

Chemical Profiles and Anti-inflammatory Activity of the Essential Oils from *Seseli gummiferum* and *Seseli corymbosum* subsp. *corymbosum*

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The anti-inflammatory activity of the essential oils from *Seseli corymbosum* subsp. *corymbosum* Pall. ex Sm. (SC) and *Seseli gummiferum* Boiss. & Heldr. subsp. *corymbosum* (SG) was investigated for the first time on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The main constituents (determined by GC-FID and GC-MS analyses) were germacrene D (54.1%) and sabinene (22.4%) in SG oil and β -phellandrene (29.2%), α -phellandrene (8.2%) and germacrene D (2.5%) in SC oil. SC and SG oils inhibited nitric oxide (NO) production with IC₅₀ values of 56.1 and 108.2 μ g/mL, respectively. The oils also inhibited prostaglandin E₂ (PGE₂) with IC₅₀ values of 49.4 μ g/mL (SC oil) and 95.5 μ g/mL (SG oil). The inhibitory effect of SC and SG oils was accompanied by dose-dependent decreases of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expressions in LPS-induced RAW 264.7 cells. The research of the reporter gene assay on nuclear factor κ B (NF- κ B) showed that SC and SG oils inhibited NF- κ B transcriptional activity. The obtained results suggest that SC and SG oils exert the anti-inflammatory effects in LPS-stimulated RAW 264.7 cells by suppressing NF- κ B activation.

Keywords: Apiaceae, *Seseli*, Essential oil, COX-2, iNOS, NF- κ B.

Apiaceae (Umbelliferae) family is represented by almost 455 genera and about 3750 species [1]. *Seseli* belongs to Apiaceae genus that is represented by 12 taxa (11 species and 1 subspecies) in the flora of Turkey [2]. Several *Seseli* species exhibit various healing effects. The roots of *Seseli mairei* Wolff. are used as the herbal remedy for human inflammation, swelling, rheumatism, pain and common cold [3a]. The seeds of *Seseli indicum* possess anthelmintic, carminative, stomachic and stimulant properties [3b]. The fruits of *Seseli tortuosum* are used as emmenagogue and anti-flatulence agents [3c]. The leaves of *Seseli libanotis* are consumed as vegetable in the eastern part of Anatolia [3d]. The studies on phytochemical composition and biological activity are mainly focused on *Seseli* species from Turkey including our recent research on *Seseli* coumarins, essential oils and their biological activity [4]. As part of our ongoing investigations on Turkish *Seseli* species, we undertook the present study in order to elucidate the anti-inflammatory potential of the EOs of two *Seseli* species. The focus was on *Seseli gummiferum* and *Seseli corymbosum* subsp. *corymbosum* that were subspecies in the past, but now they are identified as different species of *Seseli* [5]. For this purpose, the fruit EOs of *S. gummiferum* and *S. corymbosum* subsp. *corymbosum* were isolated and analyzed by GC-FID and GC-MS to identify their main components.

The fruit EOs of *S. gummiferum* (SG) and *S. corymbosum* subsp. *corymbosum* (SC) were isolated by hydrodistillation and analyzed (Table 1). In total, forty-four compounds were characterized, representing 95.1% (SG oil) and 93.7% (SC oil) of total chromatogram areas. The main volatile constituents were germacrene D (54.1%) and sabinene (22.4%) in SG oil as well as β -phellandrene (29.2%), α -phellandrene (8.2%) and germacrene D

(2.5%) in SC oil. Germacrene D is sesquiterpene and its biological function in plants is still not well understood, but could play a role as a precursor of various sesquiterpenes such as cadinenes and selinenes. Phellandrene isomers and sabinene are cyclic monoterpenes.

Isolated EOs were evaluated for their possible anti-inflammatory potential on the inhibitory effect of nitric oxide (NO) and prostaglandin E₂ (PGE₂) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. When lipopolysaccharide (LPS) stimulates macrophages, activated macrophages produce various pro-inflammatory mediators (such as nitric oxide (NO), prostaglandin E₂ (PGE₂) and pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin-1 β) to promote inflammatory responses. Inducible NO synthase (iNOS) produces NO during inflammation and COX-2 is responsible for the synthesis of PGE₂ in inflammatory cells [5a]. NO and PGE₂ play critical roles in the process of macrophage activation and are associated with both acute and chronic inflammation [5b,5c].

Thus, the suppression of NO and PGE₂ production (by inhibiting iNOS and COX-2 protein expression) could be an important therapeutic approach in the development of anti-inflammatory agents [5d]. Therefore, the amount of NO and PGE₂ induced by iNOS and COX-2 may reflect the degree of inflammation and may provide information to investigate the effect of pharmacological agents on the inflammatory process [5e]. It has been well established that LPS induced iNOS and COX-2 expression in macrophages is regulated by transcription factors such as nuclear factor κ B (NF- κ B) [5f].

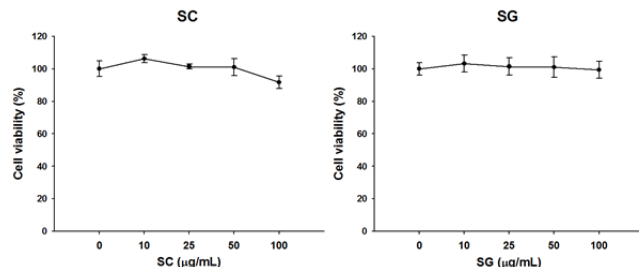
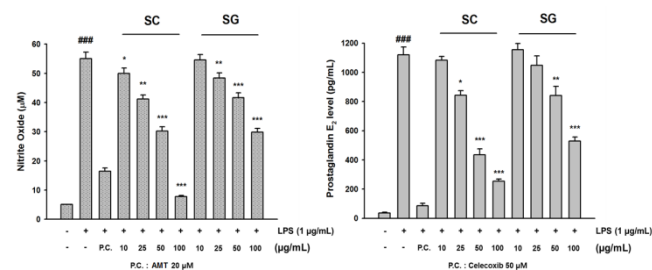
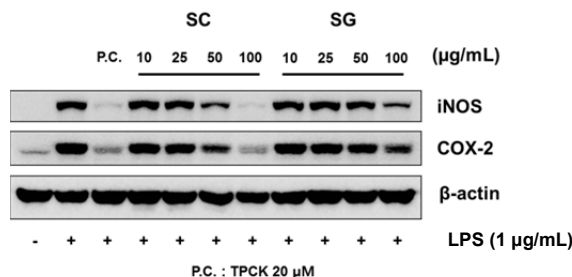
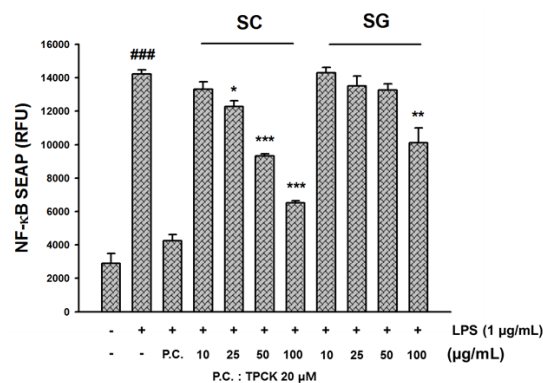
Table 1: The chemical composition of *S. gummiferum* (SG) and *S. corymbosum* (SC) oils identified by GC-FID and GC-MS analyses.

No.	Compound	RI	SG (%)	SC (%)	Identification method
1	Nonane	900	0.1	-	RI, MS, Co
2	α -Thujene	933	0.3	0.2	RI, MS, Co
3	α -Pinene	941	0.8	9.7	RI, MS, Co
4	Camphene	958	0.1	1.1	RI, MS, Co
5	Sabinene	982	22.4	4.1	RI, MS, Co
6	β -Pinene	984	1.8	4.8	RI, MS, Co
7	Myrcene	994	1.6	4.7	RI, MS
8	Isobutyl 2-methylbutyrate	1003	-	0.1	RI, MS
9	α -Phellandrene	1008	0.3	8.2	RI, MS, Co
10	α -Terpinene	1023	0.1	0.1	RI, MS, Co
11	<i>p</i> -Cymene	1032	0.3	2.6	RI, MS, Co
12	β -Phellandrene	1037	-	29.2	RI, MS
13	<i>trans</i> - β -Ocimene	1044	1.7	0.1	RI, MS
14	γ -Terpinene	1066	0.2	1.8	RI, MS, Co
15	α -Terpinolene	1093	-	0.2	RI, MS, Co
16	Fenchone	1094	0.1	-	RI, MS, Co
17	Undecane	1100	0.1	0.1	RI, MS, Co
18	Linalool	1107	0.1	0.4	RI, MS, Co
19	Terpinene-4-ol	1186	0.2	0.1	RI, MS, Co
20	Cryptone (4-isopropyl-cyclohex-2-en-1-one)	1192	-	0.2	RI, MS
21	α -Phellandrene epoxide	1209	-	0.1	RI, MS
22	5-Hydroxymethylfurfural	1259	-	0.2	RI, MS, Co
23	α -Copaene	1382	1.0	-	RI, MS, Co
24	β -Bourbonene	1389	0.2	-	RI, MS
25	β -Cubebene	1392	0.6	-	RI, MS
26	β -Elemene	1397	0.3	0.4	RI, MS, Co
27	β -Caryophyllene	1425	1.2	0.2	RI, MS, Co
28	γ -Elemene	1437	-	0.5	RI, MS
29	α -Humulene	1460	0.3	0.1	RI, MS, Co
30	<i>trans</i> - β -Farnesene	1464	-	1.1	RI, MS, Co
31	Germacrene D	1490	54.1	2.5	RI, MS
32	Bicyclogermacrene	1499	5.0	6.6	RI, MS
33	Pentadecane	1500	0.3	-	RI, MS, Co
34	Germacrene A	1508	0.4	0.4	RI, MS
35	β -Bisabolene	1512	-	0.2	RI, MS
36	δ -Cadinene	1531	0.4	-	RI, MS, Co
37	Germacrene B	1561	-	7.4	RI, MS
38	Spathulenol	1586	0.8	2.2	RI, MS
39	Carotol	1601	-	3.5	RI, MS
40	Isospathulenol	1650	-	0.2	RI, MS
41	T-Muurolol	1667	0.3	-	RI, MS
42	Heptadecane	1700	-	0.2	RI, MS, Co
43	Octadecane	1800	-	0.1	RI, MS, Co
44	Nonadecane	1900	-	0.1	RI, MS, Co
Total identified			95.1%	93.7%	

RI - relative retention indices on HP-5MS calculated against *n*-alkanes; % - area percentages calculated from GC-FID data; MS - mass spectra; Co - compound identified also by co-injection of pure and authentic standard.

The effects of SC and SG oils on NO and PGE2 production in LPS-stimulated RAW 264.7 cells: To investigate whether SC or SG oils exhibit any anti-inflammatory potential, their inhibitory effects on the production of NO and PGE2 in LPS-stimulated RAW 264.7 cells were measured. The inhibitory effect was achieved with non-cytotoxic concentrations (10-100 μ g/mL) of SC and SG oils (Figure 1). The cells were pretreated for 2 h with various concentrations of SC and SG oils followed by treatment with LPS (1 μ g/mL) for 18 h. Whereas LPS treatment dramatically increased the concentration of NO and PGE2, SC and SG oils significantly inhibited the LPS-stimulated production of NO and PGE2 in a dose-dependent manner (Figure 2). Specifically, SC oil was more effective than SG oil for inhibiting NO production and PGE2 in LPS-stimulated RAW 264.7 cells at the same concentrations (10-100 μ g/mL). These results lead us to investigate the inhibitory effects of SC and SG oils on the expression of iNOS and COX-2 enzymes which produce NO and PGE2 as key mediators of inflammation.

The effects of SC and SG oils on NF- κ B transcriptional activity in LPS-stimulated RAW 264.7 cells: NF- κ B activation is a crucial event in the activation of several inflammatory genes including iNOS and COX-2 in RAW 264.7 cells. Therefore, the effects of SC and SG oils on the LPS-stimulated activation of NF- κ B in RAW 264.7 cells were investigated. NF- κ B was detected by measuring

**Figure 1:** The effects of cell viability on *Seseli corymbosum* subsp. *corymbosum* (SC) and *Seseli gummiferum* (SG) oils in RAW 264.7 cells.**Figure 2:** The inhibitory effects of nitric oxide and prostaglandin E2 on SG and SC oils in LPS-induced RAW 264.7 cells. AMT (Anti-Aminomethyltransferase) or celecoxib was used as positive control. The values are expressed as the means $\pm$ SD from three individual experiments. * p <0.05, ** p <0.01, and *** p <0.001 indicate significance compared to LPS-treated group. (+) or (-) indicates the sample treatment.**Figure 3:** The inhibitory effects of iNOS and COX-2 protein expression on SC and SG oils in LPS induced RAW 264.7 cells. *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as positive control. (+) or (-) indicates the sample treatment.**Figure 4:** The inhibitory effects of NF- κ B transcriptional activity with SC and SG oils in LPS-induced RAW 264.7 cells. TPCK was used as a positive control. The values are expressed as the means $\pm$ SD from three individual experiments. * p <0.05, ** p <0.01, and *** p <0.001 indicate significance compared to the LPS-treated group. (+) or (-) indicates the sample treatment.

NF- κ B-dependent transcription in macrophages that were stably transfected with a reporter construct. The cells incubated with LPS showed remarkable increase of NF- κ B activity, whereas the activity was significantly inhibited by SC and SG oils in a dose-dependent manner (Figure 4).

To the best of our knowledge, this is the first report on the SC and SG EOs. The chemical composition of the oils showed similarity with other EOs of *Seseli* species from Turkey [4a,6]. For example, bicyclogermacrene (11.92%) and germacrene B (14.01%) in *S. gummiferum* subsp. *corymbosum*, and spathulenol (19.91%) in *S. gummiferum* subsp. *gummiferum* were the main constituents. In general, it is supposed that the main EO components can cause the pharmacological action. For example, bicyclogermacrene exhibited anti-inflammatory and antibacterial activities [7]. However, it should be considered that complex harmonization of terpenoids and aromatic components in the EOs can lead to synergism for emerging a biological effect [4g] in accordance with the review on antioxidant and anti-inflammatory activities of the EOs [8].

In conclusion, the EOs of two *Seseli* species significantly inhibit the expression of iNOS and COX-2 enzymes which produce NO and PGE₂ as key mediators of inflammation in LPS-stimulated RAW 264.7 cells and thus could be considered as candidate agents against inflammatory disorders. However, further investigation will be required to clarify detail mechanism of the EOs activity.

Experimental

Plant materials: *Seseli gummiferum* Pall. ex Sm. (SG) from Ankara (at 1800 m) and *Seseli corymbosum* Boiss. & Heldr. subsp. *corymbosum* (SC) from Taurus Mountains in South of Turkey (at 1650-1900 m) were collected. The voucher specimens were authenticated by Prof. H. Duman from Gazi University, Department of Biology and are deposited in Herbarium of Ankara University, Faculty of Pharmacy (AEF No: 26264, AEF No: 26262).

Essential oil distillation: Crushed fruits of both species were subjected to hydrodistillation for 3 h in a Clevenger type apparatus. The yield on a dry weight basis (v/w) of SC oil was 3.3% (from 30 g of the plant) and of SG oil was 3.2% (from 30 g of the plant).

Identification of the essential oil components: The EOs analysis was performed by GC-FID and GC-MS as described previously [9]. Quantitative results are mean data derived from triplicate GC-FID analyses. The individual peaks were identified by comparison of their retention indices (relative to C₉-C₂₅ *n*-alkanes for HP-5MS column) with those of available authentic samples and with the literature [10], as well as by comparing their mass spectra with Wiley 275 MS library (Wiley, New York, USA) and NIST98 library (Gaithersburg, Germany). The standards from Table 1 were purchased from Sigma-Aldrich (Biovit d.o.o., Zagreb, Croatia).

Cell culture: The RAW 264.7 cell line (derived from murine macrophages) was obtained from American Type Culture Collection (Manassas, VA, USA). These cells were maintained in Dulbecco's modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a 5% CO₂ incubator. The RAW 264.7 cells harboring pNF-κB secretory alkaline phosphatase (SEAP)-NPT reporter construct were cultured under the same conditions, but the culture medium was supplemented with geneticin (500 µg/mL) [11].

Viability assay: The cell viability was evaluated using the tetrazolium salt reduction (MTT) assay. RAW 264.7 cells were seeded into 96-well plates (1 × 10⁴ cells per well) and stabilized at 37°C for 24 h. The SG and SC oils, at different concentrations (10, 25, 50, and 100 µg/mL), were added to each well and then the cells were incubated for 24 h. The MTT solution (0.5 mg/mL) was added to each well and the cells were incubated for 3 h. The formazan crystals were dissolved in 100 µL of DMSO. The cell viability was

assessed by measuring the absorbance at 540 nm using an Emax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of nitric oxide (NO): The nitrite concentration in the culture medium was measured by Griess reaction test as an indicator of NO production. Briefly, RAW 264.7 cells were pretreated for 2 h with various concentrations of the EOs or AMT (Anti-Aminomethyltransferase) as positive control and then stimulated for 18 h with LPS (1 µg/mL). Then, 100 µL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) was mixed with 100 µL of the cell supernatant and the optical density at 540 nm was measured using an Emax Microplate Reader. The nitrite concentration was calculated from the standard curve generated by measuring known concentrations of sodium nitrite dissolved in the medium.

Measurement of prostaglandin E₂ (PGE₂): The PGE₂ produced from activated macrophages was quantified using an enzyme immunoassay (EIA) kit for PGE₂ (Cayman Chemical, Ann Arbor, MI, USA) in accordance with the manufacturer's instructions. Briefly, RAW 264.7 cells were pre-treated with several concentrations of the essential oils for 2 h and then activated by exposure to 1 µg/mL LPS for an additional 18 h to induce the expression of cyclooxygenase-2 (COX-2). The cell culture medium was collected and diluted 2 times with PBS, transferred to 96-well culture plate from the EIA kit that was coated with PGE₂ antibody and treated in accordance with the manufacturer's instructions. The PGE₂ produced in each sample was quantified using a standard curve. For comparison, 50 µM celecoxib (Pfizer, New York, NY, USA) was used as positive control.

Western blot analysis: RAW 264.7 cells were pretreated with the essential oils for 2 h and stimulated for 18 h with LPS (1 µg/mL). *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as positive control. After incubation, total protein extracts were lysed by 30 min incubation on ice in lysis buffer (20 mM HEPES [pH 7.6], 350 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1% NP-40, 50 mM NaF, 0.1 mM DTT, 0.1 mM PMSF and a protease inhibitor cocktail). The protein concentration was estimated using a Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Equal amounts of protein were loaded on 8% SDS-polyacrylamide gels and semi-dry transferred to nitrocellulose membranes. After the membranes were blocked by a 5% skim milk solution, the membranes were incubated overnight at 4 °C with the primary antibodies against iNOS, COX-2, and β-actin. The membranes were washed and incubated at room temperature for 1 h with the secondary antibodies which were conjugated with horseradish peroxidase (HRP). Finally, the blots were visualized using a chemiluminescence kit (Abfrontier, Seoul, Korea).

Nuclear factor-κB (NF-κB) SEAP reporter gene assay: The pNF-κB-SEAP-NPT plasmid activates the secretory alkaline phosphatase (SEAP) reporter gene in response to NF-κB activity and also encodes the neomycin phosphotransferase (NPT) gene for geneticin resistance in host cells. This plasmid was constructed and transfected into RAW 264.7 cells. Transfected RAW 264.7 cells were pretreated with the essential oils for 2 h before stimulation with LPS (1 µg/mL) for 16 h. 4-Methylumbelliferyl phosphate (4-MUP), which is a fluorescent SEAP substrate, was used to measure relative fluorescence units (RFU) by a 96-well plate fluorometer (Gemini XS, Molecular Devices; Sunnyvale, CA, USA) with the excitation wavelength of 360 nm and the emission wavelength of 449 nm. TPCK, an inhibitor of NF-κB, was used as positive control.

Statistical analysis of the assays: All data are presented as the means $\pm$ standard deviations (SD) from three different experiments. Statistical significance of the data was evaluated using one-way analysis of variance (ANOVA) followed by a Student's *t*-test, and the value of $p < 0.05$ was considered to be statistically significant.

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