Essential Oil Composition and Anti-Inflammatory Activity of *Salvia officinalis* L (Lamiaceae) in Murin Macrophages

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Abstract

**Purpose:** Sage, *Salvia officinalis* L (Lamiaceae), is widely cultivated medicinal plant for its economic importance and large content of bioactive components; therefore, in the present study, the active components (volatile compounds) and the anti-inflammatory effect of *S. officinalis* have been investigated.

**Methods:** *Salvia officinalis* samples were collected from northern and southern Albania, respectively. The crushed leaves were subjected to hydro-distillation, and the essential oils analyzed by gas chromatography GC/FID (for quantification of volatiles) and gas chromatography/mass spectroscopy (GC/MS) for identification.

**Results:** α-Thujone (30.7 %), camphor (26.6 %) and 1, 8-cineole (14.7 %) were the major components of the oil from northern Albania, while camphor (43.8 %), α-thujone (15.9 %), camphene (8.5 %) and 1,8-cineole (8.4 %) were the predominant compounds in the sample from southern Albania. The results of the anti-inflammatory tests on these essential oils using murine macrophages indicate that both oils significantly (*p* < 0.05) reduced nitric oxide (NO), and nuclear kappa B (NF-κB) production in RAW 264.7 cells.

**Conclusion:** The results indicate that NO and NF-κB production in RAW 264.7 cells are greatly decreased by the essential oil of Albanian sage. Thus, the biological properties of sage oil can be attributed to the components of the oil.

**Keywords:** Salvia officinalis, Sage oil, Camphor, Camphene, Lamiaceae, Cineole, Nuclear kappa B, Nitric oxide, α-Thujone, Volatile compound

INTRODUCTION

*Salvia* L is the largest genus of Lamiaceae (previously Labiatae) family; it is spread throughout the world, and some species are economically important because they are used as spices in food industry and flavoring agent in cosmetic industry and aromatherapy. Sage has been most commonly known as aromatic and medicinal plant native from Mediterranean countries, which is extensively cultivated due to its economic importance. Additionally, the essential oil and infusion of *Salvia officinalis* leaves have also been widely applied in traditional medicine. In this way and since ancient times, the crude herbal extracts of aromatic plants, including sage, have been used...
for different purposes, such as foods, drugs and perfumery [1].

The leaves of sage are very popular for their antioxidative properties, and have been used in the food industry and in medicine to improve human health. In traditional medicine, the plant has been used for various purposes such as anti-inflammatory, antioxidant and treatment of gastric disorders. Moreover, sage ethanol tinctures and decoctions have long been used against inflammations of oral cavity, digestive and intestinal tracts, in gastritis and tonsillitis [2,3]. Several other important biological activities of this species such as anti-bacterial, fungistatic, virustatic, astringent, hypoglycemic, eupetic, anti-hydratic and cytotoxic have also been reported [4-6].

Diverse phytochemical investigations have been performed to identify biologically active compounds responsible for the therapeutic effects of sage [6]. Consequently, various medicinally important bioactive compounds such as diterpenoids, interpenoids, flavonoids, polysaccharides, phenolic glycosides and phenolic acid derivatives have been isolated from different species of *Salvia* [5,7,8]. For example, Raal et al reported that the main compounds of sage oil from Estonia and other European countries were: 1,8-cineole, camphor, α-thujone, β-thujone, borneol and viridiflorol [9].

This study focused on the investigation of the anti-inflammatory activity of the leaves of sage in terms of LPS-stimulated macrophages. The anti-inflammatory activity of the *Salvia officinalis* has been evaluated according to their constituents and their amounts in the essential oils of the species collected from two different regions of Albania (Northern and Southern Albania).

**EXPERIMENTAL**

**Plant material**

*Salvia officinalis* L was collected from Northern and Southern Albania in the month of June, 2011. The specimens were authenticated by Prof H Duman, Department of Biology, Gazi University, Ankara (Turkey). A voucher specimen (no. AEF 26266) was deposited at the Herbarium of the Faculty of Pharmacy of Ankara University in Ankara.

Samples were coded as NAS (northern Albania Sage) and SAS (southern Albania Sage). The plants were kept under refrigerated storage at the facilities of Xherdo Co. Ltd, Albania. The samples were taken randomly from the bulk stored material and leaves were dried, and samples were ready for the distillation process.

**Essential oil distillation**

Crushed leaves of the two sage samples (NAS and SAS) were subjected to hydro-distillation for 3 h in a Clevenger (type) apparatus. The yields of essential oils on a dry weight basis were 3 and 2.3 % (v/w) for NAS and SAS, respectively. Suspensions of ~ 1 g of sage were placed in a 500 mL round flask together with 100 mL of distilled water, and 200 μL of 2-undecanone (1000 mg/L) as internal standard. Heating of the sample was continued for 1 h after getting to the boiling point. The vapours were condensed by means of a cold refrigerant. After 60 min of extraction, the solvent, 1 mL of pentane, containing the volatile compounds, was collected in a 2.5 mL vial and kept at -18 °C until GC analyses were performed. The analyses were run in triplicate.

**Gas chromatography (GC/FID and GC/MS)**

The isolation, quantification and identification of the volatile components were performed on a gas chromatograph coupled with a mass spectrometry detector (GC/MS), Saturn 2000 Varian Chrompack, with a column TRACE TR-5 (5 % phenyl methylpolysiloxane) 30 m × 0.53 mm ID × 1.0 μm film. Scanning was performed from 39 to 400 m/z in electronic impact (EI) at 70 eV, mode at 1 scan/s. Analyses were carried out using helium as carrier gas at a flow rate of 1.0 mL/min in a split ratio of 1:20 and the following program: a) 80 °C for 0 min; b) rate of 5.0 °C/min from 80 to 200 °C; c) rate of 25°C/min from 200 to 280 °C and hold for 5 min. Injector and detector were held at 200 and 300°C, respectively. The injection volume was 1 μL. The compounds were identified by using retention indices (RI), and mass spectra, with similarity indexes > 90 % (authentic chemicals and NIST05 spectral library collection; NIST, 2012) [10].

GC/MS was used for identification of the volatile compounds while GC/FID was used for semi-quantification of these compounds. The qualitative analysis performed on a gas chromatograph, Shimadzu 10, with a flame ionization detector (GC/FID). The column and chromatographic conditions were identical to those described in the previous paragraph for the GC/MS analysis. The injector temperature was 200 °C, and nitrogen was used as carrier gas (1 mL/min). The semi-quantification was obtained
from electronic integration measurements. 2-
Undecanone was added as internal standard 
(200 µg) at the beginning of the distillation
procedure to simulate the behavior of all volatile
compounds.

**MTT assay for determination of cell viability**

The measurement of cell viability of the samples 
was performed using the MTT (4,5-
dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium 
bromide) assay. Briefly, RAW 264.7 cells were 
plated at a density of 1 × 10^4 per well in a 96-well 
plate and incubated at 37 °C for 24 h. The cells 
were treated with various concentrations of the 
oils (50, 100, 200, and 500 µg/mL) or vehicle 
alone. The essential oils were first dissolved in 
dimethyl sulfoxide (DMSO) to make a 200 mg/mL 
stock concentration and further diluted with 
DMSO for working concentration (shown in Table 
1). The cell viability was measured according to 
Khan et al [11,12]. All experiments were 
performed in triplicate.

**Determination of nitric oxide in LPS-
stimulated RAW 264.7 Cells**

The NO production of SAS and NAS was 
evaluated in LPS-stimulated RAW 264.7 cells 
using the Griess reagent. Briefly, RAW 264.7 
cells were plated at a density of 1 × 10^5 per well 
in a 24-well plate and incubated at 37 °C for 24 
h. The cells were treated with various 
concentrations of NAS and SAS or vehicle alone, 
2 h before lipopolysaccharide (LPS) stimulation 
at 1 µg/mL and incubated at 37 °C for an 
additional 18 h (see Table 2). After incubation for 
18 h, NO concentration was determined 
according to Khan et al [12].

**NF-κB SEAP reporter gene assay**

NF-κB-dependent reporter gene transcription 
was analyzed according to Khan et al [11]. The 
SEAP assay was used in the determination of 
the inhibitory activity of the samples in LPS 
stimulated RAW 254.6 macrophages. In brief, 1 
× 10^5 RAW 264.7 macrophages transfeceted with 
pNF-κB–SEAP–NPT encoding four copies of κB 
sequences and the SEAP gene as a reporter 
was pre-incubated with different concentrations 
of the oils for 2 h and challenged with LPS (1 mg/mL) 
for additional 18 h. Aliquots of the cell-
free culture medium were heated at 65°C for 
5 min and given an assay buffer [2M 
diethanolamine, 1 mM MgCl2, 500 mM 4-
methylumbelliferyl phosphate (MUP)] in the dark 
37 °C for 1 h. The fluorescence from the 
products of the SEAP/MUP was measured using 
a 96-well microplate fluorometer (Gemini XS, 
Molecular Devices) at an excitation of 360 nm 
and an emission of 449 nm. In this experiment, 
N-p-tosyl-L-phenylalanyl chloromethyl ketone 
(TPCK), 30 µM, was used as a positive control.

**Statistical analysis**

Unless otherwise stated, results are expressed 
as mean ± standard deviations (SD) from three 
different experiments. One-way analysis of 
variance (ANOVA) followed by Dunnett’s t-test 
was applied to assess the statistical significance 
for differences between the study groups (SPSS 
version 10.0, Chicago, IL). A value of p < 0.05 
was chosen as the criterion for statistical 
significance.

**RESULTS AND DISCUSSION**

**Volatile composition**

Crushed leaves of *Salvia officinalis* L collected 
from Northern and Southern Albania were 
subjected to hydro-distillation, and the essential 
oils of sage were analyzed by GC/FID 
(quntification of volatiles) and GC/MS 
(identification). α-Thujone (30.7 %), camphor 
(26.6 %) and 1, 8-cineole (14.7 %) were found as 
the major components from the oil from Northern 
Albania, while camphor (43.8 %), α-thujone (15.9 
%), camphene (8.5 %) and 1, 8-cineole (8.4 %) 
were the main compounds in the sample from 
Southern Albania.

In previous studies, the essential oils of some 
sage populations from Montenegro and Serbia 
have been studied. In the oil of sage leaves 
collected from different locations, α-thujone, β-
thujone, borneol and manool were the major 
components. On the other hand, in Serbian 
population, camphor was the first major 
constituents besides thujone and 1, 8-cineole [9].

In another study, the essential oil from the sage 
dried shoots (vegetative aerial parts) was 
analyzed by GC, and GC/MS; the major 
compounds were determined as cis-thujone 
(17.4 %), α-humulene (13.3 %), 1, 8-cineole 
(12.7 %), E-caryophyllene (8.5 %) and borneol 
(8.3 %) [13]. The leaves of *S. officinalis* from 
Romania have been also analyzed and, α-
thujone (21.85 %), camphor (11.25 %), 
veridiflorol (11.71 %) and manool (9.15%) were 
detected as major constituents by GC/MS [7].

The major constituents of the essential oil from 
aerial parts of *S. officinalis* cultivated in South 
Brazil were α-thujone (24.8 %), 1, 8-cineole (14.8 
%), camphor (10.9 %), borneol (11.1 %) and β-
pinene (9.87 %) [1]. Moreover, compositions of 
the essential oils of *S. officinalis* from various 
European countries were analyzed and α-
thujone, camphor, β-thujone and 1,8-cineole were found as major constituents in most of the essential oils under analysis [9].

According to the GC/MS and GC/FID results of the present study, α-thujone (30.7 %), camphor (26.6 %) and 1, 8-cineole (14.7 %) were the major components of the essential oils of the sage samples from Northern Albania (NAS). The main constituents of the essential oil of NAS are presented in Table 1, and a model chromatogram of the NAS samples is shown in Figure 1. However, the essential oil of the Southern Albania sage (SAS) was dominated by camphor (43.8 %), α-thujone (15.9 %), camphene (8.5 %) and 1,8-cineole (8.4 %) as presented in Table 4; a model chromatogram of the SAS samples is shown in Figure 2. As some papers have previously reported, the variation in the essential oil composition is influenced by environmental (climatic, seasonal, and geographical), physiological and morphological factors [1,9]. Finally, it can be considered that the NAS sample could be classified as α-thujone chemotype, while SAS sample could be considered as a camphor chemotype.

Table 1: Essential oil composition of Salvia officinalis L. from northern (NAS) and southern (SAS) Albania

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Retention index</th>
<th>Compound</th>
<th>NAS Area (%)</th>
<th>SAS Area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>859</td>
<td>cis-Salvane</td>
<td>0.39</td>
<td>0.22</td>
</tr>
<tr>
<td>2</td>
<td>862</td>
<td>trans-2-Hexenal</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>875</td>
<td>trans-Salvane</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>4</td>
<td>937</td>
<td>α-Thujene</td>
<td>0.13</td>
<td>0.16</td>
</tr>
<tr>
<td>5</td>
<td>938</td>
<td>Tricycledene</td>
<td>0.09</td>
<td>0.23</td>
</tr>
<tr>
<td>6</td>
<td>945</td>
<td>α-Pine</td>
<td>2.02</td>
<td>3.22</td>
</tr>
<tr>
<td>7</td>
<td>960</td>
<td>Camphene</td>
<td>4.15</td>
<td>8.49</td>
</tr>
<tr>
<td>8</td>
<td>975</td>
<td>Sabine</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>9</td>
<td>996</td>
<td>β-Pine</td>
<td>2.73</td>
<td>3.36</td>
</tr>
<tr>
<td>10</td>
<td>1011</td>
<td>α-Phelandrene</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>11</td>
<td>1023</td>
<td>α-Terpinene</td>
<td>0.34</td>
<td>0.26</td>
</tr>
<tr>
<td>12</td>
<td>1029</td>
<td>p-Cymene</td>
<td>0.28</td>
<td>0.26</td>
</tr>
<tr>
<td>13</td>
<td>1033</td>
<td>Limonene</td>
<td>2.69</td>
<td>3.77</td>
</tr>
<tr>
<td>14</td>
<td>1038</td>
<td>1,8-Cineole</td>
<td>14.73</td>
<td>8.35</td>
</tr>
<tr>
<td>15</td>
<td>1057</td>
<td>γ-Terpinene</td>
<td>0.61</td>
<td>0.47</td>
</tr>
<tr>
<td>16</td>
<td>1087</td>
<td>Terpinolene</td>
<td>0.57</td>
<td>0.80</td>
</tr>
<tr>
<td>17</td>
<td>1110</td>
<td>α-Thujone</td>
<td>30.72</td>
<td>15.92</td>
</tr>
<tr>
<td>18</td>
<td>1119</td>
<td>β-Thujone</td>
<td>5.38</td>
<td>1.71</td>
</tr>
<tr>
<td>19</td>
<td>1152</td>
<td>Camphor</td>
<td>26.57</td>
<td>43.83</td>
</tr>
<tr>
<td>20</td>
<td>1186</td>
<td>Terpinen-4-ol</td>
<td>0.64</td>
<td>0.48</td>
</tr>
<tr>
<td>21-22</td>
<td>1200</td>
<td>α-Terpineol</td>
<td>nd</td>
<td>0.43</td>
</tr>
<tr>
<td>22-23</td>
<td>1418</td>
<td>Bornyl acetate</td>
<td>3.15</td>
<td>5.59</td>
</tr>
<tr>
<td>23-24</td>
<td>1453</td>
<td>trans-β-Caryophyllene</td>
<td>2.00</td>
<td>1.58</td>
</tr>
</tbody>
</table>

NIST [10]

Figure 1: Model GC/MS chromatogram of Salvia officinalis from the northern Albania sample (NAS)
Cell viability

To explore the principle mechanism underlying this anti-inflammatory effect, the action of NAS and SAS on inflammation related macrophage functions was evaluated. The cytotoxic effect of SAS and NAS in LPS-stimulated RAW 264.7 macrophages was measured using a MTT assay and results are shown in Table 2. No cytotoxic effect was observed up to 500 µg/ml in SAS and 200 µg/mL in NAS, respectively. It is apparent that non-toxic concentrations were used in the onward experiments.

Table 2: Effect of NAS and SAS on cell viability in LPS-stimulated RAW 264.7 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Essential oil concentration (µg/mL)</th>
<th>IC₅₀ (µg/mL)</th>
<th>MTT (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>NAS</td>
<td>7.46</td>
<td>14.24</td>
<td>16.16</td>
</tr>
<tr>
<td>SAS</td>
<td>4.30</td>
<td>13.92</td>
<td>13.30</td>
</tr>
</tbody>
</table>

* NAS and SAS mean northern and southern Albania samples, respectively. The data were obtained from three independent experiments and expressed as mean ± SD; * p < 0.05 indicate significant difference from the LPS-stimulated RAW 264.7 cells.

Nitric oxide production

Nitric oxide (NO) is an important pro-inflammatory mediator and responsible for various inflammatory responses. Therefore, the suppression of NO production can be essential to the development of anti-inflammatory agents. In the current study, it was found that NAS and SAS significantly inhibited LPS-induced NO production in RAW 264.7 cells. Pre-treatment with NAS and SAS considerably inhibited LPS-induced NO production (Table 3). No cytotoxic effect was observed in the tested concentrations of NAS and SAS. Hence, experimental data suggested that reducing NO production by NAS and SAS was not due to a toxic reaction of the cells. AMT (2-amino-5, 6-dihydro-6-methyl-4H-1, 3-thiazine), 10 µM, was used as positive control.

Table 3: Effect of NAS and SAS on nitrite production in LPS-stimulated RAW 264.7 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Essential oil concentration (µg/mL)</th>
<th>IC₅₀ (µg/mL)</th>
<th>Nitrite (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NAS and SAS mean northern and southern Albania samples, respectively. The data were obtained from three independent experiments and expressed as mean ± SD; * p < 0.05, ** p < 0.01, and *** p < 0.001 indicate significant difference from the LPS-stimulated RAW 264.7 cells.

NF-κB transcription factor

NF-κB transcription factor has been evidenced to play a significant role in LPS-induced expression of pro-inflammatory mediators, including NO and PGE₂ [11]. In order to investigate the molecular mechanism of these essential oils mediated inhibition of NO and PGE₂, NF-κB transcription activity was measured using the reporter gene assay. RAW 264.7 cells were stably transfected with NF-κB-SEAP-NPT plasmid containing four copies of κB sequence fused to SEAP as the reporter [12]. LPS treatment of the transfected cells for 18 h increased the SEAP expression (Table 4). Pretreatment with SAS and NAS significantly inhibited LPS-induced SEAP expression in a concentration-dependent manner; the maximum inhibition at 500 µg
SAS/mL at and 200 μg NAS/mL, respectively (Table 4). As a positive control, TPCK also showed a significant inhibitory effect on NF-κB activation at the transcription level.

**Table 4:** Effect of NAS and SAS on NF-κB in LPS-stimulated RAW 264.7 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Essential oil concentration (µg/mL)</th>
<th>IC50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>NAS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NAS and SAS mean northern and southern Albania samples, respectively. The data were obtained from three independent experiments and expressed as mean ± SD; * p < 0.05, ** p < 0.01 and *** p < 0.001 indicate significant difference from the LPS-stimulated RAW 264.7 cells.

In order to link all data generated in this study, it can be stated that the essential oil of SAS (southern Albania sage) led to higher cell viability (Table 2), lower inhibition percentages of nitric oxide production (Table 3) and also lower inhibition of NF-κB transcription activity (Table 4) than NAS (northern Albania sage). The volatile compositions of SAS and NAS led to the classification of these sage samples as camphor and α-thujone chemotypes according to the predominant volatile compound. Therefore, our recommendation is to use camphor chemotypes of sage (e.g. SAS) in medicinal treatments due to their higher anti-inflammatory activity.

**CONCLUSION**

Therefore, while NAS samples have α-thujone (30.17 %) as the most abundant constituent, camphor (43.8 %) was predominant in SAS samples. However, it is obvious that the content of α- and β-thujone and camphor are effective for determining the quality of sage oil quality. This is important since the high quality of the oil are due to their contents of thujone and camphor, and because the biological properties of sage oil are mainly attributable to camphor, 1, 8-cineole and α- and β-thujone. Furthermore, the essential oils of Albanian sage possess significant anti-inflammatory but the chemotypes with camphor predominating are highly recommended.

**REFERENCES**