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Identification of 1,8-Cineole, Borneol, Camphor, and Thujone as Anti-inflammatory Compounds in a *Salvia officinalis* L. Infusion Using Human Gingival Fibroblasts

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ABSTRACT: Drinking or gargling *Salvia officinalis* L. infusion (sage infusion) is thought to soothe a sore throat, tonsillitis, and inflamed, red gums, although structure-based scientific evidence for the key anti-inflammatory compounds in sage infusion is scarce. Human gingival fibroblasts (HGF-1) were treated with sage infusion (SI) or SI fractions containing either its volatile components and water (aqueous distillate, AD) or its dry matter (DM) for six hours. SI, AD, and DM reduced a mean phorbol-12-myristate-13-acetate/ionomycin (PMA/I)-stimulated release of the pro-inflammatory interleukins IL-6 and IL-8 by more than 50% ($p < 0.05$). Cellular uptake experiments and subsequent GC-MS analysis using stable-isotope-labeled internal standards revealed the presence of 1,8-cineole, borneol, camphor, and α-/β-thujone in SI-treated cells; LC-MS analysis demonstrated the presence of rosmarinic acid. A significant, more than 50% mean inhibition of PMA/I-induced IL-6 and IL-8 release was demonstrated for the volatile compounds 1,8-cineole, borneol, camphor, and thujone, but not for the nonvolatile rosmarinic acid when applied in concentrations representative of sage infusion. Therefore, the volatile compounds were found to be more effective than rosmarinic acid. 1,8-Cineole, borneol, camphor, and α-/β-thujone chiefly contribute to the anti-inflammatory activity of sage infusion in human gingival fibroblasts.

KEYWORDS: *HGF-1 cells, 1,8-cineole, interleukins, rosmarinic acid, Salvia officinalis L., terpenes*

INTRODUCTION

The herbal species *Salvia officinalis* L. (Lamiaceae) is known for its anti-inflammatory properties, in particular when applied locally to treat ailments of the oral cavity and the throat. Aqueous *Salvia officinalis* L. leaf extract, sage infusion, is recommended by the European pharmacopoeia as an oral rinse to relieve inflammation and pain in diseased gingival tissues.[1] The overall anti-inflammatory principle in *S. officinalis* L. is hypothesized to be ursolic acid.[2,3] Besides the nonvolatile compound ursolic acid, other components present in the essential oil, such as α-humulene, β-caryophyllene, and 1,8-cineole,[4,5] have been demonstrated to possess a potent anti-inflammatory activity by means of *in vitro* experiments and *in vivo* studies.[6−8]

However, no systematic study has been reported to identify the most potent anti-inflammatory compound in *S. officinalis* L. infusion with respect to pro-inflammatory marker modulation in human gingival tissues as an experimental model of periodontal disease.

Periodontal disorders are the most common cause of tooth loss in adults. Thus, inflammation of gingival tissues is recognized as a major public health problem worldwide.[9,10] The term periodontal disease describes several pathological conditions, which affect both the teeth and the supporting tissues around the teeth. Progression of inflammation is influenced by genetics, the presence of potentially pathogenic microorganisms, and lifestyle factors.[10,11] Virulence factors secreted by periodontal bacteria are the primary causes for inducing the host response, including the production of pro-inflammatory cytokines, that leads to the inflammation of the gingiva and the destruction of periodontal tissues.[12] Human gingival fibroblasts play an important role in modulating the host defense against periodontal bacteria[13,14] with the capacity to secrete pro-inflammatory mediators such as IL-1α, IL-1β, IL-6, IL-8, and TNF-α.[12,14−17]

The gingiva exposed to tisanes like *S. officinalis* L. infusion is the first tissue interacting with the undigested and non-metabolized aqueous extract of this dietary herb. Oral mucosal and gingival cells are, therefore, widely used for studying immune-modulatory activities of food extracts or components thereof.[18,19] Recent investigations of our group identified α-terpineol as a potent anti-inflammatory component of orange juice.[20,21] In those studies, α-terpineol reduced the release of IL-6 after treatment of the cells with the pro-inflammatory agents phorbol-12-myristate-13-acetate and ionomycin.

In the presented work, an activity-guided approach is applied to determine the impact of *Salvia officinalis* L. infusion and its components on the release of IL-6 and IL-8 from human gingival fibroblasts in order to identify the most active component.

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MATERIALS AND METHODS

Chemicals. Unless stated otherwise, cell culture materials, chemicals, and reagents were purchased from Sigma-Aldrich (Vienna, Austria).

Dried *Salvia officinalis* L. leaves were purchased from a local company (Waldland, Oberwaltenreith, Austria). The cultivar *Salvia officinalis* L. 'Extrakta' was grown in the area of Krems and Horn, Lower Austria, Austria. The package size was 250 g, and the plant material was stored under vacuum in the dark at room temperature.

Fractionation of Sage Infusion. The *S. officinalis* L. infusion was prepared with 1.5 g of dried leaves infused with hot water (95 °C; 150 mL).[22] The mixture was immediately sealed and kept in the dark for 10 min. The aqueous extract was then strained and cooled to 4 °C. The solvent-assisted flavor evaporation (SAFE) technique[23] was applied to separate the aqueous leaf extract (100 g) into the dry-matter fraction (DM), containing the nonvolatile sage infusion constituents, and the aqueous fraction (AD), containing the volatile components and water. The yield of both fractions was determined by weight and was 97.7 ± 2.88 g for the AD and 1.45 ± 2.19 g for the DM.

Identification of Volatile Compounds Present in the Aqueous Fraction by GC-MS. The AD was extracted three times with methyl *tert*-butyl ether (MTBE).[21] The organic layers were combined, dried over anhydrous sodium sulfate, and concentrated using a rotary evaporator (Büchi, Flawil, Switzerland) operated at 36 °C with a pressure of 50 kPa. The residue was filled up with MTBE to a final volume of 1.5 mL. GC-MS analyses were performed on an Agilent 6890N GC system equipped with a fused silica DB-5MS column, 30 m × 250 μm, film thickness 0.25 μm (Agilent Technologies, Vienna, Austria), connected to a 5973 mass selective detector (Agilent Technologies, Vienna, Austria) that was operated in electron impact (EI) mode at 70 eV. A sample volume of 1 μL was injected in the pulsed splitless mode at 50 °C column temperature. The oven was held at this temperature for 2 min, raised to 80 °C at a rate of 6 °C/min, heated at 0.5 °C/min to 84 °C, held at this temperature for 7 min, raised to 280 °C at 40 °C/min, and held for 3 min. Helium was used as a carrier gas at a constant flow rate of 1.1 mL/min. Individual compounds were identified based on their mass spectrometry fragmentation pattern using the ChemStation G1707 database (Agilent Technologies, Vienna, Austria) and after spiking the sample with commercially available reference standards.

Synthesis of Stable-Isotope-Labeled Internal Standards for Quantification of 1,8-Cineole, Borneol, Camphor, and α- and β-Thujone. For identification of reaction products, MS fragmentation patterns and NMR spectra were obtained. ^{1}H and ^{13}C one- and two-dimensional NMR spectra were recorded with a Bruker Avance III 500 MHz instrument at 500.32 (^{1}H) and 125.81 (^{13}C) MHz at ambient temperature. For all analyses, $(CD_3)_2CO$ with 0.03% TMS was used to dissolve the compound.

$[^2H_3]$-1,8-Cineole, $[^2H_1]$-isoborneol, and $[^2H_1]$-thujol were used as internal standards to quantify the respective volatile compounds in sage infusion (data not shown). In preceding GC-MS analyses, the absence of unlabeled isoborneol and thujol in sage infusion and cell extracts was confirmed (Figures 1−4). Therefore, no mass spectral overlap between the singly labeled internal standards and naturally occurring isotopologues of isoborneol and thujol could invalidate the quantitative results.

Synthesis of $[9\text{-}^2H_3]$-1,8-cineole was carried out as published previously.[24]

For the quantification of camphor and borneol, $[^2H_1]$-isoborneol was synthesized as internal standard according to the reaction scheme shown in Figure 2.[25] A total amount of 0.80 g of lithium aluminum deuteride (Carl Roth, Karlsruhe, Germany) was equilibrated with MTBE under a nitrogen atmosphere in a reflux condenser equipped with a calcium chloride tube. A total of 2.67 g of 1R-(+)-camphor (Alfa Aesar, Karlsruhe, Germany) was added. This reaction mixture was stirred at room temperature for 4 h to generate tetracamphyl-lithium aluminate. Hydrolysis was induced by addition of ice cold deuterated water (Roth) to yield $[^2H_1]$-isoborneol, $[^2H_1]$-borneol, aluminum hydroxide, and lithium hydroxide. This solution was then extracted with MTBE three times (3 × 10 mL). The combined organic phases were washed with saturated sodium chloride solution (3 × 10 mL), dried over Na_2SO_4, and concentrated using a rotary evaporator (36 °C, 219 hPa). The reaction product $[^2H_1]$-isoborneol was, with an amount of 95%, the most abundant among the reaction products.

$[^2H_1]$-Isoborneol: yield 97% (2.59 g; 16.8 mmol; white powder). The analyte was characterized by MS-EI (70 eV): *m/z* (%) 140 (10), 137 (15), 122 (17), 110 (19), 95 (100), 82 (10), 67 (10), 55 (9), 41 (10).

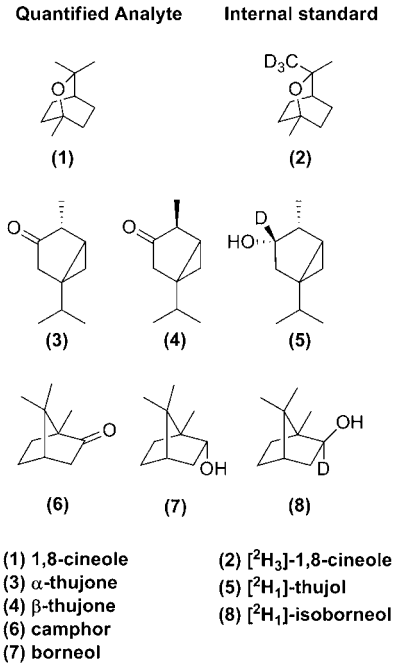

Figure 1. Volatile compounds and isotope-labeled internal standards used in the quantification of selected sage infusion constituents.

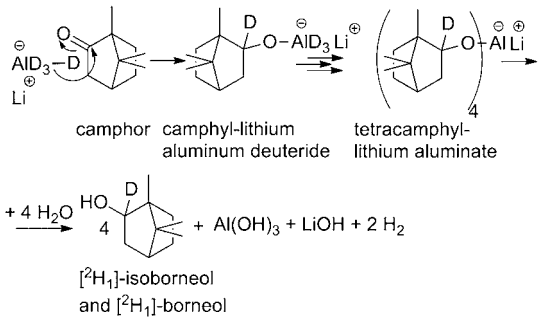

Figure 2. Synthesis of $[^2H_1]$-isoborneol by deuteration of camphor.

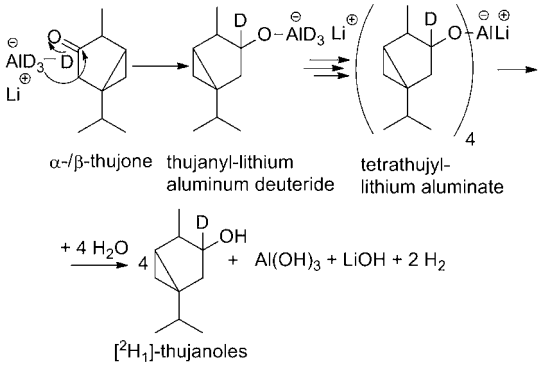

Figure 3. Synthesis of $[^2H_1]$-thujol by deuteration of α- and β-thujone.

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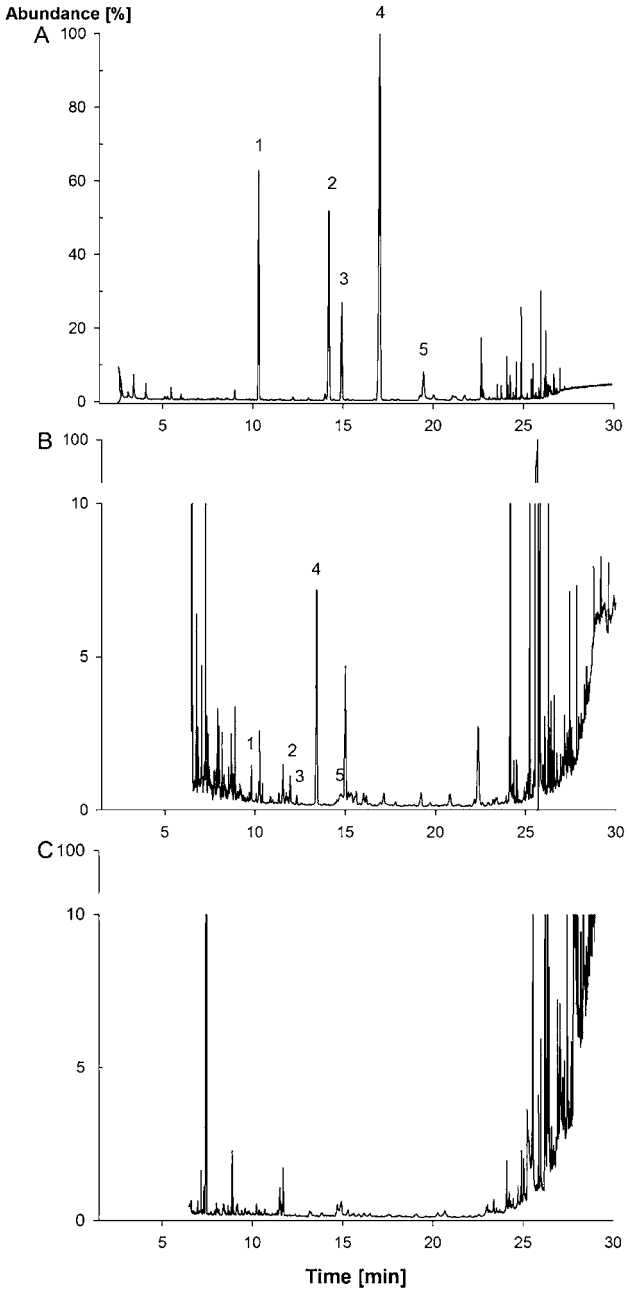

Figure 4. Gas chromatogram of volatiles isolated from either sage infusion (A) or HGF-1 cells with (B) or without (C) exposure to sage infusion for 6 h. The differences in retention times for the individual peaks can be explained by different temperature profiles applied during the GC-MS analyses of the sage infusion (A) and cell culture samples (B, C). Numbers represent the individual sage infusion components 1,8-cineole (1), α-thujone (2), β-thujone (3), camphor (4), and borneol (5).

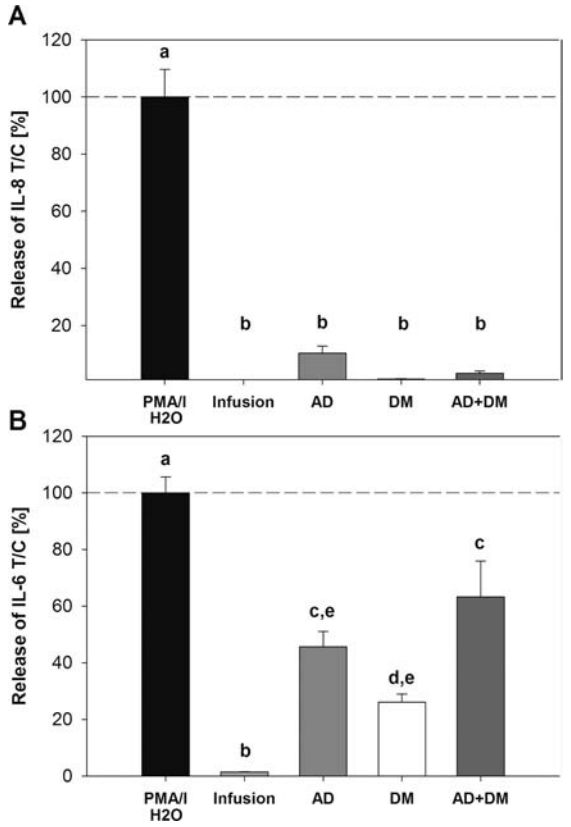

Figure 5. Release of IL-6 and IL-8 from PMA/I-stimulated HGF-1 cells after a 6 h exposure to a 1:2 dilution of the sage infusion or the sage infusion fractions. Values were normalized to positive control (PMA/I H_2O; cells incubated with PMA/I and water only). Mean ± SEM (n = 3). [a–e]Letters indicate significant differences between groups calculated by one-way ANOVA with the Student–Newman–Keuls posthoc test ($p < 0.05$).

$[^2H_1]$-isoborneol was identified by NMR analyses. ^1H NMR: δ = 3.73 ppm (s, 1 H, OH), 1.71 ppm (dd, 2 H, CH J = 8.50 and 3.76 Hz), 1.69 ppm (m, 1 H, CH), 1.64 ppm (m, 1 H, CH), 1.47 ppm (dt, 1 H, CH), 1.04 ppm (s, 3 H, CH), 0.97 ppm (dt, 1 H, CH and m, 1 H, CH), 0.88 ppm (s, 3 H, CH), 0.82 ppm (s, 3 H, CH). ^{13}C NMR: δ = 48.5 ppm (C), 45.1 ppm (CH), 40.4 ppm (CH), 38.6 ppm (C), 33.7 ppm (CH), 27.1 ppm (CH), 25.8 ppm (C), 20.0 ppm (CH), 19.7 ppm (CH), 11.0 ppm (CH).

For quantification of α- and β-thujone, $[^2H_1]$-thujol was synthesized according to the scheme shown in Figure 3.[25] A total amount of 0.36 g of lithium aluminum deuteride was mixed with anhydrous MTBE under a nitrogen atmosphere using a calcium chloride tube. After equilibration, a total of 1.2 g of a mixture of stereoisomeric thujone (95% α-thujone and 5% β-thujone) was added with stirring. After 4 h at room temperature, deuterated water was added cautiously until the hydrogen generation stopped. The reaction mixture was extracted with MTBE three times (3 × 10 mL). The organic extract was washed with saturated sodium chloride solution three times (3 × 10 mL), dried over anhydrous sodium sulfate, and concentrated to isolate stereoisomeric $[^2H_1]$-thujanoles. With an amount of 62%, $[^2H_1]$-thujol was the most abundant reaction product besides $[^2H_1]$-neothujol, $[^2H_1]$-isothujol, and $[^2H_1]$-neoisothujol, at 30%, 4.7%, and 2.6%, respectively.

The synthesis yield for $[^2H_1]$-thujol was 53% (640 mg; 4.15 mmol; white oil). The analyte was characterized by MS-EI (70 eV): m/z (%) 137 (33) M^+, 122 (75), 110 (32), 95 (100), 94 (97), 81 (41), 67 (25), 55 (34), 43 (52). $[^2H_1]$-thujol was identified by NMR analyses. ^1H NMR: δ = 3.15 ppm (s, OH), 1.92 ppm (q, CH), 1.80 and 1.60 ppm (d, 2 H, CH, J = 12.0 Hz), 2.01 and 1.52 ppm (d, 2 H, CH, J = 13.7 Hz), 1.31 ppm (m, CH), 1.25 ppm (m, 1 H, CH), 0.96 ppm (d, 3 H, CH, J = 6.94 Hz), 0.93 ppm (d, 3 H, CH, J = 2.68 Hz), 0.91 ppm (d, 3 H, CH, J = 2.99 Hz). ^{13}C NMR: δ = 78.7 ppm and 70.7 ppm (C), 44.6 ppm (CH), 35.2 ppm (CH), 34.2 ppm (C), 33.3 ppm (CH), 32.9 ppm (CH), 32.7 ppm (CH), 19.7 ppm (CH), 15.4 ppm (CH), 14.4 ppm (CH).

Quantification of 1,8-Cineole, Borneol, Camphor, and α- and β-Thujone in the *Salvia officinalis* L. Infusion Using Stable-Isotope-Labeled Internal Standards. A freshly prepared sage infusion was cooled to 4 °C, and a defined amount of 100 g was used for SAFE distillation. The AD was extracted with MTBE three times. The combined organic layers were concentrated to 100 mL using a rotary evaporator. A total of 1.5 mL of this concentrate was

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spiked with the internal standards [$^2H_3$]-1,8-cineole (1.78 μg), [$^2H_1$]-isoborneol (73.9 μg), and [$^2H_1$]-thujol (1.27 μg). GC-MS analysis was performed as described above using the selected ion monitoring (SIM) mode. The response factor for each unlabeled compound (Table 1) was determined by analyzing mixtures of known amounts of the labeled and unlabeled compounds in three different mass ratios (3:1, 1:1, 1:3). Response factors were calculated according to Horst and Rychlik.[24]

Table 1. Isotopically Labeled Standards and Response Factors Used for the Quantitation of the Most Abundant Sage Infusion Constituents

internal standard	ion (m/z)[a]	quantified analyte	ion (m/z)[b]	RF[c]
[$^2H_3$]-1,8-cineole	157	1,8-cineole	154	1.00
[$^2H_1$]-thujol	110	α-thujone	110	1.32
		β-thujone	110	0.87
[$^2H_1$]-isoborneol	140	camphor	137	1.35
		borneol	139	0.99

[a]Ion selected to monitor the area of the internal standard. [b]Ion selected to monitor the area of the analyte. [c]RF: response factor.

Since the isotope-labeled compounds were added after SAFE distillation and ether extraction due to their limited amounts available, a recovery experiment of nonlabeled 1,8-cineole, α- and β-thujone, camphor, and borneol, equilibrated in water and subjected to the SAFE distillation and ether extraction, was performed. In this experiment, mean recoveries of 31.3%, 45.4%, 39.8%, 53.0%, and 21.2% were calculated for 1,8-cineole, α- and β-thujone, camphor, and borneol, respectively. These values indicate a rather low recovery for each of the target compounds, which is likely due to a loss during SAFE distillation and the subsequent ether extraction. For quantification of the target compounds in the infusion, the results calculated using the RF factor were corrected by the respective yield to compensate for the losses.

Identification and Quantification of Rosmarinic Acid Present in the Dry Matter Fraction of *Salvia officinalis* L. Infusion Using LC-MS and HPLC-DAD. The nonvolatile part of the distilled sage infusion was subjected to freeze-drying. The residue was first homogenized with 15 mL of methanol under reflux and magnetic stirring for 2 h at 60 °C. The suspension was centrifuged at 4500*g* for 5 min, and the supernatant was collected. The remaining plant material was resuspended in 15 mL of *n*-hexane and extracted at 64 °C for 2 h under reflux. The *n*-hexane solution was centrifuged at 4500*g* for 5 min, and the supernatant was collected. The methanol and *n*-hexane fraction were combined and evaporated to dryness using a rotary evaporator. The residue was resuspended in methanol by sonication and filtered to remove precipitates. The solution was filled up with methanol to yield a final volume of 3 mL.

LC-MS analysis of the nonvolatile sage infusion extract was performed on a Dionex UltiMate 3000 LC System (Thermo Fisher Scientific, Vienna, Austria), connected to a mass sensitive detector operated in the ESI mode (LCQ Fleet, Thermo Fisher Scientific, Vienna, Austria). Separation was performed on a Luna C-18 column (3 mm × 250 mm, 5 μm pore size, Phenomenex, Aschaffenburg, Germany) at ambient temperature and an injection volume of 20 μL. The method was adapted from Ben-Farhat et al.:[5] The mobile phase was acetonitrile (A) and acidified water containing 5% formic acid (B) with a flow rate of 0.4 mL/min. The gradient was as follows: 0 min 25% A, hold for 2 min; 32 min 100% A, hold for 10 min; 47 min 25% A, hold for 10 min. A commercially available standard (Carl Roth, Karlsruhe, Germany) was used to identify rosmarinic acid by comparison of retention time and mass spectra.

For quantification of rosmarinic acid, a sample volume of 10 μL was injected into a Dionex UltiMate 3000 LC System (Thermo Fisher Scientific, Vienna, Austria) equipped with a DAD detector set to 330 nm wavelength. The operation conditions were equal to the protocol mentioned for LC-MS analysis.[5] Rosmarinic acid was quantified in the nonvolatile sage infusion extract using the external calibration with linear regression equation $y = 918.91x + 120.69$ ($r^2 = 0.990$) with a calculated recovery of 97%.

Cell Culture. Human gingival fibroblasts (HGF-1, ATCC CRL-2014, P 15) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 20% fetal bovine serum (Thermo Fisher Scientific, Vienna, Austria), 4% glutamine, and 1% penicillin-streptomycin at 37 °C and 5% CO_2. For the experiment, cells at passages 18 to 23 were incubated with DMEM supplemented with 4% glutamine.

Cell Viability. HGF-1 viability was evaluated by applying the WST-1 test using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate (Roche, Mannheim, Germany) as substrate. Formazan formation was measured 2.5 h after addition of WST-1 at 440 nm with wavelength correction set at 690 nm. For this assay 5000 HGF-1 cells were seeded in one well of a 96-well plate to reach confluence within 5 days. Confluent cells were exposed to test substance with 1 ng/mL phorbol-12-myristate-13-acetate (PMA) plus 100 ng/mL ionomycin (I) for 6 h at 37 °C and 5% CO_2 before the tetrazolium salt was added. Cytotoxic effects of sage infusion, sage infusion fractions, and the components 1,8-cineole (Carl Roth, Karlsruhe, Germany), (−)-borneol (Alfa Aesar, Karlsruhe, Germany), (+)-camphor (Alfa Aesar, Karlsruhe, Germany), a mixture of stereoisomeric thujones (95% α-thujone mixed with 5% β-thujone), and rosmarinic acid (Carl Roth, Karlsruhe, Germany) on HGF-1 cells were excluded prior to any cytokine experiment by applying the representative concentrations according to 1:2 diluted sage infusion. No cytotoxic effects of the individual compounds were observed at their natural content in 1:2 diluted sage infusion (data not shown).

Measurement of IL-6 and IL-8 Secretion from HGF-1 Cells. For measurement of interleukin release, 15 000 cells per well were seeded in a 24-well plate until confluence was reached, about five days before the experiment. A total of 1 ng/mL PMA and 100 ng/mL ionomycin were added to 300 μL of cell culture medium per well for 6 h at 37 °C and 5% CO_2. Cells were treated with PMA/I for 6 h, as this exposure time was demonstrated to result in a pronounced stimulation of pro-inflammatory IL-6 and IL-8 in human buccal cells[21] and gingival fibroblasts.[26]

For sample analyses, HGF-1 cells were co-incubated with either sage infusion (150 μL sage infusion plus 150 μL cell culture medium, corresponding to a 1:2 dilution), sage infusion fractions (reconstituted dry matter or aqueous distillate equal to 1:2 diluted sage infusion), or one of the quantified individual compounds according to its content present in 150 μL of sage infusion. Plates were sealed with foil to protect the volatile compounds from evaporation. After the 6 h incubation period, cell culture supernatants were collected and centrifuged for 30 s at 10000*g* to remove cell debris. Samples were stored at −80 °C until cytokine measurement was performed.

Measurement of IL-6 and IL-8 by Means of Luminex xMAP Magnetic Bead Technology. The Millilex MAP Kit (Merck Millipore, Vienna, Austria) was applied to quantify IL-6 and IL-8 in the cell culture supernatants. This kit uses an immunoassay on the surface of fluorescent-coded magnetic microspheres (beads) coated with specific detection antibodies. The beads were incubated overnight together with 25 μL of sample, following the manufacturer's protocol. Each sample was measured in triplicate using samples from three different passages.

Uptake Experiments of Sage Infusion Constituents in HGF-1 Cells. To analyze the cellular uptake of the sage infusion constituents by HGF-1 cells, cells were seeded into 30 cell culture dishes (150 mm diameter) and grown to confluence. Cells were exposed to either incubation medium alone or a 1:2 diluted sage infusion (525 mL sage infusion plus 525 mL incubation media) for 6 h at 37 °C and 5% CO_2. Cell culture dishes were sealed with parafilm to protect volatile compounds from evaporation. Each plate was washed with phosphate-buffered saline (PBS) and lysed by addition of 750 μL of lysis buffer containing 50 mM Tris, 25 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% IGEPAL supplemented with 1 mM PMSF, 1 mM sodium *ortho*-vanadate, and protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl

fluoride hydrochloride, bestatin hydrochloride, leupeptin, E-64, aprotinin, pepstatin A, and phosphoramidon disodium salt) at pH 7.4. The cell lysate was homogenized using a 0.8 mm syringe and centrifuged at 4500*g* for 10 min, forming a pellet and a supernatant. The incubation medium and the PBS washing solution were combined and stored at −80 °C until further analyses. All three fractions (the combined incubation medium plus the PBS wash solution, the cell pellet, and the supernatant) were subjected to the extraction procedures described for sage infusion using the SAFE apparatus.

GC-MS analyses were accomplished with slightly different operating conditions compared to the sage infusion: injection volume 5 μL in the pulsed splitless mode, column temperature held at 50 °C for 2 min, raised to 98 °C at a rate of 6 °C/min, heated at 1 °C/min to 110 °C, finally increased at 25 °C/min to 280 °C, and held isothermically for 5 min. Helium was used as a carrier gas at a constant flow rate of 1.1 mL/min.

The GC-MS chromatograms obtained for the sage infusion and the lysate prepared from cells with and without infusion treatment are shown in Figure 4A, B, and C, respectively. The differences in retention times for the individual peaks can be explained by different temperature profiles applied during the GC-MS analyses of the sage infusion and cell culture samples.

Quantification of the volatile compounds 1,8-cineole, borneol, camphor, and α- and β-thujone was carried out as described above for the sage infusion aqueous distillate using GC-MS. HPLC-DAD analyses for quantification of rosmarinic acid were applied according to the conditions used for sage infusion dry matter extract mentioned above.

Statistical Analysis. Measurements of the pro-inflammatory markers IL-6 and IL-8 were performed in samples from three different cell culture passages ($n = 3$) with at least three technical replicates each (trp = 3), and the PMA/I-induced level was normalized to 100%.

The normalized values from each set of replicates were used to determine the mean ± standard error of the mean (SEM) after application of the Nalimov outlier test. Differences between solvent control and PMA/I treatment were calculated by Student's *t*-test. Comparison between data sets from test substances and controls was calculated by applying one-way ANOVA with posthoc analysis using the Student−Newman−Keuls significance test. In all of the applied statistical tests, a value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Effect of Sage Infusion and Sage Infusion Fractions on Inflammatory Marker Release in PMA/I-Treated HGF-1 Cells. For the identification of the key immune-modulatory compounds in *S. officinalis* L. infusion, human gingival fibroblasts were co-incubated with phorbol-12-myristate-13-acetate and ionomycin (PMA/I), a combination known to induce the release of the pro-inflammatory interleukin IL-6 in human buccal cells,[21] and the infusion or quantified compounds thereof. HGF-1 cells were treated with PMA/I and the test substance for 6 h, since the cellular inflammatory response was shown to be strongly enhanced when exposed to pro-inflammatory agents over this time of incubation.[26]

As a result, treatment of HGF-1 cells with 1 ng/mL PMA and 100 ng/mL ionomycin for 6 h increased the release of the pro-inflammatory markers IL-6 and IL-8 by up to 6- and 24-fold, respectively, compared to control cells incubated with water or ethanol without the addition of PMA/I (Table 2).

The immune-modulatory effect of a *S. officinalis* L. infusion was then tested by applying a 1:2 dilution thereof to HGF-1 cells concomitantly with PMA/I for 6 h. Here, an anti-inflammatory activity of sage infusion was demonstrated by a significant reduction of 99.2 ± 0.12% and 98.5 ± 0.06% for the PMA/I-stimulated IL-8 and IL-6 release, respectively (Figure 5A, B).

In order to identify the most anti-inflammatory active compounds, the sage infusion was fractionated into an aqueous distillate (AD), containing volatiles and water, and the non-volatile dry matter (DM) fraction using the SAFE method. In accordance with the effect demonstrated for the sage infusion, treatment with a 1:2 dilution of AD, DM, and the recombined fractions (AD+DM) decreased the release of both inflammatory markers compared to the PMA/I-stimulated cells (Figure 5A, B).

Table 2. IL-6 and IL-8 Release from HGF-1 Cells Stimulated with or without PMA/I in the Presence or Absence of a Solvent[a]

	IL-6 (pg/mL)	IL-8 (pg/mL)
solvent control (water)	13.7 ± 3.46 a	5.64 ± 1.90 a
PMA/I (water)	41.1 ± 5.78 b	137 ± 19.0 b
solvent control (ethanol)	4.84 ± 1.28 a	7.41 ± 3.48 a
PMA/I (ethanol)	30.5 ± 5.20 b	89.2 ± 17.0 b

[a]Different solvents had to be used since sage infusion and sage infusion fractions were applied as aqueous extract, whereas the individual sage infusion constituents were dissolved in ethanol. The mean ± SEM amount of each cytokine present in the medium after treatment is shown for each cytokine. Letters a and b indicate significant differences between groups calculated by Student's *t*-test versus positive control ($n = 3$).

Due to the potent effect of both the AD and the DM fraction, it remained unknown if the strong anti-inflammatory activity of the *S. officinalis* L. infusion is due to compounds present in the volatile or the nonvolatile fraction.

Uptake of Sage Infusion Constituents in HGF-1 Cells and Quantification in the Infusion in Order to Calculate the Recovery of the Compounds. Previous investigations using epithelial buccal cells revealed that α-terpineol, a volatile compound present in orange juice, is able to attenuate IL-6 production through binding to the extracellular IL-6-receptor (IL-6R).[21] It is also known that IL-6 exerts its activity after internalization of the IL-6/IL-6R complex upon ligand binding.[27] Therefore, ligand binding to the soluble and membrane-bound IL-receptors is an important target in modulating periodontal disease.[12,28,29] However, it remains unknown whether anti-inflammatory active compounds exert their immune-modulatory activity through binding to IL-6 membrane receptor protein or to the soluble IL-6 receptor, resulting in internalization of the receptor-ligand complex. Furthermore, due to the lipophilic properties of volatile compounds, also passive diffusion into the cell resulting in modulation of inflammatory cascades might be involved.[30]

To gain information about the volatile and nonvolatile immune-modulating compounds present in *S. officinalis* L. infusion interacting with gingival fibroblasts, an uptake experiment using a 1:2 diluted sage infusion was completed.

After 6 h of treatment with sage infusion and/or medium, HGF-1 cells were washed and lysed. The combined supernatants (incubation media and washing solutions), the lysate, and the cell pellet were fractionated into AD and DM prior to quantitative analyses using GC-MS and HPLC-DAD. Cells incubated with medium only were analyzed as a control.

The chromatograms in Figure 4 show that several volatile sage infusion constituents were present in the infusion-exposed HGF-1 cells, whereas none of the compounds could be detected in untreated control cells. The additional peaks obtained in the MS spectra of infusion-treated cells were assigned to 1,8-cineole, borneol, camphor, and α- and β-thujone. Camphor was the most abundant volatile compound that was quantified in the HGF-1 cell lysate, with an absolute

Table 3. Total Recovery of Sage Infusion Compounds Detected in the Supernatant of Lysed HGF-1 Cells (n = 1, trp = 9) and the Incubation Media (n = 1, trp = 9) Containing 525 mL of Sage Infusion after a 6 h Incubation Period, Calculated from the Total Amount Quantified in the Infusion (n = 3, trp = 3)

	1,8-cineole	α-thujone	β-thujone	camphor	borneol
added to the cell (mg)	3.67 ± 0.71	20.9 ± 3.46	5.51 ± 0.45	6.03 ± 0.61	2.66 ± 0.23
cell pellet (μg)	0.01 ± 0.001	0.08 ± 0.01	0.03 ± 0.01	0.17 ± 0.02	<LOD[a]
cell lysate (μg)	0.21 ± 0.01	0.56 ± 0.03	0.22 ± 0.01	2.97 ± 0.17	0.25 ± 0.25
cell culture media (μg)	157. ± 5.08	427 ± 26.3	181 ± 11.9	922 ± 54.0	98.4 ± 93.4
recovery absolute (μg)	157	428	181	925	98.7
recovery relative (%)	4.28	2.05	3.29	15.3	3.71

[a]LOD, limit of detection for borneol: 0.056 μg.

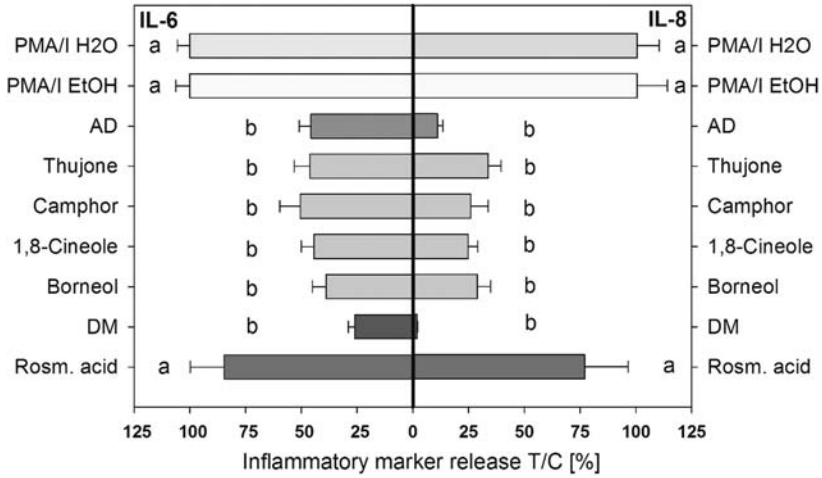

Figure 6. Release of IL-6 and IL-8 from PMA/I-stimulated HGF-1 cells exposed to sage infusion AD, DM, or one of the individual compounds dissolved in ethanol according to the amount present in the 1:2 diluted sage infusion. Values from sage infusion fractions were normalized to positive control containing PMA/I and water only (PMA/I H_2O). Values from cells treated with each of the individual compounds were normalized to cells incubated with PMA/I and ethanol (PMA/I EtOH). Mean ± SEM (n = 3). [a,b]Letters indicate significant differences between controls, fractions, and the individual compound groups calculated by one-way ANOVA with the Student−Newman−Keuls test ($p < 0.05$).

amount of 2.97 μg (Table 3). Values for 1,8-cineole, borneol, and α- and β-thujone, at 0.21, 0.56, 0.22, and 0.25 μg, respectively, were in the same range but considerably lower compared to the amount quantified for camphor.

To calculate the recovery of these compounds in HGF-1 cells, three stable-isotope-labeled internal standards were synthesized for quantification of 1,8-cineole, borneol, camphor, and α- and β-thujone (Figure 1) in the sage infusion. The stable isotope [$^2H_3$]-1,8-cineole was used to quantify 1,8-cineole, whereas borneol and camphor were quantified using [$^2H_1$]-isoborneol. The internal standard [$^2H_1$]-thujol was selected to quantify both α-thujone and β-thujone. Synthesis of [$^2H_1$]-thujanol was carried out using a mixture of α- and β-thujone; thus, the reaction product contained four stereoisomeres of [$^2H_1$]-thujanol with [$^2H_1$]-thujol being the most pronounced (Figure 3).

The percent recovery of the individual volatile compounds in the cell pellet, the cell lysate, and the combined cell culture medium containing the sage infusion and washing solutions showed the highest value for camphor, with a total recovery of 15.3%. The percent recoveries found for 1,8-cineole, borneol, and α- and β-thujone were in the range of 1−4% (Table 3). The recovery values obtained for the target compounds in this experiment are lower compared to previous findings of our group, which may be due to diffusion of the volatile compounds into the headspace of the cell culture dishes and evaporation during the 6 h incubation period at 37 °C.[21]

On the basis of the GC-MS analyses of the *S. officinalis* L. infusion, α-thujone was identified as the most abundant volatile compound with a total amount of 20.9 mg in the amount of 525 mL infusion added to HGF-1 cells. In comparison, total amounts of camphor and β-thujone added were 6.03 and 5.51 mg, respectively. For 1,8-cineole and borneol, the total content in the *S. officinalis* L. infusion added to the cell culture medium was 3.67 and 2.66 mg, respectively.

The amounts of the individual volatile compounds in the here tested sage infusion were comparable to previous investigations of volatile constituents in the aqueous extract from *S. officinalis* L. leaves.[4,24,31] All together, the five monoterpenes 1,8-cineole, borneol, camphor, and α- and β-thujone made up 72% of the total volatile fraction. Thus, these compounds quantified in the sage infusion represent the most abundant volatile constituents of the sage infusion studied here.

In earlier investigations on *S. officinalis* L. infusions, several phenolic acids and flavonoids were identified as nonvolatile components, with rosmarinic acid being the most abundant phenolic compound, with a concentration of 9.50−10.0 mg/g *S. officinalis* L. plant material.[31,32]

To investigate whether the potent anti-inflammatory activity of the sage infusion DM fraction applied to HGF-1 cells is caused by rosmarinic acid, the DM fraction of the pellet and the lysate of cells, exposed to the *S. officinalis* L. infusion, was analyzed. Using HPLC-DAD measurements, rosmarinic acid was quantified in the cell lysate with a total amount of 18.1 ± 1.14 mg.

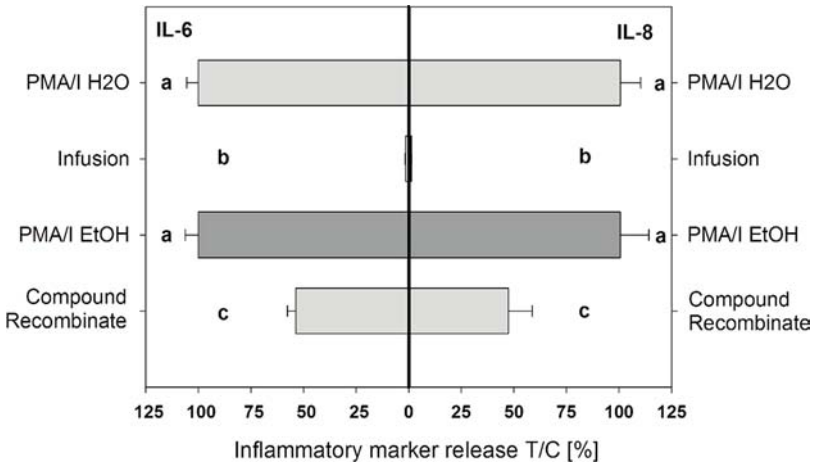

Figure 7. Release of IL-6 and IL-8 from PMA/I-stimulated HGF-1 cells after a 6 h exposure to 1:2 diluted sage infusion or a mixture of 1,8-cineole, borneol, camphor, thujone, and rosmarinic acid according to their natural concentration in the 1:2 diluted infusion. Values from the sage infusion were normalized to the positive control containing PMA/I and water only (PMA/I H_2O). Values from cells treated with the compound mixture were normalized to cells incubated with PMA/I and ethanol (PMA/I EtOH). Mean ± SEM (n = 3). [a−c]Letters indicate significant differences between controls and treatment groups calculated by one-way ANOVA with the Student−Newman−Keuls posthoc test ($p < 0.05$).

Subsequent LC-MS analyses revealed that rosmarinic acid was also present in the DM fraction of the *S. officinalis* L. infusion tested here.

The total amount of rosmarinic acid in the incubation media after application of the 525 mL infusion applied in our study was calculated to be 51.6 ± 11.2 mg. This concentration is comparable to respective concentrations found in the literature.[31,32] However, taking into account the rosmarinic acid content of 2.33 ± 1.14 mg that was determined in the cell culture medium containing the sage infusion, a percent recovery of 4.6% was determined. Although this recovery seems low, our results clearly demonstrate that the most abundant components detected in *S. officinalis* L. infusion are predominantly located in the lysate of human gingival fibroblasts.

Anti-inflammatory Activity of the Sage Infusion Constituents 1,8-Cineole, Borneol, Camphor, Thujone, and Rosmarinic Acid in HGF-1 Cells. To determine whether 1,8-cineole, borneol, camphor, thujone, and rosmarinic acid are responsible for the anti-inflammatory activity of the sage infusion, and to determine if one of these compounds is chiefly responsible for the anti-inflammatory effect, PMA/I-stimulated HGF-1 cells were exposed to each of these compounds according to their natural content in the 1:2 diluted infusion. The results, shown in Figure 6, demonstrate that treatment of the cells with either 1,8-cineole, borneol, camphor, or thujone significantly decreased the release of both interleukins with a mean inhibition of 67−76% and 50−61% for PMA/I-stimulated IL-8 and IL-6 secretion. Remarkably, all of the compounds were equally effective. For food-representative concentrations of borneol, camphor, and thujone, this result is original, while 1,8-cineole has been reported as an anti-inflammatory agent in different biological test systems including animal studies and human interventions.[6,7,33,34] For expamle, when 1,8-cineole was applied at a physiological concentration of 1.5 μg/mL to LPS-stimulated monocytes, it significantly inhibited TNF-α, IL-1β, IL-6, and IL-8 by 99%, 84%, 76%, and 65%, respectively.[7] In comparison, incubation of HGF-1 cells with the sage infusion representative 1,8-cineole concentration of 3.50 μg/mL reduced the PMA/I-induced IL-8 and IL-6 secretion by 75.9 ± 4.35% and 55.7 ± 5.73%, respectively (Figure 6). Thus, 1,8-cineole might be one of the compounds chiefly responsible for the anti-inflammatory activity of *S. officinalis* L. infusion in PMA/I-stimulated HGF-1 cells. However, since borneol, camphor, and thujone were equally effective in this model system, these compounds should also be taken into account for future studies on anti-inflammatory effects of volatile constituents present in *S. officinalis* L. infusions.

To investigate the potent activity of the DM fraction of sage infusion, HGF-1 cells were treated with rosmarinic acid, the most abundant nonvolatile compound in this fraction.

In contrast to the potent anti-inflammatory activity observed for the terpenes, rosmarinic acid did not affect the IL-6 and IL-8 release in PMA/I-stimulated cells (Figure 6). Thus, no anti-inflammatory effect could be demonstrated for rosmarinic acid when applied to HGF-1 cells in a sage infusion representative concentration of 49.1 μg/mL for 6 h. In contrast, a 4 h incubation with 1 μg/mL of rosmarinic acid significantly decreased IL-1β, IL-6, and tumor necrosis factor (TNF)-α in gingival tissues collected from the buccal gingiva or interdental papilla of healthy volunteers after pretreatment with 10 μg/mL of *E. coli*-LPS for 24 h.[19] Our results, therefore, do not exclude an anti-inflammatory activity of rosmarinic acid.

To clarify whether the strong anti-inflammatory effect of the *S. officinalis* L. infusion was due to the combination of the individual infusion constituents tested here, recombinate experiments using a combination of 1,8-cineole, borneol, camphor, thujone, and rosmarinic acid, according to their natural concentration in 1:2 diluted sage infusion, were performed (Figure 7). Although incubation with the compound recombinate reduced the PMA/I-stimulated IL-6 and IL-8 release by 53.1 ± 11.3% and 46.4 ± 3.97%, it was less effective than the total tea infusion (Figure 7). Here, we cannot exclude that the presence of rosmarinic acid, the compound for which no anti-inflammatory effect was demonstrated in the human gingival fibroblasts, attenuated the potent anti-inflammatory effect of 1,8-cineole, borneol, camphor, and thujone. However, since the strong anti-inflammatory effect of the total *S. officinalis* L. infusion cannot be solely explained by the combination of 1,8-cineole, borneol, camphor, thujone, and rosmarinic acid, other compounds might further contribute to its anti-inflammatory

activity. Another nonvolatile compound, luteolin, has also been quantified in aqueous *S. officinalis* L. extracts in its glucosidic forms.[31,32] Thus, luteolin might also exert an anti-inflammatory effect, although results on the immune-modulatory activity of luteolin are conflicting.[35,36]

Salvia officinalis L. infusion can be seen as a potent agent for the reduction of PMA/I-stimulated inflammatory cytokines released by gingival fibroblasts. The volatile aroma compounds 1,8-cineole, borneol, camphor, and α- and β-thujone are key determinants of the anti-inflammatory activity of a sage infusion in gingival cells, although nonvolatile constituents may contribute to the anti-inflammatory effect of sage infusion.

Even though the high anti-inflammatory potential of *S. officinalis* L. is not completely understood, these results underline the health beneficial potential of tisanes and dietary herbs.

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ABBREVIATIONS USED

HGF-1, human gingival fibroblasts; PMA/I, phorbol-12-myristate-13-acetate/ionomycin; SAFE, solvent-assisted flavor evaporation; SI, sage infusion; AD, aqueous distillate; DM, dry matter; RF, response factor; IL-6R, interleukin-6 receptor; MTBE, methyl *tert*-butyl ether; TNF-α, tumor-necrosis factor-α

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