



In vitro anti-inflammatory activity of terpenes via suppression of superoxide and nitric oxide generation and the NF- κ B signalling pathway

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Abstract

Background and Aims Terpenes are considered the main components of essential oils and an important source for the identification of novel lead molecules. This study aimed to investigate the in vitro anti-inflammatory activity of *L*-carveol, *L*-carvone, and *m*-cymene (monoterpenes) and of valencene and guaiene (sesquiterpenes).

Methods The influence on intracellular nitric oxide (NO) and pro- and anti-inflammatory cytokine (TNF- α , IL-1 α and IL-10) production and on nuclear factor kappa B (NF- κ B) activity was determined using Griess reagent, immunoenzymatic assay kits (ELISA) and chemiluminescence measurements in cell-based assays, respectively. Antioxidant activity was assayed through the protective effect against cellular oxidative damage produced by superoxide anion production (O_2^-) and hydrogen peroxide on macrophages and by the quenching activity of the NO radical.

Results and Discussion Terpenes reduced the pro-inflammatory cytokines TNF- α and IL-1 α and increased the production of IL-10. In addition, the terpenes, especially guaiene ($53.3 \pm 2.4\%$) and *m*-cymene ($38.1 \pm 0.6\%$), significantly inhibited NO production in a macrophage cell culture-based assay, whereas no effect was observed in the scavenging activity of this radical. *L*-carveol and *m*-cymene significantly inhibited O_2^- production with reductions of approximately $68.6 \pm 2.2\%$ and $48.2 \pm 4.2\%$, respectively, at a concentration of 10 μ M. Moreover, these terpenes were verified to suppress NF- κ B activity. The results indicate that these terpenes have therapeutic potential and may be used to suppress inflammatory diseases or as a leading compounds.

Keywords Lead compounds · Inflammation · NF- κ B · Nitric oxide · Superoxide anion · Terpenes

Abbreviations

IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
NO	Nitric oxide
NF- κ B	Nuclear factor kappa B

O_2^-	Anion superoxide
ATCC	American Type Culture Collection

Introduction

Acute inflammation is a host-protective reaction that is based on a well-organized response to infection and tissue injury. This process begins and is maintained through the action of exogenous and endogenous mediators, and it causes, as its main objective, tissue repair and the restoration of homeostasis. Unresolved inflammation has long been recognized to play a key role in the development of chronic diseases, including arthritis, autoimmune diseases and asthma (Kolaczowska and Kubek 2013; Hamidzadeh et al. 2017).

Macrophages are considered the primary contributors to potentially pathological inflammatory processes. They are highly responsive cells responsible for the uptake of

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apoptotic cells, the synthesis and release of many inflammatory mediators (ex: nitric oxide and cytokines) and the protection of the body from infection (Feghali and Wright 1997; Hamidzadeh et al. 2017). Another important fact related to the inflammatory response of macrophages is the activation of nuclear factor kappa B (NF- κ B), which is involved in cell activation, immune responses, cell proliferation and inflammatory processes. When activated, NF- κ B induces the expression of pro-inflammatory genes, including chemokines, cytokines (such as TNF- α and IL-1) and cyclooxygenase-2 (COX-2), and the activation of iNOS. Although inflammation is a protective process in the body, an excessive acute inflammatory response or chronic inflammation can cause irreversible tissue damage, organ failure or even death (Feghali and Wright 1997; Lawrence 2009; Deutschman and Tracey 2014; Baker et al. 2011; Zhang et al. 2017).

Non-steroidal anti-inflammatory drugs were discovered more than 100 years ago, and they remain at the top of the list for the pharmacological management of acute and chronic pain and inflammatory diseases. However, they are commonly associated with a high incidence of adverse events, particularly in the gastrointestinal tract and cardiovascular system (Brune and Patrignani 2015; Pereira-Leite et al. 2017). Therefore, there is growing interest in identifying new drugs with greater therapeutic efficacy and reduced toxic effects. Within the broad range of compounds obtained naturally, terpenes have been gaining importance. They represent the great majority of components identified in essential oils, with monoterpenes and sesquiterpenes prevailing, and are found in many plants in nature (Wagner and Elmadfa 2003). Terpenes, which comprise a diverse class of naturally occurring organic compounds, display a wide array of very important pharmacological properties and demonstrated the ability to suppress NF- κ B signalling, the key transcription factor in the pathogenesis of inflammatory diseases and cancer development (Salminen et al. 2008; Yadav et al. 2010). The monoterpene genipin and the sesquiterpene (–)- α -bisabolol exert their anti-inflammatory effects by downregulating expression of iNOS and NO production in LPS-stimulated RAW 264.7 macrophages and simultaneously, blocked the degradation of I κ B β protein, inactivated AP-1 and inhibited the NF- κ B signalling (Koo et al. 2004, Kim et al. 2011). Thus, terpenes, especially mono- and sesquiterpenes, are of great interest as lead structures that may be used as templates for the development of new drugs by the pharmaceutical industry.

Among these promising lead compounds are L-carveol, L-carvone, *m*-cymene, guaiene and valencene. L-carveol and L-carvone are natural unsaturated, monocyclic monoterpene found at high concentration in spearmint essential oil (*Mentha spicata*). L-carvone showed depressant effects and antinociceptive activity (De Sousa et al. 2007; Gonçalves

et al. 2008), and antimicrobial (Hussain et al. 2010) and chemopreventive activity through the induction of apoptosis in cancer cells (Patel and Thakkar 2014). Carveol demonstrated nematocidal (Echeverrigaray et al. 2010), repellent (Omolo et al. 2004) and cancer chemopreventive activity (Crowell et al. 1992) and inhibited the function of human α 7 nicotinic acetylcholine receptors (Lozon et al. 2016). Cymene is a monocyclic monoterpene, found in over 100 plant species as example in plants of the genus *Protium*, *Thymus* and *Artemisia* and shows a range of biological activity including antioxidant, anti-inflammatory, and antimicrobial (Marchese et al. 2017). Valencene is a sesquiterpene common found as an aroma component of citrus fruit and citrus-derived odorants that show to inhibit the production and expression of the pro-inflammatory cytokines IL-1 β and IL-6 in LPS-stimulated RAW 264.7 cells (Yang et al. 2016) and increase the pro-oxidative effect of doxorubicin in CaCo-2 cells (Ambrož et al. 2015). Guaiene has been isolated from a variety of plant sources and first isolated from guaiac wood oil from *Bulnesia sarmientoi* demonstrated to inhibit NO generation in RAW 264.7 cells without cytotoxic effects (Mendes et al. 2013).

Although there are some general reports demonstrating the therapeutic potential of the proposed terpenes for study, the anti-inflammatory properties and their mechanisms of action have not been addressed until now. Therefore, in the continuous search of new lead compounds, the aim of the present investigation was to study the *in vitro* anti-inflammatory effects of L-carveol, L-carvone, *m*-cymene, guaiene and valencene using various chemical and cell-based assays.

Materials and methods

Chemicals and biochemicals

Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, L-carveol, L-carvone, *m*-cymene, valencene and guaiene (purity \geq 95%) was purchased from Sigma Aldrich[®] Chemical Co., St. Louis, MO, USA. Terpenes were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in the growth medium for cell-based assays at the desired concentration. DMSO concentration in the wells was kept under 0.25%. IL-1 α , IL-10 and TNF- α ELISA kits were obtained from eBioscience, San Diego, CA, USA. Coomassie protein assay reagent was purchased from Thermo Scientific (Rockford, IL, USA). Sodium nitroprusside dihydrate (SNP—Neon Comercial Ltda), sulforhodamine B sodium salt (SRB), NG-methyl-L-arginine acetate salt (L-NMMA), nitroblue tetrazolium (NBT), hydrogen peroxide solution (H₂O₂), gallic acid, N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and human tumour necrosis factor- α (all from Sigma, USA) were also utilized. The Dual-Luciferase[®] Reporter Assay System

10-Pack (Promega Corporation, USA) was used. Other reagents used were of analytical grade and were obtained from various commercial sources.

Measurement of nitrite with sodium nitroprusside

To determine the NO scavenging activity of terpenes, sodium nitroprusside (SNP) was used following the methodology previously described by Bates et al. (1991) with modifications. The SNP solution was diluted in 2.5 mM water, mixed with different concentrations of the sample (0.0625–2.0 mM) dissolved in ethanol and incubated at room temperature (37 °C) for 10 min. Nitrite was determined with the Griess reaction (Green et al. 1982) by adding 150 µL of Griess reagent [equal volumes of 1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl)ethylenediamine solution] to the mixture. Gallic acid (1.5 mM) dissolved in ethanol was used as the positive control. Absorbance was measured in a microplate reader at 540 nm with a calibration curve with sodium nitrite standards.

Cell lines

RAW 264.7 murine macrophages (ATCC[®] TIB-71[™]), MIAPaCa-2 human pancreatic carcinoma (ATCC[®] CRL-1420[™]), Swiss 3T3 albino mouse fibroblasts (ATCC[®] CCL-92[™]) and Hepa-1c1c7 murine hepatoma cells (ATCC[®] CRL-2026[™]) and Human embryonic renal cells transfected with the luciferase-expressing gene (Panomic, Fremont, CA) (retroviruses) were purchased from the Cell Bank of Rio de Janeiro, Brazil. Cells were cultured in Dulbecco's modified Eagle medium (Sigma Aldrich[®] Chemical Co., St. Louis, MO, USA) or high-glucose modified culture medium (DMEM-Gibco-BRL Life Biotechnologies, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS-Sigma Aldrich[®] Chemical Co., St. Louis, MO, USA), 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (Sigma Aldrich[®] Chemical Co., St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO₂.

MTT assay

The MTT assay was used to investigate cellular viability (Mosmann 1983). Briefly, cells were seeded at a density of 7 × 10⁴ cells mL⁻¹ in 96-well plates and cultured for 24 h in the presence or absence of increasing concentrations (1–200 µM) of L-carveol, L-carvone, *m*-cymene, guaiene and valencene. After a 24-h incubation, 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (2 mg mL⁻¹) was added to each well, and the formazan crystals formed were dissolved with DMSO. The optical density was measured at 595 nm using a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular

Devices Spectra, USA). The experiments were carried out in triplicate at a minimum.

Sulforhodamine B assay (SRB)

The SRB assay was used to examine cell viability (Skehan et al. 1990). Briefly, non-cancerous RAW 264.7 macrophages and human hepatocellular carcinoma (HepG2-Luc) cells were seeded at a density of 2 × 10⁵ and 10 × 10⁴ cells mL⁻¹, respectively, in 96-well plates and cultured for 24 h in the presence or absence of increasing concentrations (1–100 µM) of the samples. After the incubation period, cells were fixed by adding 10% trichloroacetic acid (TCA), and the plate was incubated at 4 °C for 30 min. Thereafter, the plate was gently washed, dried and stained with 100 µL of SRB (0.4%). After incubation for 30 min, the plate was washed and allowed to dry at 45 °C. The protein-bound dye was dissolved in 10 mM Tris base solution (pH 10.0), and the optical density was measured at 515 nm using a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). The experiments were carried out in triplicate at a minimum.

Superoxide anion assay

The superoxide anion assay was used to examine the inhibitory effects of samples on superoxide anion (O₂⁻) production in LPS-activated RAW 264.7 macrophages (Choi et al. 2006). Briefly, RAW 264.7 cells were seeded at a density of 2 × 10⁵ cell mL⁻¹ in 96-well plates and grown for 24 h in a humidified incubator with 5% CO₂ at 37 °C. Then, cells were treated with 1 µg mL⁻¹ LPS in the presence or absence of increasing concentrations (1–100 µM) of the samples for 20 h. Gallic acid (50 µM) was used as a positive control. After incubation, the supernatant was removed, 100 µL of NBT (1 mg mL⁻¹) was added to each well, and the plates were incubated for an additional 2 h. Then, cells were washed with methanol and dried for 20 min at 37 °C. Formazan crystals were dissolved with DMSO and potassium hydroxide (KOH). The optical density was measured at 620 nm using a microplate reader (Mults-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). The experiments were carried out at in triplicate at a minimum.

Hydrogen peroxide assay

The hydrogen peroxide assay was used to examine the protective effect of the samples against oxidative cell death caused by hydrogen peroxide (H₂O₂) (Annan and Houghton 2008; Adetutu et al. 2011). Briefly, RAW 264.7 cells were seeded at a density of 1 × 10⁵ cells mL⁻¹ in 96-well plates and cultured in a humidified incubator with 5% CO₂ at 37 °C

for 20 h. After incubation, H₂O₂ (500 μM) was added to each well in the presence or absence of increasing concentrations (1–100 μM) of the samples for 24 h. After, cytotoxicity was evaluated by MTT assay as previously described.

Nitric oxide analysis

The nitrite concentration in the culture medium was measured as an indicator of nitric oxide (NO) production (Green et al. 1982; Kondratyuk et al. 2012). Briefly, RAW 264.7 cells were seeded at a density of 2×10^5 cells mL⁻¹ in 96-well plates and cultured in a humidified incubator with 5% CO₂ at 37 °C for 24 h. The cells were pre-treated with phenol red-free medium, followed by 1 μg mL⁻¹ LPS in the presence or absence of increasing concentrations (1–100 μM) of samples for 20 h. After the incubation, NO production was spectrophotometrically evaluated by measuring nitrite concentrations via the Griess reaction. Absorbance was measured in a microplate reader at 540 nm against a calibration curve with sodium nitrite standards. Under the same experimental conditions, cellular viability was examined in parallel by the SRB assay.

Measurement of cytokines

TNF-α, IL-1α and IL-10 were quantified in the supernatant of RAW 264.7 murine macrophages unstimulated or stimulated with LPS by ELISA using specific antibodies (purified and biotinylated) and cytokine standards, according to the manufacturer's instructions (eBioscience, San Diego, California, USA). Optical densities were measured at 450 nm in a microplate reader (Multis-Mode Microplate Reader, Filter Max F5, Molecular Devices Spectra, USA). Cytokine levels were expressed in pg, and the sensitivities were > 10 pg mL⁻¹.

Luciferase NF-κB assay

An NF-κB luciferase assay was used to monitor changes in the NF-κB pathway (Kondratyuk et al. 2012; Homhual et al. 2006). Human embryonic kidney (HEK) 293 cells (Panomic, Fremont, CA) were seeded into a sterile 96-well plate at 2×10^5 cells per well and grown to approximately 80% confluence by incubating for 48 h. After incubation, the medium was replaced, and the cells were treated with various concentrations (1–100 μM) of test compounds and incubated for an additional 6 h with or without TNF-α (2 ng mL⁻¹). Then, cells were washed with PBS, and the luciferase assay was performed using the Luc assay system from Promega (Madison, WI) following the manufacturer's instructions. The results are presented as the percentage of NF-κB inhibitory activity.

Statistical analysis

Statistical analyses were performed using GraphPad software (San Diego, CA, 176 USA). Data are presented as the mean ± standard deviation (SD), and statistical comparisons were carried out using one-way analysis of variance (ANOVA) followed by Dunnett's post-test or two-way-ANOVA when appropriate. The level of significance was $p < 0.05$.

Results and discussion

Acute inflammation is a protective reaction of the microcirculation initiated after infection and/or injury. Unresolved or uncontrolled inflammation is considered a central pathological process associated with many diverse disease states. The clinically available anti-inflammatory drugs suffer from the disadvantage of side effects, and in the case of biologics, a high cost of treatment. Continued basic research in the identification of bioactive lead compounds offers a great hope in the identification and development of new drugs for treating inflammatory diseases (Gautam and Jachak 2009; Fullerton and Gilroy 2016; Dang and Süßmuth 2017).

At first, the cytotoxic effects of the studied terpenes were evaluated by MTT and SRB assays in the non-cancerous 3T3 fibroblasts and RAW 264.7 macrophage cell lines and in human hepatocellular carcinoma (HepG2-Luc), human pancreatic carcinoma (MIAPaCa-2) and hepatoma cancer cells (Hepa-1c1c7). No cytotoxic effects were observed in any tested cell line at concentrations up to 200 μM (data not shown). Therefore, further cell-based assays were performed using concentrations of 1–100 μM which exhibited no cytotoxic effects on macrophages, as well as frequently observed in previous *in vitro* studies using terpenes (Koo et al. 2004; Kim et al. 2011; Mendes et al. 2013).

Activation of macrophages by bacterial LPS induces the production of reactive oxygen species (ROS), which play an important role in the progression of many inflammatory diseases and can produce various degenerative conditions characterized by DNA, protein, lipid, cellular damage, and inhibit cellular functionality (Valko et al. 2007). During inflammation, ROS such as the superoxide radical (O₂⁻), peroxynitrite (ONOO), hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), and reactive nitrogen species such as nitric oxide are produced (Barreiros et al. 2006). These molecules modulate the synthesis of prostaglandins, thromboxanes and other inflammatory molecules (Coleman 2001). NO also plays a role in many important biological functions, including vasodilation, immunoregulation, inflammation and neurotransmission. If terpenes are able to scavenge some free radicals, or inhibit some oxidative enzymes they can also act as anti-inflammatory agents, because one

of the inflammatory responses is the oxidative burst that occurs in diverse cells, including monocytes, neutrophils and macrophages. The monoterpene menthol demonstrated the ability on the reduction of superoxide anion production and its effects were correlated to a decrease in superoxide dismutase (SOD) activity, the key enzyme responsible for catalysing the dismutation of superoxide into oxygen and hydrogen peroxide (Rozza et al. 2014; Filippin et al. 2008). Therefore, inflammation was first evaluated through the production of the superoxide anion production and cellular oxidative damage in response to hydrogen peroxide exposure in RAW 264.7 macrophages. Figure 1 summarizes the results of superoxide anion production after cellular exposure to the studied terpenes. The monoterpenes L-carveol and *m*-cymene resulted in reductions in O₂⁻ production of 68.6 ± 2.2% and 55.7 ± 4.3% (10 μM) and 48.2 ± 4.2% and 51.5 ± 3.8% (100 μM) (Fig. 1). Gallic acid (50 μM) was used

as a positive control and inhibited 58.9 ± 5.2% of O₂⁻ production (*p* < 0.05). All tested terpenes did not exhibit protective effects against cellular oxidative damage induced by hydrogen peroxide in RAW 264.7 macrophage cells at concentrations of 1–100 μM (Fig. 2).

Excessive nitric oxide (NO) production, as a consequence of iNOS induction in activated macrophages, has been linked to different diseases, and it is considered a pro-inflammatory mediator that induces inflammation (Coleman 2001). Preliminary results showed that there was no detectable NO production in resting RAW 264.7 cells and the cells treated with the test compounds alone, indicating that the test compounds do not stimulate NO production. As shown in Fig. 3, terpenes efficiently suppressed lipopolysaccharide-induced NO release. NO reductions of 35.5 ± 0.8%, 44.1 ± 1.5% and 38.1 ± 0.6% were observed with 100 μM L-carvone, L-carveol and

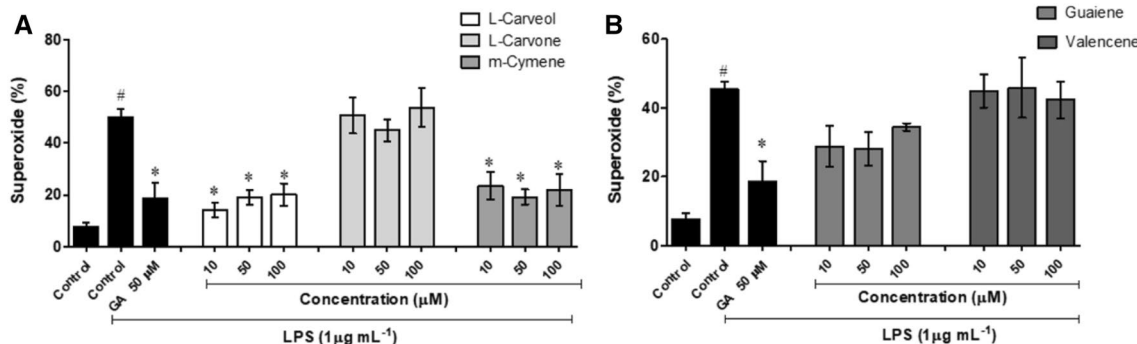


Fig. 1 Effect of terpenes on superoxide anion production in vitro. RAW 264.7 macrophages were exposed to terpenes (10, 50 and 100 μM) for 2 h, followed by the addition or not of LPS (1 μg mL⁻¹). Fifty micromolar gallic acid (GA) was used as a positive control. Superoxide anion production was determined after 24 h using the

colorimetric NBT reagent. **a** Monoterpenes: L-carveol, L-carvone and *m*-cymene. **b** Sesquiterpenes: guaiene and valencene. The results are expressed as the mean ± SD of three independent experiments. *Significant at *p* < 0.05 compared to control + LPS. #Control versus control + LPS

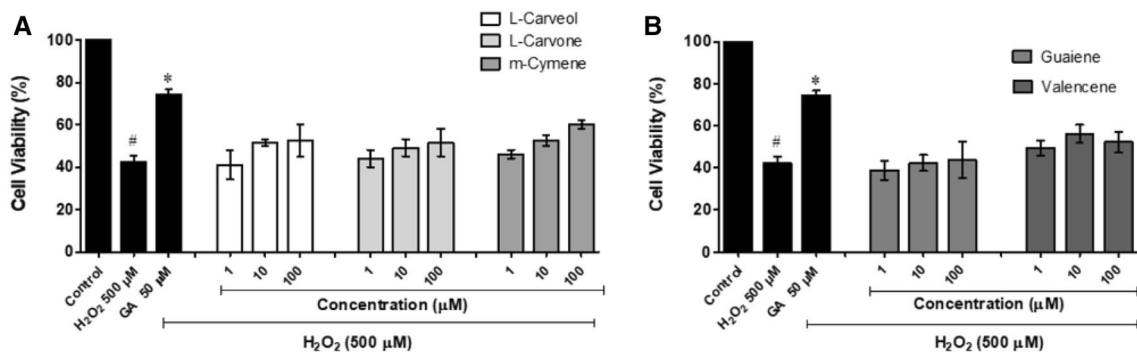


Fig. 2 Protective effect of terpenes against hydrogen peroxide (H₂O₂) induced damage. RAW 264.7 macrophages were exposed to terpenes (1, 10 and 100 μM) for 2 h, followed by the addition or not of H₂O₂ (500 μM). Fifty micromolar gallic acid (GA) was used as a positive control. Cell viability was determined using 3-(4,5-dimethyl-thiazol-

2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. **a** Monoterpenes: L-carveol, L-carvone and *m*-cymene. **b** Sesquiterpenes: guaiene and valencene. The results are expressed as the mean ± SD of three independent experiments. *Significant at *p* < 0.05 compared to 500 μM H₂O₂. #Control versus 500 μM H₂O₂

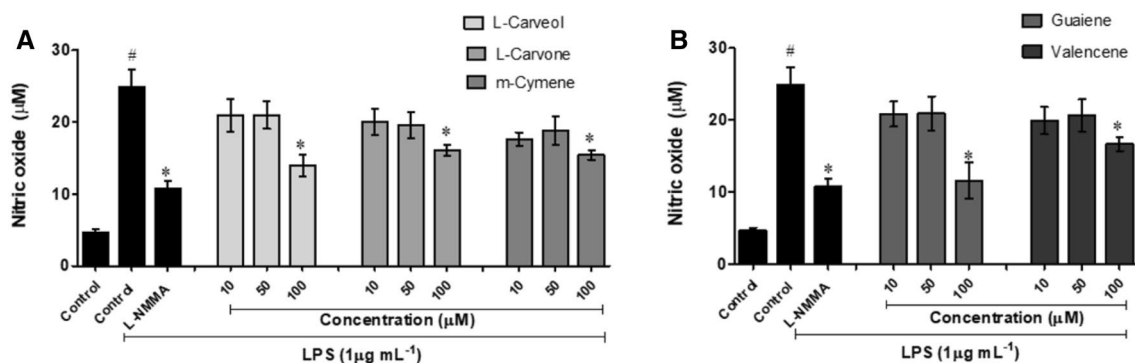


Fig. 3 Terpenes reduce the production of nitric oxide (NO) in vitro. RAW 264.7 macrophages were treated with or without terpenes, and after 2 h, they were stimulated with LPS. **a** Monoterpenes: L-carveol, L-carvone and *m*-cymene. **b** Sesquiterpenes: guaiene and valencene. NO production was measured 24 h later using Griess's reagent as

described in the Materials and Methods section. L-NMMA (500 µM) was used as a positive control. The results are expressed as the mean ± SD of three independent experiments. *Significant at $p < 0.05$ compared to control + LPS. #Control versus control + LPS

m-cymene, respectively. The sesquiterpenes guaiene and valencene were tested at a concentration of 100 µM and showed an inhibition of $53.3 \pm 2.4\%$ and $33.0 \pm 0.9\%$, respectively (Fig. 3). It should be noted that guaiene elicited the highest inhibition of NO, with inhibition similar to that of 500 µM L-NMMA ($57.0 \pm 1.7\%$), an iNOS inhibitor. Terpenes did not influence the NO production in unstimulated RAW 264.7 macrophages (data not shown). No terpenes exhibited NO scavenging activity (data not shown) at concentrations up to 2 mM.

These results suggest that inhibition of NO may be due to a reduction in the activity of iNOS without affecting cellular viability. Corroborating with our findings, Mendes et al. (2013) evaluated guaiene-type sesquiterpenes and demonstrated that adding a hydroxyl group or aglycone or changing the position of the double bond to guaiene increased the inhibitory potential towards NO.

Upon tissue injury or LPS exposure, macrophages rapidly produce large amounts of inflammatory cytokines that can initiate a cascade of inflammatory mediator release, leading to whole tissue destruction (Hamidzadeh et al. 2017). The early release of cytokines holds great potential as a target for new therapeutic strategies (Kopf et al. 2010; Schett et al. 2013; Hamidzadeh et al. 2017). Therefore, the effects of terpenes and dexamethasone (10 µM) on LPS-induced inflammation in RAW 264.7 macrophages were evaluated by measuring the production of TNF-α, IL-1α and IL-10. As shown in Figs. 4 and 5, stimulation with LPS for 24 h significantly increased the release of pro-inflammatory cytokines (TNF-α and IL-1α), indicating that an inflammatory response was induced in RAW 264.7 cells. Interestingly, higher concentrations of IL-10 were induced by the five terpenes than by the LPS control (Fig. 4, 5). Dexamethasone significantly reduced TNF-α and IL-1α production but did not influence the concentration of IL-10.

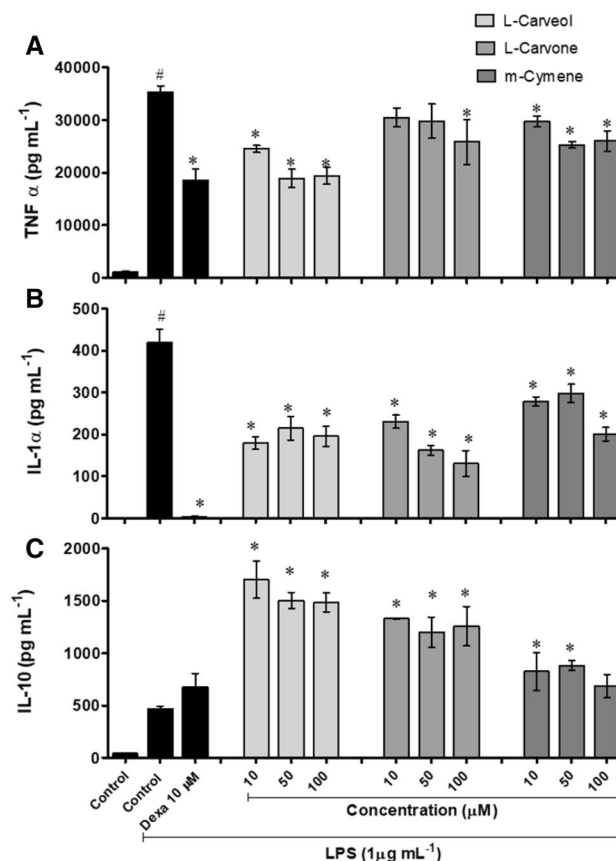


Fig. 4 Monoterpenes reduce the concentration of pro- and anti-inflammatory cytokines (TNF-α, IL-1α and IL-10) in vitro. RAW 264.7 macrophages were exposed to terpenes, and after 2 h, they remained unstimulated or were stimulated with LPS. **a** TNF-α, **b** IL-1α and **c** IL-10. Cytokine levels were measured 24 h later using ELISA kits as described in the materials and methods section. The results are expressed as the mean ± SD of three independent experiments. *Significant at $p < 0.05$ compared to control + LPS. #Control versus control + LPS

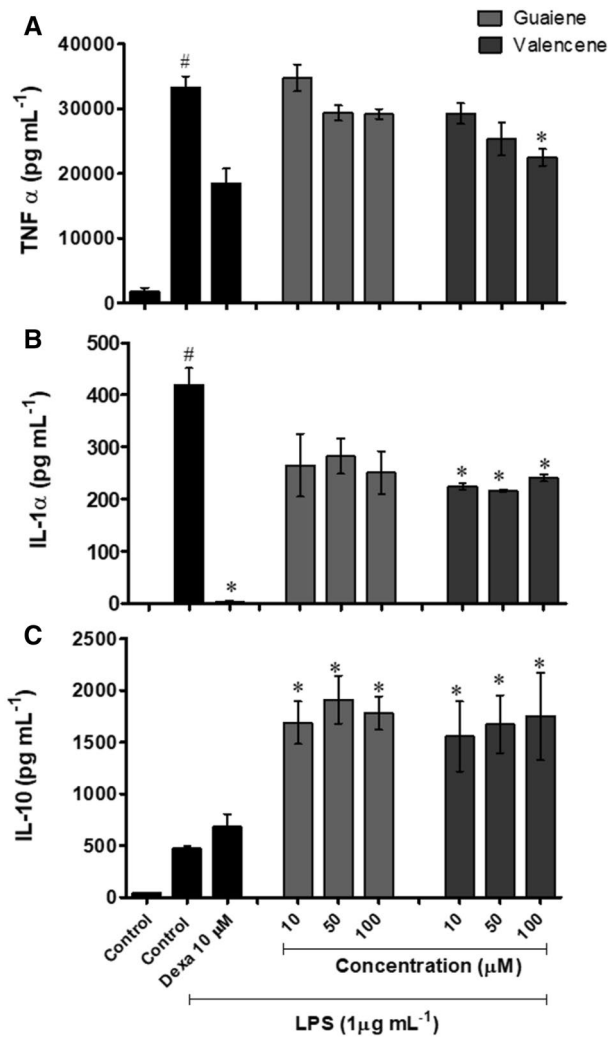


Fig. 5 Sesquiterpenes reduce the concentration of pro- and anti-inflammatory cytokines (TNF- α , IL-1 α and IL-10) in vitro. RAW 264.7 macrophages were exposed to terpenes, and after 2 h, they remained unstimulated or were stimulated with LPS. **a** TNF- α , **b** IL-1 α and **c** IL-10. Cytokine levels were measured 24 h later using ELISA kits as described in the materials and methods section. The results are expressed as the mean \pm SD of three independent experiments. *Significant at $p < 0.05$ compared to control + LPS. #Control versus control + LPS

As shown in Fig. 4, when macrophages were treated with up to 100 μ M of the monoterpenes, significant reductions in IL-1 α and TNF- α concentrations were observed. L-carveol showed the greatest reduction in TNF- α production, with $46.3 \pm 7.0\%$ and $44.8 \pm 6.5\%$ inhibition at 50 and 100 μ M, respectively (Fig. 5a). Similar results were observed with dexamethasone. A significantly decreased concentration of IL-1 α was detected after monoterpene treatment at 10–100 μ M (Fig. 4b). L-carveol (100 μ M) and L-carvone (100 μ M) showed inhibition values of $52.5 \pm 7.5\%$ and $69.1 \pm 12.7\%$, respectively. Interestingly, unlike the other monoterpenes, L-carveol resulted in greater inhibition at

10 μ M ($57.5 \pm 6.8\%$). Production of IL-10 was also influenced by monoterpenes. Surprisingly, IL-10 production was higher at the lower tested concentrations (10 and 50 μ M). L-carveol and L-carvone induced a significant increase of 3.6- and 2.8-fold compared to the LPS control (Fig. 4c).

The sesquiterpenes guaiene and valencene exhibited different patterns of IL-1 α and TNF- α production in LPS-stimulated macrophages (Fig. 5). Only valencene caused a significant decrease in IL-1 α and TNF- α production (Fig. 5). Although guaiene did not influence IL-1 α and TNF- α , a significant effect was observed on IL-10 production, which increased by approximately 4.1- (at 50 μ M) and 3.8-fold (at 100 μ M) compared to that induced by the LPS control (Fig. 5c). Valencene provoked a significant increase of approximately 3.7-fold at 100 μ M compared to the LPS control (Fig. 5c). Mono- and sesquiterpenes did not influence the TNF- α , IL-1 α and IL10 production NO production in unstimulated RAW 264.7 macrophages (data not shown).

Thus, the inhibitory effect of the studied terpenes on the production of these early pro-inflammatory cytokines, especially TNF- α and increased IL-10 production further emphasize their anti-inflammatory properties. TNF- α has long been considered a key rate-limiting step in the development of many inflammatory diseases and a highly effective therapy for rheumatoid arthritis (Choi et al. 2006). Reactive oxygen and nitrogen species together of cytokines, in particular the TNF- α can act as modulators of protein and lipid kinases, and transcription factors, including nuclear factor- κ B (NF- κ B). This well-known transcription factor plays an important role in controlling the inflammation process and regulating immune cells, especially macrophages. NF- κ B induces the transcription of pro-inflammatory mediators, such as inducible NO synthase (iNOS), cyclooxygenase (COX)-2, TNF- α , IL-1 and IL-6 (Surh et al. 2001; Hamidzadeh et al. 2017). Thus, the inhibitory effects of terpenes on inflammatory mediators prompted us to further investigate their ability to influence the NF- κ B signalling pathway. Figure 6 summarizes the results after the treatment of HEK 293 cells with the studied terpenes at 50 and 100 μ M. TPCK was used as a positive control at 833.3 μ M, and the NF- κ B inhibitory activity observed was $38.1 \pm 0.2\%$. The suppression of NF- κ B activation was observed with the four tested terpenes, as shown in Fig. 6. Cellular viability was maintained above 98%.

Yang et al. (2016) demonstrated that valencene inhibits the expression/secretion of I-CAM and I κ B and the nuclear translocation of NF- κ B in keratinocyte cells (HaCaT), in addition to reducing IL-1 β and IL-6 in macrophages. Other compounds with promising anti-inflammatory activity have also been shown to inhibit the production of inflammatory mediators such as pro-inflammatory cytokines and NO, and their effects are mediated by expression of NF- κ B (De Stefano et al. 2007; Low et al. 2015). Thus,

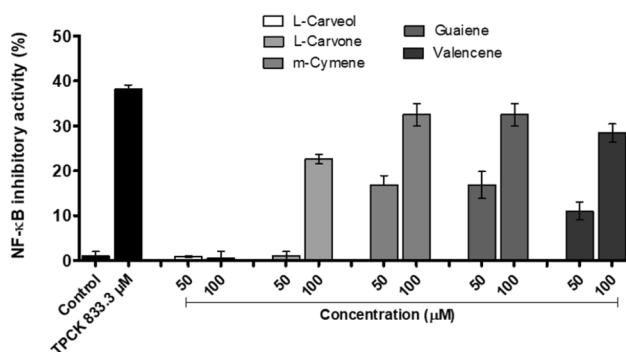


Fig. 6 NF- κ B inhibitory activity of studied terpenes. Human embryonic kidney (HEK) 293 cells were exposed to terpenes (50 and 100 μ M) and incubated for 6 h with or without TNF- α (2 ng mL $^{-1}$). The results are presented as the percentage of NF- κ B inhibitory activity and are expressed as the mean \pm SD of two independent experiments

we considered that the NF- κ B signalling pathway might be involved in the terpene-mediated down-regulation of NO and cytokines. These results demonstrated that the molecular mechanism by which terpenes inhibit the production of these inflammatory mediators might be partially related to inhibition of NF- κ B activation. Taken together, these results suggest that the studied terpenes may be effective agents for inhibiting inflammation.

Conclusion

The present research suggests that studied terpenes exhibit anti-inflammatory activity by inhibiting key mediators responsible for activating the inflammatory process. Using cell-based assays terpenes may exert their activities via the suppression of pro-inflammatory enzymes (such as iNOS and NADH/NADPH oxidase), inflammatory mediators (TNF- α and IL-1 α) and NF- κ B signalling. Altogether, these results emphasize the biological importance of essential oil-derived terpenoids and provide the first insight of this approach to identifying novel natural NF- κ B inhibitors for the development of new therapeutic strategies to treat inflammatory diseases.

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Compliance of ethical standards

Conflict of interest The authors have no conflicts of interest to report.

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