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Original Article

# Chemical composition and anti-inflammatory activities of essential oil from *Trachydium roylei*

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ABSTRACT

Chemical composition, anti-inflammatory activity, and cytotoxicity of essential oils obtained from the aerial parts of *Trachydium roylei* were investigated in this study. The chemical composition of *T. roylei* essential oil was analyzed using gas chromatography mass spectrometry. Fifty-nine components, representing 98.87% of the oils, were characterized. The oils were predominated by aromatic compounds and monoterpene hydrocarbons, and the main components were myristicin (25.35%), β-phellandrene (22.95%), elemicine (7.69%), isoelemicin (5.48%), and cedrol (5.26%). The anti-inflammatory activity of the oil in *lipopolysaccharide*-stimulated murine RAW 264.7 cells was evaluated. The oils downregulated the production of proinflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-1β, and IL-6, and significantly increased the anti-inflammatory cytokine IL-10 levels. Results indicated that the oils effectively inhibited the secretion of nitric oxide and prostaglandin E2 in *lipopolysaccharide*-stimulated macrophages. Western blot analyses were performed to determine whether the inhibitory effects of the oils on proinflammatory mediators (nitric oxide and prostaglandin E2) were related to the modulation of *inducible nitric oxide synthase* and cyclooxygenase-2 expression. These findings suggest that *T. roylei* essential oils exert an anti-inflammatory effect by regulating the expression of inflammatory cytokines.

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## 1. Introduction

Inflammation is a local tissue defensive reaction in response to infection and tissue injury, leading to local accumulation of plasma and blood cells [1] and the release of inflammatory mediators, which finalize the restoration of tissue structure and function. However, if inflammation remains untreated, the condition can be detrimental and may lead to an onset of certain diseases, such as asthma, rheumatoid arthritis,

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atherosclerosis, diabetes, and cancer [2]. Therefore, anti-inflammatory agents may be helpful in the treatment of inflammatory disorders [3]. Traditionally, the pharmacotherapy of inflammatory conditions is based on the use of steroidal anti-inflammatory drugs (SAIDs) or non-SAIDs (NSAIDs). The use of SAIDs, such as glucocorticoids, as anti-inflammatory agents are now controversial because of their multiple side effects, including osteoporosis, myophagism, and metabolic disturbance [4]. Prolonged use of NSAIDs, such as aspirin, ibuprofen, and indomethacin, can cause serious gastrointestinal toxicity [5]. Some NSAIDs have also been linked to increased blood pressure, considerably increased risk of congestive heart failure, and occurrence of thrombosis [6]. Hence, the discovery of novel and safer anti-inflammatory drugs is constantly needed. Interest in traditional medicinal plants, which are important sources of new chemical substances with potential therapeutic effects, has been growing [7]. Essential oils (EOs) extracted from various herbs and spices have been used in medicine, food, and cosmetics primarily because of the continuous discovery of their multifunctional properties. The anti-inflammatory effects of many EOs have also been investigated [8,9].

*Trachydium*, which is classified from the tribe *Smyrnieae* of the *Apiales*, is a genus of perennial herbs. This genus comprises approximately 10 species that are widespread from Siberia to the Himalayan region [10]. *Trachydium roylei* is a 4–10 cm tall perennial herb with a light and camphor-like odor and is usually acaulescent. This species is primarily distributed in the alpine grasslands of Central Asia, and it provides a long reputed medicinal value in traditional local medicine for the treatment of hypertension, fever, inflammation, and scabies.

However, the chemical composition and biological activities of *T. roylei* have not been described. Therefore, in the current study, the EOs of *T. roylei* were investigated for their chemical composition and anti-inflammatory activity. To our knowledge, this study is the first to report on the anti-inflammatory activity of *T. roylei* extract.

## 2. Methods

### 2.1. Plant material and chemicals

The aerial parts of *T. roylei* were collected from the Pamirs, Xinjiang Region, China, in June 2014. The plant specimens were identified [10]. The plants were dried under shade at room temperature. The voucher specimen (number IBSC0462255) was deposited in the South China Botanical Garden, Chinese Academy of Sciences.

Dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT), and phosphate-buffered saline were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium and fetal bovine serum were obtained from Invitrogen-Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were purchased from R&D Systems Inc. (St. Louis, MO, USA), and ELISA kits for IL-10 were purchased from BioLegend (San Diego, CA, USA). Antibodies against cyclooxygenase-2 (COX-2) and *inducible nitric oxide synthase* (iNOS) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody against β-actin was purchased from Chemicon (Temecula, CA, USA). Other chemicals and reagents were of analytical grade.

### 2.2. Isolation of EOs

Air-dried plant materials (300 g) of *T. roylei* were chopped and subjected to hydrodistillation for 5 hours using a Clevenger-type apparatus. The obtained oils were dried with sodium sulfate for 24 hours, filtered, and stored at 4°C in sealed brown glass vials until analysis.

### 2.3. Gas chromatography analysis

An Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA), which comprised an HP-5 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness) and an flame ionization detector detector, was used for gas chromatography (GC)–flame ionization detector analysis. Helium gas at a constant flow rate of 1 mL/min was used as the carrier gas. Injector and mass transfer line temperatures were set at 250°C and 280°C, respectively. The EO solution (1 μL) in hexane was injected and analyzed under the following column conditions: initial column temperature of 40°C for 1 minute, increased to 250°C at a 3°C/min heating ramp, and subsequently maintained at 250°C for 20 minutes.

### 2.4. GC–MS analysis

The EOs were quantitatively and qualitatively analyzed using a GC–mass spectrometry (MS) 6890-5975 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-5 MS fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 μm film thickness). For GC–MS detection, an electron ionization system with 70 eV of ionization energy was used. Helium gas was used as the carrier gas with a constant flow rate of 1 mL/min. Injector and mass transfer line temperatures were set at 250°C and 280°C, respectively. One microliter of EO solution in hexane was injected and analyzed under the following column conditions: initial column temperature at 40°C for 1 minute, increased to 250°C at a 3°C/min heating ramp, and maintained at 250°C for 20 minutes. Kovats indices were calculated for all volatile components using a homologous series of n-alkanes ($C_8$–$C_{25}$) in a HP-5 MS column. The major oil components were identified by coinjection with standards (whenever possible) and confirmed with the Wiley version 7.0 (John Wiley & Sons Inc.,Hoboken, NJ, USA) and National Institute of Standards and Technology version 2.0 GC-MS library(the National Institute of Standards and Technology (NIST) is an agency of the U.S. Department of Commerce) using the Kovats indices. The relative concentration of each EO compound was quantified on the basis of the peak area integrated in the analysis program.

### 2.5. Cell culture

RAW 264.7 cells belonging to a murine macrophage cell line were purchased from the China Center for Type Culture Collection (Shanghai, China). These cells ($2 \times 10^5$) were cultured in a 96-well plate containing Dulbecco's modified Eagle's medium (Sigma) and supplemented with 10% fetal bovine serum in a $CO_2$ incubator (5% $CO_2$) at 37°C.

### 2.6. Cell viability

Cells were preincubated for 24 hours in a $CO_2$ incubator, pretreated with several EO concentrations (0 mg/mL, 1.25 mg/mL, 2.5 mg/mL, 5.0 mg/mL, 10 mg/mL, 20 mg/mL, and 40 mg/mL) for 1 hour, and costimulated with 100 ng/mL lipopolysaccharide (LPS) for 24 hours at 37 °C. Afterwards, the cells were washed twice with phosphate-buffered saline and incubated with 100 mL of 0.5-mg/mL MTT for 2 hours to determine the cell viability. The medium was discarded, and 100 μL of dimethyl sulfoxide was added. After 30-minutes incubation, the absorbance at 570 nm was determined using a microplate reader.

### 2.7. Measurement of nitric oxide, PGE2, TNF-α, IL-1β, IL-6, and IL-10 levels

RAW 264.7 cells were placed in a 12-well plate at a density of $2 \times 10^5$ cells/well and incubated for 24 hours. The cultured cells were treated with various EO concentrations (0 mg/mL, 2.5 mg/mL, 5.0 mg/mL, 10 mg/mL, and 20 mg/mL) for 1 hour and stimulated with 100-ng/mL LPS for 24 hours. The cultured media were collected after centrifugation at 2000*g* for 10 minutes and stored at −80°C until analysis. The nitrite concentration in the cultured media was measured as an indicator of nitric oxide (NO) production based on the Griess reaction [11]. The levels of PGE2, IL-1β, IL-6, IL-10, and TNF-α in cultured media were quantitated with ELISA (R&D Systems) in accordance with the manufacturer's instructions.

### 2.8. Western blot analysis

RAW 264.7 cells placed in a 12-well plate were pretreated with various EO concentrations (0 mg/mL, 2.5 mg/mL, 5.0 mg/mL, 10 mg/mL, and 20 mg/mL) for 1 hour and stimulated with LPS for 6 hours. After the incubation period, the cells were scraped from flasks and lysed in a lysis buffer [100 mL: 50mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 0.1M (volume/volume) sodium dodecyl sulfate, 1% (volume/volume) Triton X-100, 1mM phenylmethane sulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 10 μg/mL pepstain]. Samples were boiled at 100°C for 5 minutes and centrifuged at 16,627*g* for 2 minutes at 4°C. Protein extracts were run on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-*buffered* saline and Tween 20 buffer for 1 hour at room temperature. Afterward, the membranes were incubated with an appropriate dilution ratio of the relative primary antibody overnight at 4°C. The membranes were further incubated with the secondary antibody for 4 hours at room temperature and detected using an enhanced chemiluminescence reagent. The membranes were washed three times and the immunoreactive proteins were detected with an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghanshire, UK). The results of the Western blot analysis were quantified by measuring the relative intensity compared to the control and represented in the relative intensities. Mouse β-actin was simultaneously detected as an internal control to monitor the intensity. Bands for iNOS, COX2, and β-actin antibodies were recognized at ~135 kDA, ~72 kDA, and ~45 kDa, respectively.

### 2.9. Statistical analysis

Data were presented as means ± standard deviation. For statistical analysis, the data were analyzed by one-way analysis of variance followed by Duncan *post hoc* pair-wise comparisons between groups. Significant difference between groups was determined at $p < 0.05$.

## 3. Results

### 3.1. EO yield and chemical composition

The steam distillation of 300 g of dried plant material yielded 5.6 mL (1.80%, volume/weight) of yellow oils with a distinct smell. The oil samples were analyzed using GC–MS. Their components were identified in terms of their retention index values and mass spectra compared with those reported in previous studies. The GC–MS analysis results of *T. roylei* EO revealed 59 components, representing 98.87% of the oils (Table 1). The components of the EOs were 30.38% monoterpene hydrocarbon fraction, 2.22% sesquiterpene hydrocarbon fraction, 6.51% oxygenated monoterpene fraction, 14.69% oxygenated sesquiterpenoid fraction, 41.53% aromatic compounds, and 3.54% other components. The main components of the oils were myristicin (25.35%), β-phellandrene (22.95%), elemicine (7.69%), isoelemicin (5.48%), and cedrol (5.26%).

### 3.2. Cell viability

The effect of oils on RAW 264.7 cell viability was determined using MTT assay. Figure 1 shows the cell viability at 1.25-mg/mL, 2.5-mg/mL, 5-mg/mL, 10-mg/mL, 20-mg/mL, and 40-mg/mL EOs in the presence of LPS (100 ng/mL). A low oil concentration with 100-ng/mL LPS incubation for 24 hours showed no effects on cell viability. By contrast, 40-mg/mL EO significantly decreased the cell viability to ~76.3% ($p < 0.001$).

### 3.3. Effects of EOs on LPS-induced inflammatory cytokines

Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, and the anti-inflammatory cytokine IL-10 play important roles in the inflammatory process. The treatment of RAW 264.7 cells with LPS alone resulted in an increased release of pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-10) compared with that in nonactivated controls (Figure 2).

**Table 1 – Chemical components of essential oils obtained by hydrodistillation of *Trachydium roylei*.**

| Peak no. | RI[a] | Components | %RA[b] | Identification methods | Peak no. | RI[a] | Components | %RA[b] | Identification methods |
|---|---|---|---|---|---|---|---|---|---|
| Monoterpene hydrocarbons | | | 30.38 | | 30 | 1598 | Guaiol | 0.21 | MS, RI |
| 1 | 936 | α-Pinene | 1.21 | MS, RI | 31 | 1605 | Cedrol | 5.26 | MS, RI, Co |
| 2 | 966 | β-Thujene | 0.26 | MS, RI | 32 | 1621 | Fonenol | 0.21 | MS, RI |
| 3 | 978 | Pinene | 0.15 | MS, RI | 33 | 1630 | α-Acorenol | 3.13 | MS, RI |
| 4 | 986 | β-Myrcene | 1.13 | MS, RI | 34 | 1639 | Isospathulenol | 0.22 | MS, RI |
| 5 | 1008 | a-Phellandrene | 0.48 | MS, RI | 35 | 1652 | Bisabolol oxide | 0.68 | MS, RI |
| 6 | 1024 | β-Phellandrene | 22.95 | MS, RI, Co | Aromatic compounds | | | 41.53 | |
| 7 | 1040 | (E)-β-Ocimene | 0.33 | MS, RI | 36 | 1196 | Estragole | 0.21 | MS, RI |
| 8 | 1045 | (Z)-β-Ocimene | 1.05 | MS, RI | 37 | 1323 | Mesitaldehyde | 0.29 | MS, RI |
| 9 | 1059 | γ-Terpinene | 0.52 | MS, RI | 38 | 1410 | Methyleugenol | 2.01 | MS, RI |
| 10 | 1083 | Terpinolene | 2.08 | MS, RI | 39 | 1518 | Myristicin | 25.35 | MS, RI, Co |
| 11 | 1111 | 1,3,8-p-Menthatriene | 0.22 | MS, RI | 40 | 1521 | Calamenene | 0.34 | MS, RI |
| Oxygenated monoterpenes | | | 2.22 | | 41 | 1556 | Elemicine | 7.69 | MS, RI, Co |
| 12 | 1140 | E-Pinocarveol | 0.31 | MS, RI | 42 | 1657 | Isoelemicin | 5.48 | MS, RI, Co |
| 13 | 1160 | Isoborneol | 0.24 | MS, RI | 43 | 1682 | Apiol | 0.16 | MS, RI |
| 14 | 1178 | Terpinene-4-ol | 0.2 | MS, RI | Others | | | 3.54 | |
| 15 | 1187 | Crypton | 1.13 | MS, RI | 44 | 855 | Isononane | 0.16 | MS, RI |
| 16 | 1190 | α-Terpineol | 0.16 | MS, RI | 45 | 901 | Isobutyl isobutyrate | 0.22 | MS, RI |
| 17 | 1273 | Phellandral | 0.18 | MS, RI | 46 | 1006 | Isovaleric acid, isobutyl ester | 0.18 | MS, RI |
| Sesquiterpene hydrocarbons | | | 6.51 | | 47 | 1012 | 3-Carene | 0.22 | MS, RI |
| 18 | 1372 | α-Copaene | 0.33 | MS, RI | 48 | 1014 | Isobutyric acid, isopentyl ester | 0.24 | MS, RI |
| 19 | 1394 | (–)-β-Elemene | 0.37 | MS, RI | 49 | 1016 | Isobutyric acid, 2-methylbutyl ester | 0.18 | MS, RI |
| 20 | 1417 | Caryophyllene | 1.38 | MS, RI | 50 | 1018 | o-Cymene | 0.24 | MS, RI |
| 21 | 1456 | Humulene | 1.19 | MS, RI | 51 | 1043 | Benzeneacetaldehyde | 0.34 | MS, RI |
| 22 | 1480 | Germacrene D | 0.54 | MS, RI | 52 | 1101 | Isopentyl 2-methylbutanoate | 0.15 | MS, RI |
| 23 | 1503 | Bicyclogermacrene | 2.51 | MS, RI | 53 | 1103 | n-Amyl isovalerate | 0.13 | MS, RI |
| 24 | 1516 | α-Muurolene | 0.19 | MS, RI | 54 | 1105 | Isoamyl valerianate | 0.27 | MS, RI |
| Oxygenated sesquiterpenes | | | 14.69 | | 55 | 1134 | Cosmene | 0.22 | MS, RI |
| 25 | 1562 | (trans)-Nerolidol | 1.17 | MS, RI | 56 | 1230 | Cumaldehyde | 0.27 | MS, RI |
| 26 | 1566 | Spathulenol | 0.74 | MS, RI | 57 | 1242 | trans-Chrysanthenyl acetate | 0.23 | MS, RI |
| 27 | 1572 | (–)-Spathulenol | 0.79 | MS, RI | 58 | 1282 | L-bornyl acetate | 0.24 | MS, RI |
| 28 | 1578 | Caryophyllene oxide | 2.09 | MS, RI | 59 | 1357 | Citronellol acetate | 0.25 | MS, RI |
| 29 | 1594 | Isoaromadendrene epoxide | 0.19 | MS, RI | Total identified (%) | | | 98.87 | |

Co = coinjection with authentic compound; MS = mass spectrum; RI = retention index.

[a] Retention index relative to n-alkanes on HP-5 MS capillary column.

[b] Relative area (peak area relative to the total peak area).

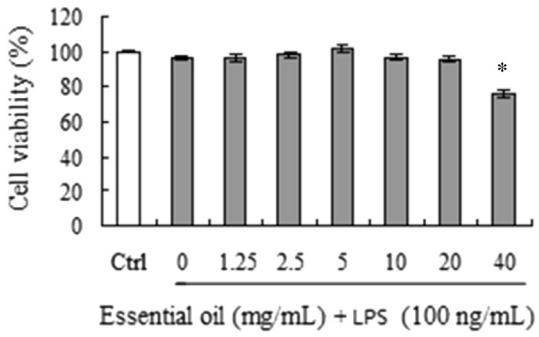


**Figure 1 – Growth inhibitory effect of *Trachydium roylei* essential oil in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Values are expressed as mean ± standard deviation ($n = 3$). * $p < 0.001$ compared with the LPS-only treatment group. Ctrl = control.**

The increased levels of TNF-α (Figure 2A), IL-1β (Figure 2B), and IL-6 (Figure 2C) in RAW 264.7 cells by LPS stimulation remarkably decreased in a dose-dependent manner after the cells were exposed to EOs ($p < 0.05$). By contrast, the anti-inflammatory cytokine IL-10 level significantly increased in a dose-dependent manner after the cells were exposed to the EOs ($p < 0.05$; Figure 2D).

### 3.4. *Effects of EOs on LPS-induced production of NO and PGE2*

The production of NO and PGE2 was examined in RAW 264.7 cells stimulated with LPS for 24 hours in the presence or absence of EOs. The production of NO, which was measured as nitrite, was increased by LPS treatment. By contrast, EOs (2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) significantly reduced the NO levels in LPS-stimulated cells in a dose-dependent manner ($p < 0.05$; Figure 3A). The increased PGE2 production by LPS in RAW 264.7 cells was also significantly suppressed by the EOs (2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL; Figure 3B).

### 3.5. *Effects of EOs on LPS-induced protein expression of iNOS and COX-2*

To investigate whether the inhibitory effects of EOs on the production of NO and PGE2 were mediated by inhibition of gene expression, we evaluated iNOS and COX-2 using Western blot analysis. Macrophages were treated with EOs (2.5 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) to examine the protein expression of inflammation-associated molecules triggered by LPS (Figure 4). In these experiments, LPS-activated macrophages increased the protein expression levels of COX-2 and iNOS compared with those in the untreated control group. By contrast, EO treatment downregulated the expression of these LPS-stimulated proteins in a concentration-dependent manner ($p < 0.05$).

## 4. Discussion

As natural volatile substances from plants, EOs may represent an alternative source of anti-inflammatory agents because they are not usually extracted as chemically pure substances,

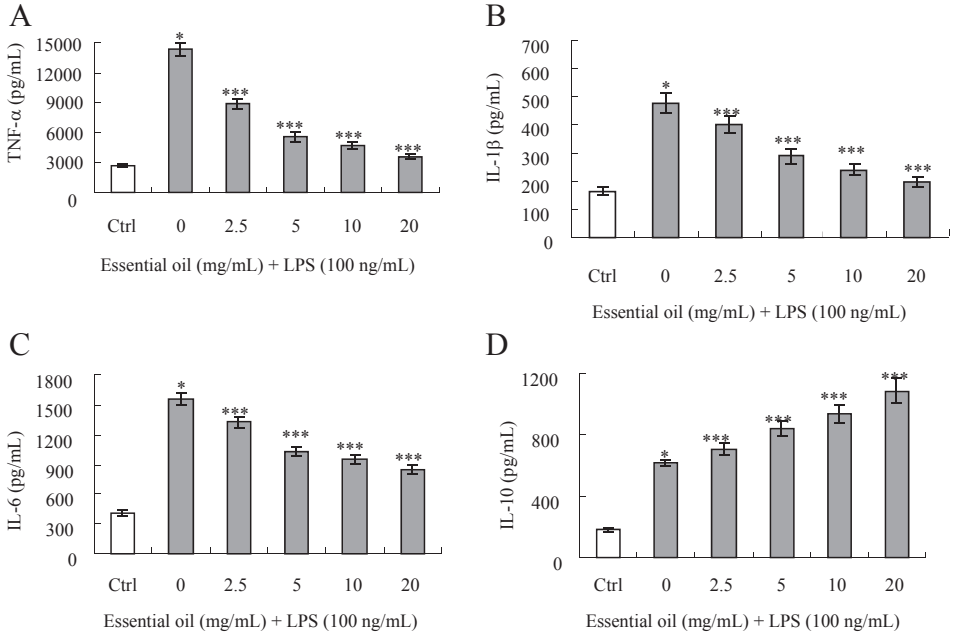


**Figure 2 – (A) Effects of *Trachydium roylei* essential oil on lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α production of RAW 264.7 macrophages; (B) effects of *T. roylei* essential oil on LPS-induced interleukin (IL)-1β production of RAW 264.7 macrophages; (C) effects of *T. roylei* essential oil on LPS-induced IL-6 production of RAW 264.7 macrophages; and (D) effects of *T. roylei* essential oil on LPS-induced IL-10 production of RAW 264.7 macrophages. Values are expressed as mean ± standard deviation ($n = 3$). * Indicates $p < 0.001$ compared with the control group. ** $p < 0.01$ compared with the LPS-only treatment group. *** $p < 0.001$ compared with the LPS-only treatment group.**

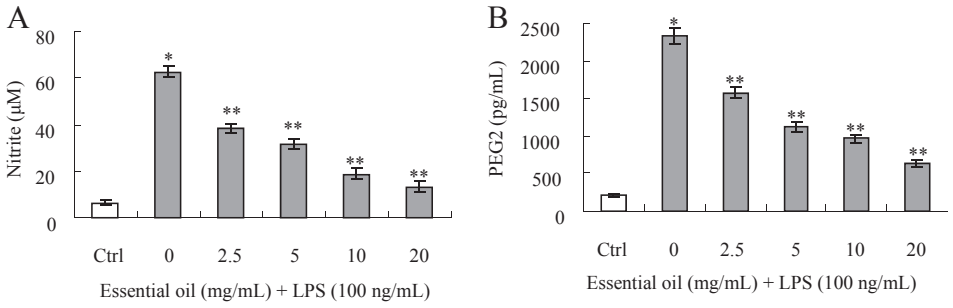


**Figure 3 – (A) Effects of *Trachydium roylei* essential oil on lipopolysaccharide (LPS)-induced nitric oxide production of RAW 264.7 macrophages; and (B) effects of *T. roylei* essential oil on LPS-induced prostaglandin E2 (PGE2) production of RAW 264.7 macrophages. Values are expressed as mean ± SD ($n = 3$). * Indicates $p < 0.001$ compared with the control group. ** Indicates $p < 0.001$ compared with the LPS-only treatment group. Ctrl = control.**

but these oils consist of mixtures containing many bioactive compounds that are biodegradable into nontoxic products and potentially suitable for use in integrated management programs [12]. In the present study, the chemical composition of *T. roylei* EO and its anti-inflammatory effects on LPS-stimulated RAW 264.7 macrophage cells were investigated. The results of phytochemical analysis demonstrated that *T. roylei* EO is predominantly composed of aromatic compounds and monoterpene hydrocarbons, and the major chemical compounds detected were myristicin and β-phellandrene (Table 1). To our knowledge, this study is the first to assess the chemical composition and bioactivity of the genus *Trachydium*.

Inflammation is a bodily response to harmful stimuli such as injury and infection [13]. Various inflammatory models allow evaluation of test compounds and provide further understanding about the inflammatory process. Macrophages are essential for innate immunity and play an important role in both host defense mechanism and inflammation [14]. Pathogenic bacteria and other infectious agents can stimulate macrophages directly, thereby initiating the release of proinflammatory mediators, such as NO, PGE2, TNF-α, and IL-1β, to sustain inflammation and the immunological response [15,16]. However, overproduction of these mediators can harm tissues and organisms and has been associated with the pathogenesis of various inflammatory-related diseases, such as rheumatoid arthritis, diabetes, inflammatory bowel disease, atherosclerosis, and cancer [17]. In many studies, anti-inflammatory compounds have been investigated for their potential inhibitory effects *in vitro* using LPS-stimulated RAW 264.7 macrophages. LPS, a component of the outer membrane of gram-negative bacteria, can activate murine macrophages [18] and induce an oversecretion of various inflammatory and toxicity-mediating molecules, such as TNF-α, IL-6, eicosanoids, and NO [19]. Therefore, this cell system is an excellent model for drug screening and subsequent evaluation of potential inhibitors against iNOS and NO production [20].

To further understand the molecular mechanisms of the anti-inflammatory activity of *T. roylei* EO, we investigated the effects of such EOs on the secretion of NO, PGE2, TNF-α, IL-1β, IL-6, and IL-10, as well as on the expression of iNOS and COX-2 in LPS-induced RAW 264.7 macrophages. Furthermore, these oils were found to be safe for mammalian macrophages at concentrations ranging from 1.25 mg/mL to 20 mg/mL.

To analyze the anti-inflammatory mechanism mediated by *T. roylei* EO, we investigated the effects of these EOs on LPS-

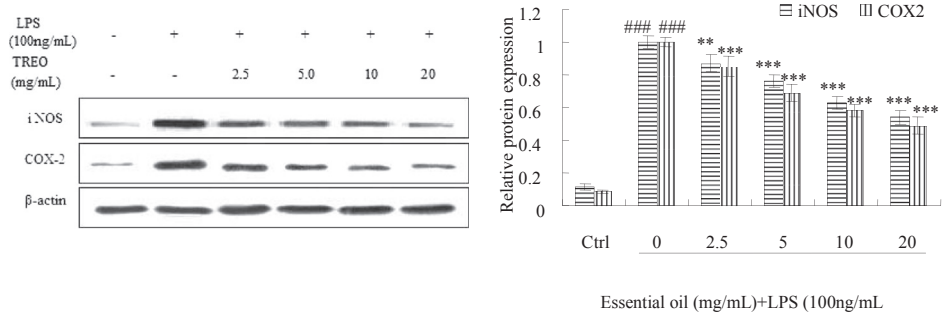


**Figure 4 – Inhibitory effect of *T. roylei* essential oil on protein expression of iNOS and COX-2 in LPS-stimulated RAW 264.7 cells. The results presented are representative of three independent experiments. Values are expressed as mean ± SD ($n = 3$). ### indicates $p < 0.001$ compared with the control group. **, and *** indicate $p < 0.01$, and $p < 0.001$, respectively, compared with the LPS-only treatment group. TREO=essential oil from Trachydium roylei; Ctrl=control.**

induced cytokine production, including proinflammatory cytokines, such as IL-1β and IL-6, as well as TNF-α and the anti-inflammatory cytokine IL-10, which are regarded as crucial anti-inflammatory targets [21]. Proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are produced primarily by activated monocytes or macrophages. Moreover, TNF-α and IL-1β can induce and/or enhance PGE2 production in macrophages [22–24]. IL-6 is characterized as an inflammatory factor because it synergistically augments the inflammatory actions of IL-1 in human synovial cells [25]. The treatment of RAW 264.7 cells with LPS alone resulted in a significant increase in proinflammatory cytokine production compared with that in the control group ($p < 0.001$). By contrast, *T. roylei* EO significantly reduced these cytokine levels ($p < 0.001$; Figures 2A–C). IL-10 is generally considered as an anti-inflammatory and immunosuppressive cytokine, and its inhibitory effect on the production of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, has been demonstrated in numerous studies [26–28]. In the current study, EOs from *T. roylei* significantly increased the levels of anti-inflammatory cytokine IL-10 (Figure 2D). Therefore, the regulation of cytokines observed in this study may reflect one of the mechanisms underlying the anti-inflammatory effect of *T. roylei* EO.

Inducible COX-2 is responsible for the high prostaglandin levels observed in several inflammatory pathologies. Similarly, iNOS produces large amounts of NO and is thought to play a central role in inflammatory diseases [29,30]. Numerous studies have reported that NO and PGE2 participate in inflammatory and nociceptive events [31]. Additionally, our results indicated that *T. roylei* EO effectively inhibited the secretion of NO and PGE2 in LPS-stimulated macrophages (Figure 3). Western blot analyses determined whether the inhibitory effects of the EOs on proinflammatory mediators (NO and PGE2) were related to the modulation of iNOS and COX-2 expression levels (Figure 4). In addition, according to the previous literature, the anti-inflammatory mechanisms of some components found in the oil, such as myristicin, have been studied. Myristicin has anti-inflammatory properties related with its inhibition of NO, cytokines, chemokines, and growth factors in double stranded RNA-stimulated macrophages via the calcium pathway [32].

## 5. Conclusion

In conclusion, our study is a pioneer report about the chemical composition and bioactivities of *T. roylei* EOs. This study showed that the anti-inflammatory activity of *T. roylei* EOs depends on its ability to regulate the expression of NO, PGE2, TNF-α, IL-1β, IL-6, and IL-10. Overall, this study suggests *T. roylei* EO as an effective agent to treat inflammatory diseases.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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