The potential of *Salvia officinalis* as a suppressor of cell proliferation in animal feed and human nutrition: an experimental study

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Abstract: The objective of this study was to examine the in vitro cytotoxic activities of *Salvia officinalis* (sage) oil on human immortalized keratinocyte (HaCaT) cell lines by using an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (MTT) cytotoxicity assay after the *Salvia officinalis* oil administration in different doses and time-points. In vitro cytotoxic activities of *Salvia officinalis* oil on HaCaT cell lines were assessed, and MTT assays were used to determine cell viability. The HaCaT cells (100 µL) were cultured in 96-well plates at 2 × 104 cells per well and treated with different concentrations of *Salvia officinalis* oil (25 µM, 50 µM, 100 µM, 150 µM, and 200 µM) for durations of 24, 48, and 72 h. Cell death was determined by collecting and staining with 0.4% Trypan blue for 5 min at room temperature before microscopic examination. At 24 h, there was a significant difference between the 25 and 200 µM doses ($P = 0.034$). For the 72 h *Salvia officinalis* oil application, there was a significant difference between the 50 and 200 µM doses only ($P = 0.002$). On the other hand, for 48 h *Salvia officinalis* oil application, there were significant differences between 25 and 150 µM, 25 and 200 µM, 50 and 150 µM, 50 and 200 µM, 100 and 150 µM, and 100 and 200 µM doses ($P = 0.003$, $P = 0.000$, $P = 0.005$, $P = 0.001$, $P = 0.028$, and $P = 0.003$, respectively). We concluded that *Salvia officinalis* oil may help the living organism to have a healthy digestive system by stopping excessive cell proliferation via the apoptotic pathway in the gastrointestinal system and, thus, may be used in both animal feed and human nutrition.

Key words: Cytotoxins, apoptosis, lamiaceae, cell line, cell viability, cell proliferation

1. Introduction:
The therapeutic significance of plants is well known. This can lead us to look for a new natural compound for preventing some cellular processes [1,2]. Natural plant products are used as alternative or complementary modalities by people or animals all over the world, and this is an increasing trend [3–5]. *Salvia officinalis*, which has been cultivated since ancient times and known as sage, is a native aromatic perennial plant [6]. Pharmacologic studies have reported that it contains various types of secondary metabolites, including alpha and beta thujones, flavonoids, and phenolic acids [7,8]. Compared to other species of Salvia, *Salvia officinalis* contains the highest amount of essential oils (1–2.8%) [9]. The current literature has proven that this plant has antimicrobial, antioxidant, hypoglycemic, antidiabetic, antiinflammatory, and anticancer properties. Additionally, due to their intense antioxidant activity [10,11], these polyphenols are useful not only for combating several degenerative diseases including Alzheimer's and various cellular processes [12–17] but also helpful as dietary supplements in animal nutrition [18]. In studies in which *Salvia officinalis* was used as a dietary supplement, several features such as having the highest activity of glutathione peroxidase in the liver, reducing the side effects of oxidative processes in the duodenal mucosa due to beneficial effect on the glutathione peroxidase activity and adjustment of lipid peroxidation in the kidney tissue, have been demonstrated [10,11,19,20].

On the other hand, human immortalized keratinocyte (HaCaT) cells have been widely used to study the epidermal homeostasis and its pathophysiology, especially in chronic inflammatory skin diseases such as psoriasis [21,22]. Polyphenols in *Salvia officinalis* are in contact with some immunological agents to cope with oxidative stress and inflammation. They scavenge oxygen radicals such as peroxide, hydroperoxides, or lipid peroxyl, consequently inhibiting the oxidative stress and affecting the T-helper cell cytokines to prevent inflammation [23,24]. Furthermore, they are involved in a number of cellular signaling pathways such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt, and

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tumor necrosis factor-related apoptosis-inducing ligand. They also affect cell proliferation, differentiation, and regulation steps and cause expression of some agents and modulation of cellular events [6,25].

The purpose of this study was to investigate the in vitro cytotoxic effects of *Salvia officinalis* oil on HaCaT cell lines by using an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (MTT) cytotoxicity assay after administering *Salvia officinalis* oil in different doses and at different time points.

2. Materials and methods

2.1. Plant material

The harvesting of *Salvia officinalis* was done from spontaneously growing samples found in middle Anatolia. The sample collection was done in May 2016, and sample identification was made by the Department of Aromatic and Medicinal Plants Research, National Institute of Agricultural and Food Technology (INIA) (Erzurum, Turkey). A sample was stored at the INIA for internal control. Samples were dried and kept in an oven heated to 35°C to prevent climatic influences until analysis.

2.2. Extraction process

A 100 g sample was used for essential oil extraction. The oil was obtained by hydrodistillation of the dried ground material in a Clevenger-like apparatus for 2 h at atmospheric pressure. Time was measured starting with the falling of the first drop of the distillate. Two replications were performed for the process. The weight of the essential oil yield was measured. The effects of *Salvia officinalis* at varying concentrations (25, 50, 100, 150, and 200 μM) and at various time points (24, 48, and 72 h) on the proliferation of HaCaT cells were determined using the MTT assay.

2.3. Cell culture

The nontumorigenic HaCaT cell line was acquired from the Cell Culture and Biological Resources Unit at Yeditepe University. These cells were seeded at a concentration of 5,000 cell/well on 96-well plate (BIOFIL, TPC, Switzerland) and conserved in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO, USA), which included HEPES (Sigma) buffer along with 10% heat-inactivated fetal bovine serum (Hyclone Lab., Logan, UT, USA), 100-μg/mL streptomycin (Sigma), and 100-U/mL penicillin (Sigma). These were incubated in disposable plastic tissue culture flasks in a 5% CO₂/95% air incubator at 37°C. After incubation, the culture media were removed; the cells were washed with PBS and MTT cell proliferation assay was performed.

2.4. Cell viability (MTT) assay

MTT assays were used to determine cell viability. The HaCaT cells (100 μL) were cultured in 96-well plates at 2 × 104 cells per well and treated with different concentrations of *Salvia officinalis* oil (Group 1: 25 μM; Group 2: 50 μM; Group 3: 100 μM; Group 4: 150 μM; and Group 4: 200 μM) for durations of 24, 48, and 72 h. Following treatment, each well was filled with 10 μL of MTT reagent (5 mg/mL) and incubated for 4 h at 37°C. The medium was then eliminated and 150 μL of DMSO was added to make the MTT formazan soluble. The absorbance of solubilized MTT formazan products was measured by an ELISA plate reader (Biotek, USA) at 590 nm at 24, 48, and 72 h.

Cell death was determined by collecting and staining with 0.4% of Trypan blue for 5 min at room temperature before microscopic examination. Viable cells were counted via Trypan blue exclusion. Dead cells that stained blue were deemed positive and summed against the total.

2.5. Statistical analysis

Statistical Package for the Social Sciences SPSS version 22 (IBM Corp., Armonk, NY, USA) was used for data analysis. As descriptive statistics, numerical variables were summarized as mean (± standard deviation). Comparisons between the groups were made with the one-way ANOVA test. A P-value below 0.05 was considered as statistically significant.

3. Results

The kinetic study revealed that *Salvia officinalis* inhibits cell growth in a dose- and time-dependent way in the HaCaT cells. Cell viability decreased in all *Salvia officinalis* oil doses, and the high cytotoxic dose was 200 μM for all time points. On the other hand, *Salvia officinalis* oil showed its effect with minimum cytotoxicity for 72 h in all doses on HaCaT cells. The Figure and *Salvia officinalis* oil showed its effect with minimum cytotoxicity for 72 h in all doses on HaCaT cells. The Figure and Table 1 show the mean values of cell viability in all experimental groups with standard deviations.

After the statistical analyses were performed, it was seen that there were significant differences between groups concerning the dose intervals and time points. For 24-h *Salvia officinalis* application, there was a significant difference between the 25 and 200 μM doses (P = 0.034). For the 72-h *Salvia officinalis* application, there was a significant difference between only 50 and 200 μM doses (P = 0.002). On the other hand, for 48-h *Salvia officinalis* oil application, there were significant differences between the 25 and 150 μM, 25 and 200 μM, 50 and 150 μM, 50 and 200 μM, 100 and 150 μM, and 100 and 200 μM doses (P = 0.003, P = 0.000, P = 0.005, P = 0.001, P = 0.028, and P = 0.003, respectively). The significance (P) values of all variables are given in Table 2.

4. Discussion

In this study, we examined the impact of *Salvia officinalis* oil on the cell viability of human HaCaT keratinocytes. When the literature was checked regarding the effects of *Salvia officinalis* oil on HaCaT keratinocyte cells, no it
was determined that no previous scientific studies had been performed on HaCaT cells; however, there have been some studies carried out on the role of *Salvia officinalis* in preventing chronic inflammation [26,27]. If any natural compounds have inhibitory features on the proliferation and modulation of keratinocyte differentiation, they may be beneficial for the treatment of psoriasis by controlling keratinocyte growth. According to our findings, *Salvia officinalis* could significantly suppress the proliferation of HaCaT cell line in vitro employing the MTT assay. Such antiproliferative activity of *Salvia officinalis* oil was reported in Jiang et al.'s study [6]. They applied *Salvia officinalis* on HepG2 liver carcinoma cells and demonstrated the cytotoxic, antioxidant, and antiproliferative effects on cancer cells in a time- and dose-dependent manner.

It is well established that epidermal keratinocyte hyperproliferation results from the abnormal expression of several regulatory molecules related to proliferation such as IFNγ, IL-4, IL-17A, and IL-22. Apoptotic defects are thought to play a crucial role in the pathophysiology of psoriasis [21,28]. To date, cytotoxicity studies have been gaining importance. In this study, we detected that there was a dose-dependent decrease in cell viability following *Salvia officinalis* oil application. The optimal nontoxic dose was statistically identified as 100 µM, and the optimal time point was detected as 72 h. Kozics and his colleagues examined the viability of HepG2 cells by 24-h treatment using various concentrations of *Salvia officinalis* extract as 5.7 mg·mL\(^{-1}\) [29]. On the other hand, Zare Shahneh et al. applied *Salvia officinalis* extract on 6 different cell lines including human breast cancer, human prostate cancer, human leukemic monocyte lymphoma (Raji), non-Hodgkin’s B-cell lymphoma (Raji), human acute myelocytic leukemia (KG-1A), and mouse fibrosarcoma; they reported a range of IC\(_{50}\) 40-167 µg·mL\(^{-1}\) [30]. They also stated that 200 and 300 µg/mL of *Salvia officinalis* extract after incubation for 24 h was the effective dose for Raji, U937, KG-1A, and HUVEC cell lines. In addition, El Hadri et al. reported that *Salvia officinalis* showed cytotoxic activity in murine macrophage RAW264.7 and colon

<table>
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<tr>
<th>Time of measurement</th>
<th>Group 1 (25 µM)</th>
<th>Group 2 (50 µM)</th>
<th>Group 3 (100 µM)</th>
<th>Group 4 (150 µM)</th>
<th>Group 5 (200 µM)</th>
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<td>Mean ± SD</td>
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<tr>
<td>24 h</td>
<td>0.075 ± 0.004</td>
<td>0.073 ± 0.002</td>
<td>0.072 ± 0.005</td>
<td>0.071 ± 0.005</td>
<td>0.062 ± 0.008</td>
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<td>48 h</td>
<td>0.053 ± 0.008</td>
<td>0.050 ± 0.005</td>
<td>0.047 ± 0.004</td>
<td>0.031 ± 0.005</td>
<td>0.026 ± 0.005</td>
</tr>
<tr>
<td>72 h</td>
<td>0.158 ± 0.008</td>
<td>0.185 ± 0.032</td>
<td>0.148 ± 0.013</td>
<td>0.152 ± 0.019</td>
<td>0.112 ± 0.024</td>
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Figure: Results for the mean values of cell viability in all experimental groups by using the MTT assay. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide.

Group 1: treated with 25 µM *Salvia officinalis* oil; Group 2: treated with 50 µM *Salvia officinalis* oil; Group 3: treated with 100 µM *Salvia officinalis* oil; Group 4: treated with 150 µM *Salvia officinalis* oil; Group 5: treated with 200 µM *Salvia officinalis* oil.
cancer at IC50 73.3 μg mL⁻¹ [9]. Similar to our findings, a study reported that 100 mg/mL for 24 h application of Salvia officinalis was the most effective dose on the HepG2 cell line [6]. Additionally, a study performed by Khare et al. evaluated the antiwrinkle potential of Salvia officinalis on Col-I, Ela-I, and Hya-I cell lines and reported that Salvia officinalis had inhibitory effects of antiaging enzymes by modulating some cellular processes [31].

One limitation of this study was the lack of information on the crucial parameters related to inflammation and the oxidative stress processes. Experiments studying molecular, immunological, and biochemical parameters are needed to further elucidate this issue and to determine the possible beneficial effects of fresh Salvia officinalis tea.

5. Conclusion
This study showed that Salvia officinalis oil used in cell culture inhibited excessive cell proliferation via the apoptotic pathway. We conclude that Salvia officinalis oil may help the living organism to have a healthy digestive system by stopping excessive cell proliferation via the apoptotic pathway in the gastrointestinal system and, thus, may be used in both animal feed and human nutrition. Furthermore, Salvia officinalis oil may contribute to the treatment of several diseases in the oral mucosa called “tumor-like masses,” “proliferative lesions,” or “benign soft tissue lesions,” which may lead to nutritional disorders.

In light of this information, the detailed explanation of the underlying cellular and biochemical mechanisms for the bioactive polyphenolic compounds of Salvia officinalis should be developed for the successful treatment of chronic inflammatory skin diseases such as psoriasis. Therefore, further studies should examine the mechanisms of antiinflammatory, antioxidant, and apoptotic effects of Salvia officinalis extracts on different cell lines, including HaCaT cells.

Conflict of interest
The authors declare no conflict of interest.

References


