevention.

The aim of the present study was to investigate the potential effects of essential oils and compounds obtained from MC fruit on sepsis and multiple organ damage at molecular and cellular levels on in vitro sepsis model. With this aim in mind, this study examined the effects of LPS on endothelial cell damage in human umbilical cord vein endothelial cells (HUVECs).

Materials and Methods

Chemicals

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (100 U penicillin/mL, 100 mg streptomycin/mL), trypsin Gibco were obtained from Invitrogen Inc. (Grand Island, New York, USA), L-glutamine (CAS No. 56-85-9), 1.8 cineole (CAS No. 470-82-6, 99%), α-pinene (CAS No. 7785-70-8, 98%), α-terpineole (CAS No. 98-55-5% 90), LPS (*E. coli* O55: B5), Tween 80 and ethanol (≥ 99.5%) were obtained from Sigma-Aldrich® (St. Louis, MO, USA).

Plant Material and Essential Oil Analysis Studies

The MC sample was obtained from the Finike District of Mediterranean Region in July 2016. The essential oil found in the fruits of the MC plant was obtained by hydrodistillation technique using the Clevenger device (Ankara, Turkey, Biokim). 100 g of the fruit sample was heated in the glass balloon of the Clevenger apparatus with 1000 mL of water for 2 h. The essential oil obtained was cooled and condensed by the device and accumulated in the graduated pipe of the device.

Protocol for Cell Culture and Proliferation Analysis

In this study, human umbilical cord endothelial cell line (HUVEC, PCS-100-013TM) was used. The HUVEC cell line was purchased from the American Type Culture Collection (ATCC, USA) and replicated from stocks available from the Cell Culture Laboratory at Atatürk University, Faculty of Medicine, Medical Pharmacology Department.

The HUVEC cell line is grown in medium containing 89% DMEM, 10% FBS, 1% PSA and 2% L-Glutamine. When the cells in the culture dishes were 80% confluent, the cells were resected with the cell scraper. Cells were collected by centrifugation technique and cell solution was prepared at 1/1 with 0.1% trypan blue dye. 10 µL of this mixture was placed on a Cedex device (INNOVATIS) and cell count was performed.

To determine the most effective dose on the HUVEC cell line, 500,000 HUVEC cells were cultured in each of the e-plates (DMEM medium added). Stock solutions for MC essential oil and 1.8 cineole, α-pinene, α-terpineole

compounds in Tween-80 were prepared. Then different concentrations of 4 mg/mL, 2 mg/mL, 1 mg/mL, 100 µg/mL were prepared for MC essential oil and different consentraions of 10^{-3} M, 10^{-4} M, 10^{-5} M, 10^{-6} M were prepared for 1.8 cineole, α-pinene, α-terpineole compounds. Equal volumes of 100 µL of different concentrations prepared were dispensed to the cells. For each dose, three wells were cultured and they were incubated in the incubator containing 5% CO_2 at 37 °C. IC50 values were automatically calculated using the xCELLigence® system.

The measurements of electrical impedance are carried out via interdigitated micro-electrodes integrated into the bottoms of cell culture E-plates. The impedance data are displayed as a cell index value, providing quantitative information about the cell number, viability and morphology. Usually, the real time monitoring of cell proliferation and viability by electrical impedance measurements is known to correlate well with other cell number and viability assays. At the beginning of the experiments, cells were incubated on the xCELLigence station inside the incubator for 24 h. Every 10 min, electrical impedance was measured to assess cell proliferation and adhesion. On the next day, cell medium was removed and the wells were washed with PBS once. Groups in the in vitro sepsis model induced by 1 µg/mL LPS were determined as control, LPS, MC, MC+LPS, 1.8 cineole, 1.8 cineole+LPS, α-pinene, α-pinene+LPS, α-terpineole, α-terpineole+LPS. After 24 h, the cells were normalized. Compounds and essential oil were pipetted onto the cells in the e-plate wells (except the control and LPS group) at the IC50 value determined and LPS was given after 1 h. The electrical impedance was measured at 10-min intervals. In summary, the electrical impedance of the cells was measured for 96 h on four consecutive days. Measurement data were displayed as impedance change over time, referred to as cell index. The growth curves were normalized to the point of time before addition of the essentially oil and its compounds. All experiments were performed in duplicate and repeated twice.

Molecular Analysis

Total RNA Extraction and cDNA Synthesis

Total RNA was purified using a RNeasy Mini Kit (Qiagen, Hilden, Germany) in a QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA samples were then copied to reverse DNA using the High Capacity cDNA Reverse Replication Kit (Applied Biosystems, Foster City, Calif.). Then, total 10 mL RNA was treated with 2 mL 10X RT buffer, 0.8 mL 25X deoxynucleotide mix, 2 mL 10X RT randomize primer, 1 mL MultiScribe reverse transcriptase, and 4.2 mL diethylpyrocarbonate water. The RT was incubated for 120 min at 37 °C, for 10 min at 25 °C and then kept at 85 °C for 5 min using the Applied Biosystems Veriti (TM) Thermal Cycler (Applied Biosystems Foster City, CA). cDNA concentration and properties were counted and evaluated using the Epoch Spectrophotometer System and Take3 Plate (Biotek, Winooski, Vermont, USA).

Relative Measurement of Gene Expression

eNOS, TNF-α, IL-1β, IL-6 mRNA expression analysis was performed with the StepOne Plus Real-time polymerase chain reaction (PCR) system (Applied Biosystems Foster City, CA) using cDNA synthesized from HUVEC RNA. The results were compared with the control group and revealed as relative floor. Three replications were performed for the whole cDNA sample obtained using β-actin as the control gene. RT-PCR reaction was prepared by adding 9 µL cDNA (100 ng), 1 µL Primer Perfect Probe mix and 10 µL QuantiTect Probe PCR Master mix (Qiagen, Hilden, Germany) in appropriate plates and total volume was completed to 20 µL in each well. -qPCR The prepared reaction plate was carried out as at 50 °C 2 min, at 95 °C 10 min one cycles and at 94 °C 15 s, at 60 °C 60 s 40 cycles. All data were calculated by comparing with the control group according to the $2^{-\Delta\Delta Ct}$ method.

Calculation of IC50 Values

50% inhibitory concentration (IC50) values that inhibit the proliferation of 50% of HUVEC cells and MC essential oil and 1.8 cineole, α-pinene, α-terpineole components in vitro conditions were calculated automatically by the device in the xCELLIGENCE analysis.

Statistical Analysis

GraphPad 5.0 Prism (La Jolla, CA) software was used for data analysis and graphical drawing. The results are shown as mean ± SD. Intergroup comparisons were analyzed by one-way ANOVA and post-hoc test of Tukey. Statistical analysis (*: according to the 24th hour control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, δ: according to the 48th hour control group, $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$, σ: according to the 24th hour LPS group, $^{\sigma}p < 0.05$, $^{\sigma\sigma}p < 0.01$, $^{\sigma\sigma\sigma}p < 0.001$; Θ: according to the 48th hour LPS group, $^{\Theta}p < 0.05$, $^{\Theta\Theta}p < 0.01$, $^{\Theta\Theta\Theta}p < 0.001$, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$) accepted.

Findings

IC50 Values of MC Volatile Oil and Its Compounds

IC50 values of MC volatile oil and of 1.8 cineole, α-pinen ve α-terpineol compounds which are the major components of this volatile oil are indicated in Table 1.

Table 1 IC50 value of compounds

Compound name	IC50 value
Myrtus communis L. essential oil	6.2×10^{-4} g/mL
1.8 Cineole	10^{-5} M
α-Pinene	10^{-5} M
α-Terpineole	10^{-5} M
LPS	1 µg/mL

xCELLigence Analysis Findings

Cells treated with MC+LPS were found to inhibit cell proliferation by 36.05% at 24 h and 85.47% at 48 h, as compared to the control group. According to this result, it has been shown that MC volatile oil cannot inhibit HUVEC cell damage caused by LPS. (Fig. 1).

In the MC and MC+LPS treated cells the proliferation at 24 h and 48 h (Fig. 2a) has statistically significantly increased when compared to MC group in terms of cell inhibition from MC+LPS group at 24 h ($p < 0.05$) and this increase reached even a higher level at 48 h ($p < 0.001$).

When cell proliferations at 24 and 48 h of the MC group were examined, no statistically significant difference was observed compared to the control group and it was shown that MC alone was not toxic to HUVEC cells (Fig. 2a, $p > 0.10$).

Cells treated with 1.8 cineole+LPS were found to inhibit cell proliferation by 18.11% at 24 h and 8.7% at 48 h, as compared to the control group (Fig. 3). According to this result, the 1.8 cineole compound is not an effect that inhibits proliferation in HUVEC cells, but is also proof that it is not cytotoxic. When 1.8 cineole was administered to cells that were toxic to LPS, only high-level cell inhibition in LPS-treated cells was discontinued and cell proliferation was shown to continue.

When 1.8 cineole, LPS and 1.8 cineole+LPS cells were compared with control cell proliferations at 24th hour, no significant difference was observed in cells

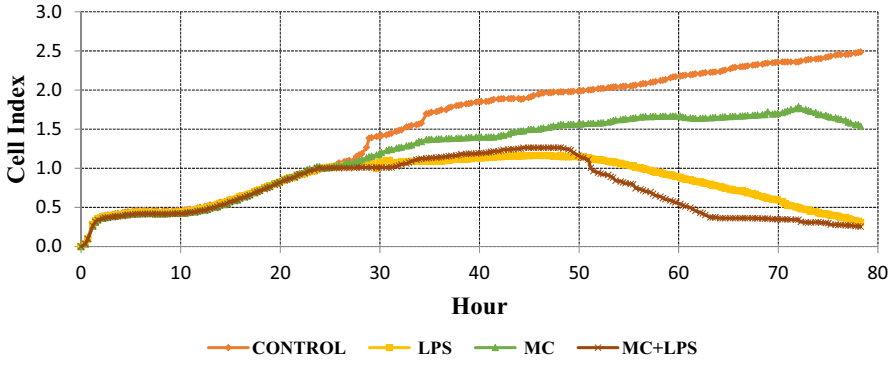


Fig. 1 The proliferative effect of the essential oil obtained from the fruit of the MC (100 µg/mL) plant on the LPS (1 µg/mL) treated cells. Cells received treatments at the 24th hour of curve. *MC Myrtus communis* L., *LPS* lipopolysaccharide

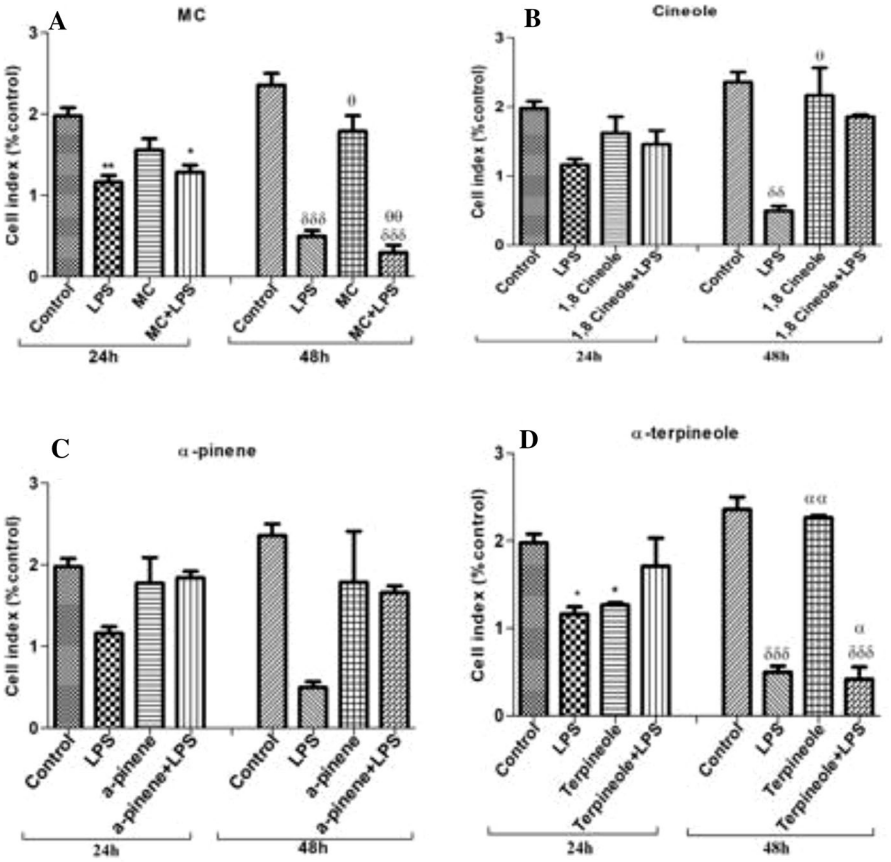


Fig. 2 **a** The proliferative effects of Essential oil MC (100 µg/mL) fruit on LPS (1 µg/mL) treated cells after 24th and 48th hours. *MC Myrtus communis* L., *LPS* lipopolysaccharide. *According to the 24th hour control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; δ: according to the 48th hour control group, $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$; Θ: according to the 48th hour LPS group, $^{\Theta}p < 0.05$, $^{\Theta\Theta}p < 0.01$, $^{\Theta\Theta\Theta}p < 0.001$. **b** The proliferative effects of 1.8 cineole (10^{-5} M) on LPS (1 µg/mL) treated cells after 24 h and 48 h. *LPS* lipopolysaccharide, $^{\delta}$according to the 48th hour control group, $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$; Θ: according to the 48th hour LPS group, $^{\Theta}p < 0.05$, $^{\Theta\Theta}p < 0.01$, $^{\Theta\Theta\Theta}p < 0.001$. **c** The proliferative effects of α-pinene (10^{-5} M) on LPS (1 µg/mL) treated cells after 24 h and 48 h. *LPS* lipopolysaccharide, δ: according to the 48th hour control group, $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$. **d** The proliferative effects of α- terpineole (10^{-5} M) on LPS (1 µg/mL) treated cells after 24 h and 48 h. *LPS* lipopolysaccharide, *: according to the 24th hour control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; δ: according to the 48th hour control group, $^{\delta}p < 0.05$, $^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$, Θ: according to the 48th hour LPS group, $^{\Theta}p < 0.05$, $^{\Theta\Theta}p < 0.01$, $^{\Theta\Theta\Theta}p < 0.001$

treated with 1.8 cineole, LPS and 1.8 cineole+LPS (Fig. 2b, $p < 0.05$). When only the 48th hour is examined; cell proliferation in the LPS-treated group decreased statistically significantly compared to the control group. In the group treated with 1.8 cineole+LPS, cell proliferation was continued, inhibition caused by LPS was stopped and results were close to the control group.

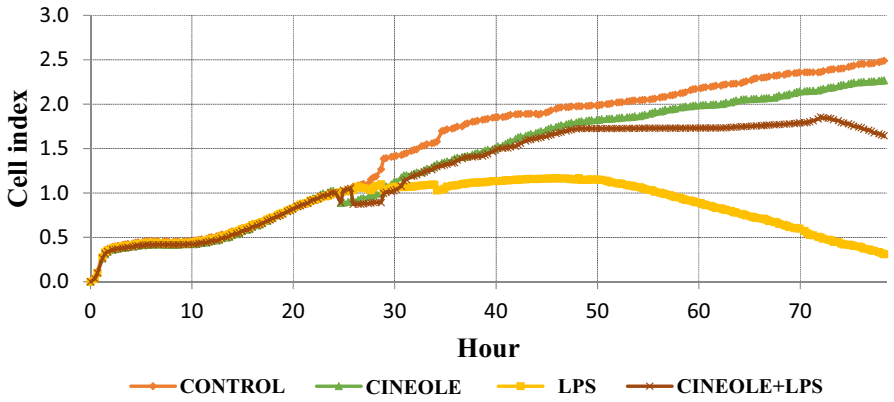


Fig. 3 The proliferative effects of 1.8 cineole (10^{-5} M) and MC essential oil combined on LPS (1 µg/mL) treated cells. Cells received treatments at the 24th hour of curve. *MC Myrtus communis* L., *LPS* lipopolysaccharide

Cells treated with α- pinene+LPS were found to inhibit cell proliferation by 20.50% at 24 h and 14.42% at 48 h, as compared to the control group. In HUVEC cells treated with α-pinene, it was observed that cell viability did not change much compared to the control. In cells treated with α-pinene+LPS, although the cell index value was slightly lowered, there was no decrease in the cell index observed only in LPS treated cells (Fig. 4).

In the α-pinene and α-pinene+LPS groups, cell proliferation at 24th and 48th hours was not statistically significant when compared to the control group (Fig. 2c, $p > 0.10$).

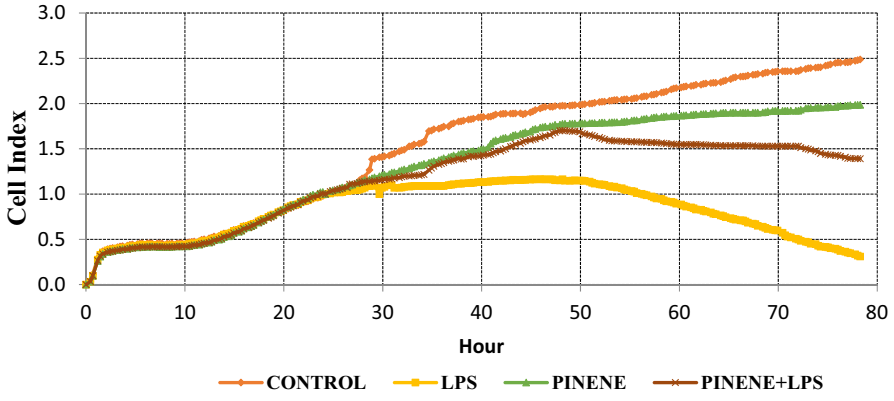


Fig. 4 The proliferative effects of α-pinene (10^{-5} M) and MC essential oil combined on LPS (1 µg/mL) treated cells. Cells received treatments at the 24th hour of curve. *MC Myrtus communis* L., *LPS* lipopolysaccharide

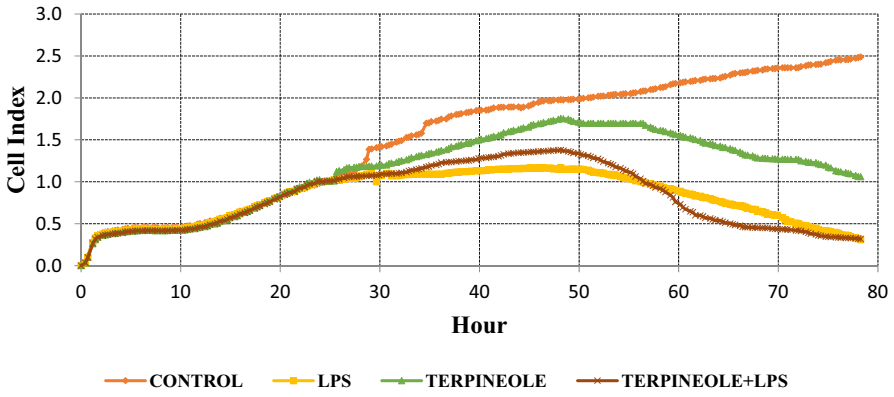


Fig. 5 The proliferative effects of α- terpineole (10^{-5} M) and MC essential oil combined on LPS (1 µg/mL) treated cells. Cells received treatments at the 24th hour of curve. *MC Myrtus communis* L., *LPS* lipopolysaccharide

Cells treated with α-terpineol+LPS were found to inhibit cell proliferation by 36.02% at 24 h and 82.53% at 48 h, as compared to the control group. In the cells treated with α-terpineol, the cell index value was significantly decreased at 48 h. α-terpineol was toxic in healthy HUVEC cells and decreased the cell proliferation (Fig. 5). It was found that HUVEC cells treated with α-terpineol+LPS significantly inhibited HUVEC cells in a time-dependent manner when compared to the control group ($p < 0.001$).

The inhibitory effect on α-terpineol+LPS group cell proliferation at 48 h (Fig. 2d, p<0.001) showed that the toxic effect of α-terpineol administered alone to HUVEC cells was removed (Fig. 2d, $p > 0.10$).

Molecular Findings

mRNA expressions of TNF-α, IL-1β, IL-6 and eNOS cytokines at 2 and 4 h after LPS administration were determined using qPCR analysis in the LPS-induced endotoxemia model in HUVEC cells.

TNF-α (Fig. 6a), IL-1β (Fig. 6b), IL-6 (Fig. 6c) and eNOS (Fig. 6d) mRNA expressions at 2 and 4 h of HUVEC cells treated with the volatile oil obtained from fruits of MC plant were compared with control group and no statistically significant difference was determined (Fig. 6, $p > 0.10$). In the LPS group, expression of TNF-α, IL-1β, and IL-6 mRNA was increased (Fig. 6, $p < 0.001$) and eNOS mRNA expression decreased at 2 h and more at 4 h. The effect of MC application on TNF-α, IL-1β, IL-6 and eNOS mRNA expressions on LPS-induced HUVEC cells was not significantly different at the 2nd and 4th hour when compared to the LPS-treated groups (Fig. 6, $p > 0.10$).

TNF-α (Fig. 7a), IL-1β (Fig. 7b), IL-6 (Fig. 7c) and eNOS (Fig. 7d) mRNA expressions at 2nd and 4th hours of the cells treated with 1.8 cineole compound which is one of the major components of volatile oil of the fruits of MC plant were compared to the control group and no statistically significant difference has been

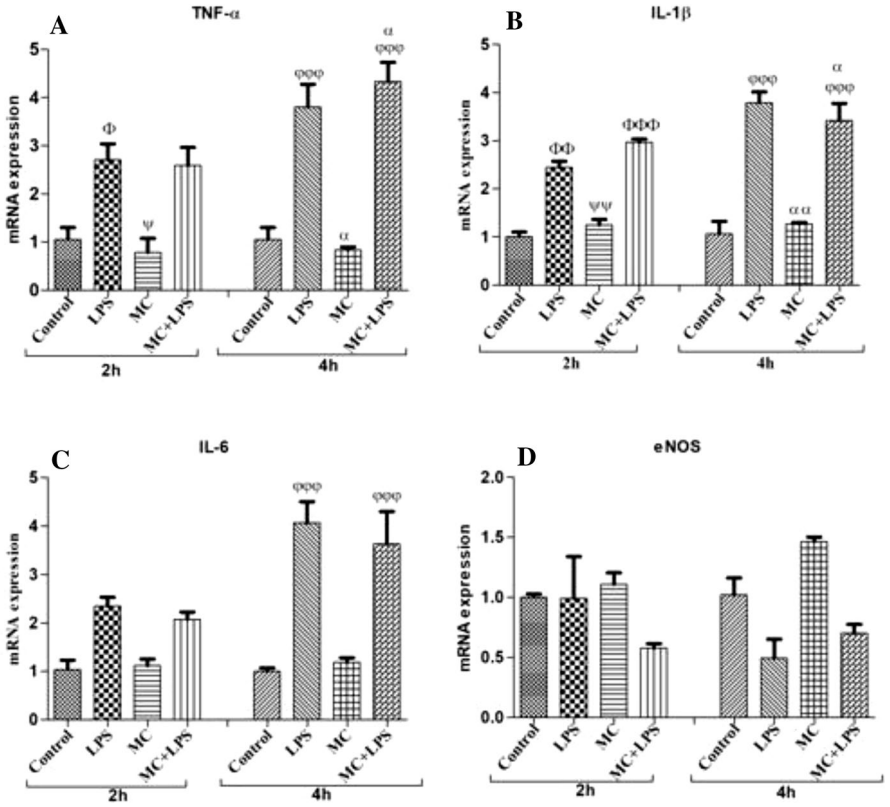


Fig. 6 **a** TNF-α mRNA expression of the essential oil obtained from the fruit of the MC plant. *MC Myrtus communis* L., *LPS* lipopolysaccharide. Φ: According to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **b** IL-1β mRNA expression of the essential oil obtained from the fruit of the MC plant. *MC Myrtus communis* L., *LPS* lipopolysaccharide. Φ: According to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **c** IL-6 mRNA expression of the essential oil obtained from the fruit of the MC plant. *MC Myrtus communis* L., *LPS* lipopolysaccharide. φ: According to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **d** eNOS mRNA expression of the essential oil obtained from the fruit of the MC plant. *MC Myrtus communis* L., *LPS* lipopolysaccharide, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$

found (Fig. 7, $p > 0.10$). In the LPS group, expression of TNF-α, IL-1β, and IL-6 mRNA was increased (Fig. 7, $p < 0.001$) and eNOS mRNA expression decreased at 2nd hours and more at the 4th hour. Provided that 1.8 cineole+LPS group's eNOS mRNA expression was statistically significant at 2nd hours ($p < 0.01$); a significant increase has been found at 4th hour when compared with the LPS administered group (Fig. 7). The effect of 1.8 cineole on LPS toxicity induced HUVEC cells on

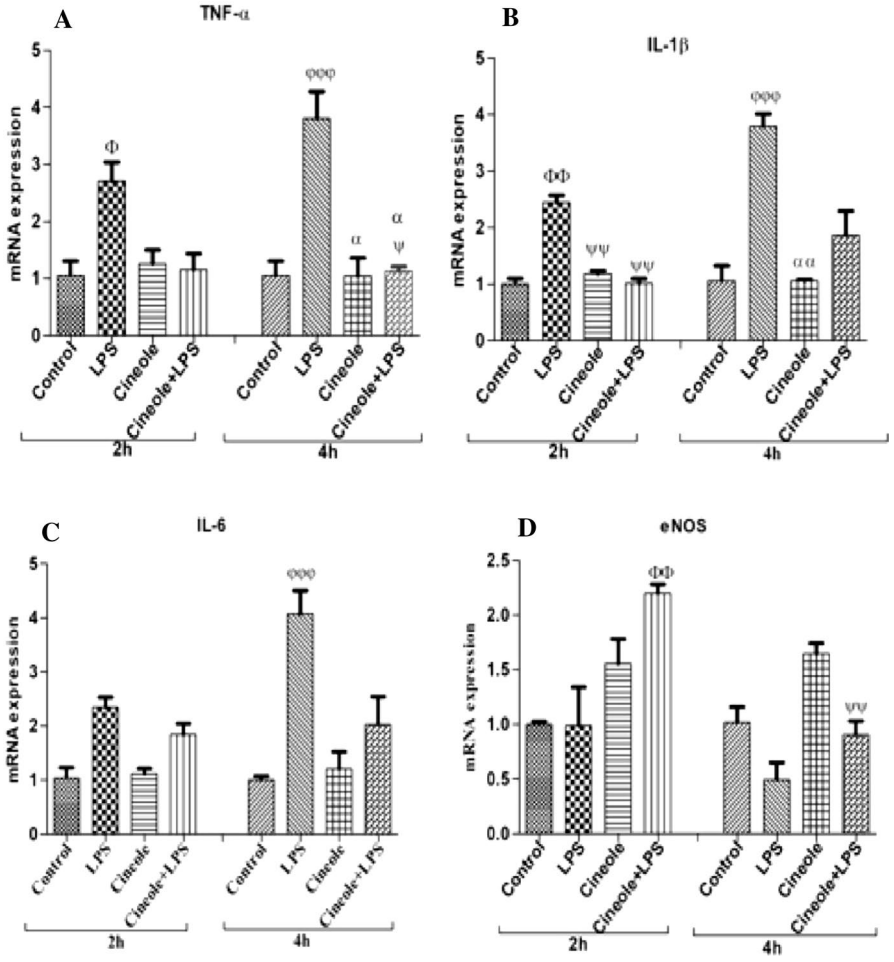


Fig. 7 **a** TNF- α mRNA expression of 1.8 cineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **b** IL-1 mRNA expression of the 1.8 cineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group, $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.001$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **c** IL-6 mRNA expression of the 1.8 cineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **d** eNOS mRNA expression of the 1.8 cineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$

TNF-α, IL-1β and IL-6 mRNA expressions was significantly reduced when compared with LPS-treated groups at 2 and 4 h. TNF-α (Fig. 8a), IL-1β (Fig. 8b), IL-6 (Fig. 8c) and eNOS (Fig. 8d) mRNA expressions at 2nd and 4th hours of the cells treated with α-pinene compound which is one of the components of volatile oil of the fruits of MC plant were compared to the control group and no statistically

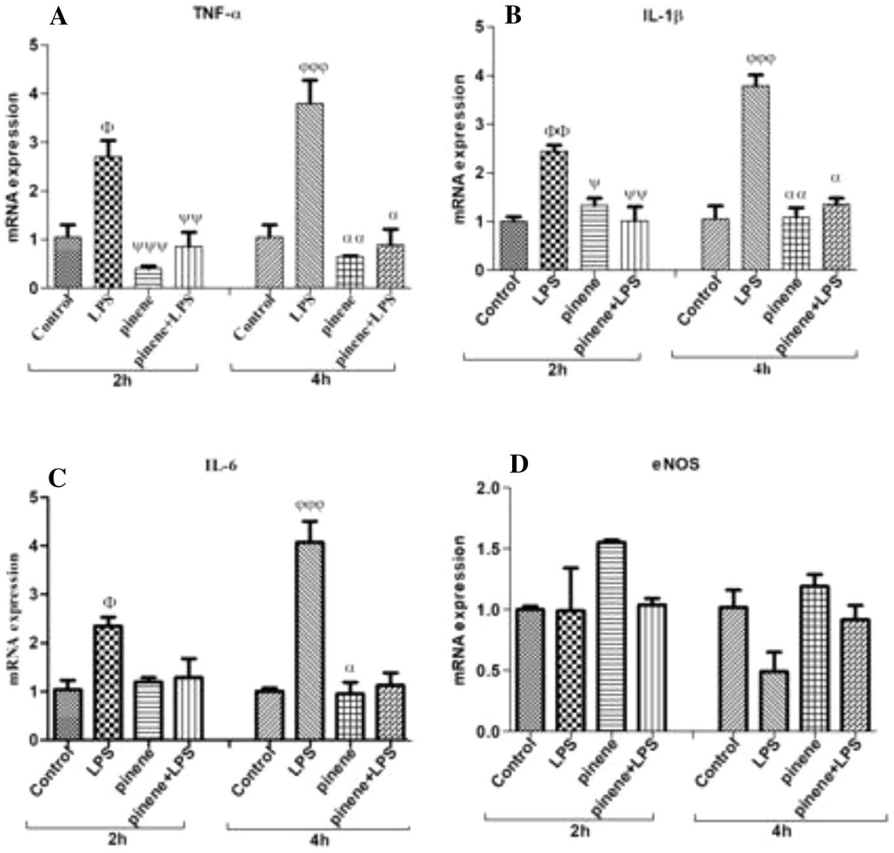


Fig. 8 **a** TNF- α mRNA expression of the α-pinene compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **b** IL-1 mRNA expression of the α-pinene compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group, $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, Ψ: according to the 2nd hour LPS group, $^{\Psi}p < 0.05$, $^{\Psi\Psi}p < 0.01$, $^{\Psi\Psi\Psi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **c** IL-6 mRNA expression of the α-pinene compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group, $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **d** eNOS mRNA expression of the α-pinene compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide

significant difference has been found (Fig. 8, $p > 0.10$). In the LPS group, expression of TNF-α, IL-1β, and IL-6 mRNA was increased (Fig. 8, $p < 0.001$) and eNOS mRNA expression decreased at 2 h and more at 4 h (Fig. 8, $p < 0.05$). The effect of α-pinene compound application on TNF-α, IL-1β, IL-6 and eNOS mRNA expressions on LPS-induced HUVEC cells was significantly different at the 2nd and 4th hour when compared to the LPS-treated groups (Fig. 8, $p < 0.01$) and there was not

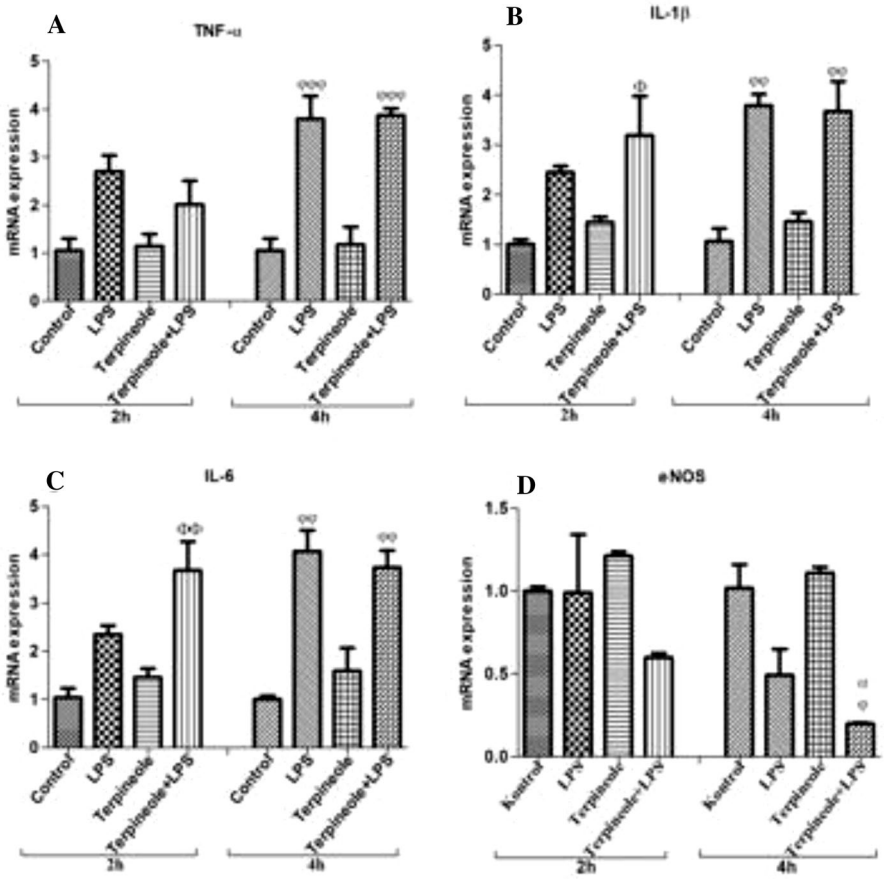


Fig. 9 **a** TNF- α mRNA expression of the α-terpineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$. **b** IL-1 mRNA expression of the α-terpineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, φ: according to the 4th hour control group $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **c** IL-6 mRNA expression of the α-terpineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, Φ: according to the 2nd hour control group, $^{\Phi}p < 0.05$, $^{\Phi\Phi}p < 0.01$, $^{\Phi\Phi\Phi}p < 0.001$, φ: according to the 4th hour control group, $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$. **d** eNOS mRNA expression of the α-terpineole compound of MC essential oil. *MC Myrtus communis* L., *LPS* lipopolysaccharide, φ: according to the 4th hour control group, $^{\varphi}p < 0.05$, $^{\varphi\varphi}p < 0.01$, $^{\varphi\varphi\varphi}p < 0.001$, α: according to the 4th hour LPS group; $^{\alpha}p < 0.05$, $^{\alpha\alpha}p < 0.01$, $^{\alpha\alpha\alpha}p < 0.001$

any significant difference in the eNOS mRNA expression (Fig. 8, $p > 0.10$). TNF-α (Fig. 9a), IL-1β (Fig. 9b), IL-6 (Fig. 9c) and eNOS (Fig. 9d) mRNA expressions at 2nd and 4th hours of the cells treated with α-terpineol compound which is one of the components of volatile oil of the fruits of MC plant were compared to the control group and no statistically significant difference has been found (Fig. 9, $p > 0.10$). In the LPS group, expression of TNF-α, IL-1β, and IL-6 mRNA was statistically increased (Fig. 9, $p < 0.001$) and eNOS mRNA expression decreased at 2 h and more at 4 h (Fig. 9, $p < 0.01$). The effect of α-terpineol compound application on TNF-α, IL-1β, IL-6 and mRNA expressions on LPS-induced HUVEC cells was not significantly different at the 2nd and 4th hour when compared to the LPS-treated groups (Fig. 9, $p < 0.10$) and there was not any significant difference in the eNOS mRNA expression at the 2nd hours but there was a significant decrease when compared to LPS treated groups at the 4th hours (Fig. 9, $p < 0.10$).

Discussion

This study examined the effects of treatments with essential oils and components of MC fruit that have previously been shown to have very high anti-inflammatory, antioxidant, and antibacterial properties on endothelial cell damage in an in vitro sepsis model at cellular and molecular levels. The results revealed that essential oil from MC fruit did not ameliorate LPS-induced cell damage. Interestingly, when we examined components of the essential oil, α-terpineol increased the cytokine damage induced by LPS without any effect on LPS-induced damage. In contrast, both the 1.8 cineole-only treatment and α-pinene-only treatment ameliorated cell toxicity and increased cytokine levels due to sepsis.

Although monoterpenes are found in many plants, the amounts and proportions vary from plant to plant. In a study that examined separate components (terpenes) of *Melaleuca alternifolia*, terbinafine-4-ol induced a strong anti-inflammatory effect (Hart et al. 2000). In the same study, although total extract of tea tree oil was toxic to monocytes at a concentration of 0.016%, terpenes-4-ol showed beneficial effects by significantly reducing the amounts of LPS-induced TNFα, IL-1β, and IL-10 in monocytes (Hart et al. 2000). There are many studies that are similar to this study. Unlike the total extract, it is important that the effects of the plant are studied separately. In our work, these studies revealed similar results.

In the in vitro sepsis model in the present study, neither the MC volatile oil-only treatment nor α-terpineol-only treatment reduced the increase in cytokine levels induced by LPS. These findings are in accordance with those of previous research. In contrast, both the 1.8 cineole treatment and α-pinene treatment significantly decreased the increase in cytokine levels due to LPS administration.

TNF-α is a potent mediator of the pathogenesis of sepsis (Özcan et al. 1996). According to previous in vitro studies of human monocyte cells exposed to LPS, 1.8 cineole application decreased LPS-induced increases in TNF-α mRNA expression (Rita et al. 2013; Juergens 2014; Zhao et al. 2014).

According to previous research, α-pinene inhibited TNF-α (Adriana et al. 2013; Bouzabata et al. 2016). Another study reported that it had an inhibitory effect on the

transcription factor NF-κB and that it contributed to pain control during the inflammation process (Adriana et al. 2013). NF-κB is involved in the regulation of genes in all cell types and in pathological events, such as inflammation (Ayaz et al. 2017; Baeuerle 1998). It induces a cellular response and regulates the expression of pro-inflammatory cytokines (Ayaz et al. 2017; Baeuerle 1998). The results of the aforementioned studies support the findings on α-pinene in the present study.

In a previous study on tea tree oil, in terms of anti-inflammatory effects, terpinen-4-ol was much more effective than α-terpineol (42% vs. 3%). Indeed, α-terpineol also did not exhibit anti-inflammatory activity (Hart et al. 2000). Another study found that α-terpineol decreased the quantity of TNF-α mRNA expression (Barreto et al. 2014). In accordance with our findings, a previous study reported that α-terpineol did not reduce LPS-induced increases in IL-1B, TNF-α, and IL-10 levels in human monocyte cells, whereas terpinen-4-ol did reduce these levels (Abe et al. 2003).

Other than TNF-α, the cytokine IL-1β plays an important role in the pathogenesis of septic shock and multiple organ failure (Ayaz et al. 2017; Madhav and Shabbir 2004). In the present study, MC volatile oil-exposed cells, high amounts of IL-1β mRNA were detected in the MC plus LPS treatment group 2 and 4 h post-treatment. The level of IL-1β mRNA expression in the 1.8 cineole and 1.8 cineole plus LPS treatment groups were the same as that in the control group 2 and 4 h post-treatment. According to a previous study, the inhibition of IL-1β by 1.8 cineole was carried out over NF-κB activation (Rita et al. 2013). In another study, 1.8 cineole decreased LPS-induced decreases in IL-1β levels (Zhao et al. 2014). In the present study, IL-1β mRNA expression 2 and 4 h post-treatment in the HUVECs treated with α-pinene was close to that in the control group. In a previous study, α-pinene inhibited the NF-κB transcription factor, which in turn inhibited IL-1β (Adriana et al. 2013). In this study, the expression levels of IL-1β mRNA 2 h post-treatment were slightly higher in the α-terpineol and α-terpineol plus LPS groups as compared with those in the control group. The expression levels 4 h post-treatment were slightly higher than those after 2 h. A previous study reported that α-terpineol had minor effects on IL-1β expression (Nogueira et al. 2014). In research on tea tree extracts containing α-terpineol and terpinen-4-ol, these compounds prevented NF-κB activation and consequently reduced IL-1β, IL-6, and IL-10 levels (Nogueira et al. 2014). The study concluded that this effect was due to terpinen-4-ol. Some studies also reported that IL-1β expression increased the toxic effects of TNF-α (Liu et al. 2017; Cuschieri et al. 2010; Randeep et al. 2011; Panacek et al. 2004; Waka et al. 2016).

IL-6 is synthesized in a very short time during inflammation. In the present LPS-induced in vitro sepsis model, IL-6 mRNA expression in HUVECs increased slightly in the MC volatile oil and MC volatile oil plus LPS groups 2 h post-treatment as compared with that in the control, and IL-6 mRNA expression levels were very high 4 h post-treatment. In intergroup comparisons of IL-6 mRNA expression in HUVECs, there was no increase in gene expression 2 and 4 h post-treatment in the 1.8 cineole and 1.8 cineol plus LPS groups as compared with that in the control. Previous studies concluded that 1.8 cineole inhibited IL-6, which served as an inflammatory mediator (Bouzabata et al. 2016; Rita et al. 2013; Juergens 2014). In

the present study, IL-6 mRNA expression 2 and 4 h post-treatment in the α-pinene-treated HUVECs was the same as that in the control group. Previous research found that α-pinene inhibited the IL-6 mediator and the NF-κB pathway (Adriana et al. 2013). In the α-terpineol plus LPS group, IL-6 mRNA expression increased 2 and 4 h post-treatment. In another study, IL-6 production inhibited TLR2/4, thereby leading to significant inhibition of IL-6 (Nogueira et al. 2014). In previous research, α-terpineol inhibited NF-κB and promoted downregulation of IL-6 production (Barreto et al. 2014).

The initial response of an organism to inflammation is NO synthesis from macrophages (Aladağ et al. 2000; Ayaz et al. 2017; Lepoivre et al. 1991; Özkan and Akgül 2010; Türköz and Özerol 1997). As demonstrated previously, NO produced by iNOS has damaging and toxic effects, whereas NO produced by eNOS is required for the protection of endothelial function (Ayaz et al. 2017). Studies showed that increased eNOS levels reduced vascular inflammation but that the amount of eNOS decreased as vascular damage increased. The important functions of eNOS in terms of vascular structures that are in a regular and healthy condition are well known (Lee et al. 2016; Varadharaj et al. 2015).

The amount of eNOS in sepsis varies, and the amount of eNOS is thought to be correlated with the prognosis (Scalia and Stalker 2002; Yamashita et al. 2000). In our study, eNOS protein expression levels decreased in the HUVECs treated with LPS, and α-terpineol from the essential oil of MC fruit did not ameliorate the reduction in the amount of eNOS in LPS-treated HUVECs. In parallel with these results, there was no increase in the cell index. In contrast, 1.8 cineole and α-pinene, which are other major components of MC fruit, significantly increased eNOS protein expression in LPS-induced HUVECs as compared with that in the LPS group. In addition, the cell index, which was decreased in the LPS group, was close to normal in the 1.8 cineole plus LPS and α-pinene plus LPS groups.

Overall, the results of the present study showed that LPS treatment of HUVECs significantly reduced cell proliferation 24 and 48 h post-treatment. The LPS treatment also led to endothelial cell damage and a significant increase in cytokine levels. At both cellular and biomolecular levels, the α-terpineol treatment had no effect on LPS-induced endothelial cell damage. In contrast, 1.8 cineole and α-pinene molecules, other major components of MC fruit, ameliorated LPS-induced HUVEC damage at cellular and biomolecular levels.

In conclusion, it is possible to link the cause of ineffectiveness of volatile oil obtained from MC plant to cell damage caused by sepsis to the synergism of various active substances in its content. This essential oil contains three major components, one of which further increases cell damage due to sepsis. The other two active molecules, on their own, have considerable therapeutic effects. Coexistence of the three components may mask the biological activity that can be very important. We anticipate that the subsequent studies on these molecules, individually or in combination, will produce far more diverse and positive results.

Acknowledgements This research was supported by Scientific Research Projects Commission with Project Number ATAUNI-BAP-2016/115. We are grateful the support of Assist. Prof. Dr. Nurcan KILIC BAYGUTALP for English reduction and revisions.

Compliance with Ethical Standards

Conflict of ınterest There is not conflict of interest for all authors.

Ethical Approval This study was approved by the Local Ethics Committee of Ataturk University, under Protocol Number 93722986.12/92.

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Affiliations

Zerrin Kutlu[1] · Mine Gulaboglu[1] · Zekai Halıcı[2] · İrfan Cınar[2] · Busra Dıyarbakır[2]

✉ Mine Gulaboglu
minegulaboglu@atauni.edu.tr

Zekai Halıcı
hzeaki@gamil.com

İrfan Cınar
irfan.nar@gmail.com

Busra Dıyarbakır
busradiyarbakir@gmail.com

[1] Department of Biochemistry, Faculty of Pharmacy, Ataturk University, 25240 Erzurum, Turkey

[2] Department of Pharmacology, Faculty of Medicine, Ataturk University, 25240 Erzurum, Turkey