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Effects of Salvia aramiensis on proliferation, caspase-related apoptosis and pro-inflammatory cytokines in lung cancer cells

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This study was conducted to determine the antioxidant and anticancer activity of *Salvia aramiensis* Rech.f., which is a member of Lamiaceae, and its phenolic compounds. For this purpose, the phenolic compounds from the *S. aramiensis* methanol extract (SAME) were identified by LC-MS-MS. Antioxidant activities of SAME were determined by *in vitro* DPPH scavenging and metal chelating assays. The antiproliferative effect of SAME on lung cancer cells (A549) was determined by using the MTT assay. In addition, the levels of the pro-inflammatory cytokines IL6 and IL8, and apoptotic marker caspase-3 were determined using ELISA assays. As a result of LC-MS-MS screening, luteol was detected as the main compound of SAME. The DPPH scavenging and metal chelating activities of SAME were insignificant. In addition, it was observed that SAME reduces the viability of A549 cells. At doses of 25, 50, and 100 µg/mL, IL6 and IL8 levels increase, but at 200 µg/mL, a considerable decrease was observed. Caspase-3 levels also decreased at doses of 100 and 200 µg/mL. As a result, although SAME has weak antioxidant activities, it has significant antiproliferative and pro-inflammatory cytokine suppressing effects. Due to the decreased levels of caspase 3, the cytotoxic effect of SAME may be mediated by a caspase independent pathway.

Keywords: Anticancer, Antioxidant, Caspase-3, Inflammatory cytokines, Phenolic compound, *Salvia aramiensis*

Salvia genus is one of the most important members of Lamiaceae. Numerous species within this genus are ingested primarily as tea due to their medicinal properties and flavour. There are approximately 900 species of the genus *Salvia*, which is represented by numerous species in the world[1]. According to İpek & Gürbüz[2], there are 97 taxa belonging to the genus *Salvia* in Turkey, 51 of which are endemic. Due to their bioactivities and active compounds, *Salvia* species are consistently utilized in research by scientists throughout the world. The primary reason why *Salvia* species are utilized in these studies is due to the abundance of chemical compounds they contain. This genus contains an abundance of phenolic and terpene compounds. Although the bioactivities of numerous *Salvia* species have been reported[3-10], many *Salvia* species, including *S. aramiensis*, have not been adequately evaluated, particularly with regard to their anticancer activity.

Lung cancer is the most prevalent form of the disease. It is the leading cause of mortality for both women and men[11]. Although a variety of chemicals are utilized in chemotherapy, the number of these chemicals is insufficient and novel anticancer agents are required. In this regard, it is essential to identify plants that contain phytochemicals with minimal or non-toxic effects relative to synthetic compounds. In phytochemical research, it is crucial to focus on cancer cells in particular. Compounds with the fundamental properties of inhibiting inflammation, proliferation, migration, invasion, and activating cell death pathways such as apoptosis in cancer cells are sought. Apoptosis is programmed cell death, and crucial enzymes such as caspases are responsible for causing cell death[12].

S. aramiensis is a perennial semi shrub form that is naturally distributed in Turkey. It can be about 1 m. tall and shows frequent branching at the top. The leaves are simple, narrowed, oblong or ovate in shape. Its flowers can be lilac, purple or white. *S. aramiensis* is a species that flowers in May and June and grows naturally in turkish pine forests and rocky areas. The upper parts of the body are finely hairy and almost hairless towards the top. Smaller leaves are usually clustered at the base of the flower shoots compared to those on the stem. These leaves become smaller and sparse during the flowering period[13].

As far as our literature survey, there are no reports about anticancer activity of *S. aramiensis*. Also, very little is known about its antioxidant activity and phenolic compounds. Hence, in this study, the

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antioxidant, antiproliferative, apoptosis induction, and inflammatory cytokine suppressing properties of SAME and its phenolic compounds were evaluated.

Material and Methods

Collection and extraction of *S. aramiensis*

In June 2021, *S. aramiensis* was collected from the Amanos Mountains of Islahiye district of Gaziantep province. It was identified by Associate Professor Mustafa Pehlivan and added to his personal herbarium with the voucher number MPH209-9. *S. araminesis* was washed with distilled water. Before the extraction, *S. araminesis* was desiccated on blotting paper in the open air and away from sunlight and then ground in a mortar. Then, *S. aramiensis* was exposed to 200 mL of methanol in a beaker for 72 h. The solution was then filtered, and the solvent was evaporated using a rotary evaporator. The methanol extract was kept at +4°C until the tests began.

Determination of phenolic contents

Total phenolic component in SAME were determined according to the method developed Ratkevicius *et al.*[14],. Homogenized extract was centrifuged at 10000 rpm for 10 min and 50 µL of the supernatant was extracted. Final volume was adjusted to 1 mL by adding 3% sodium carbonate and 0.3 N Folin-Ciocalteau, and the mixture was kept at room temperature for 2 h. At 765 nm, these samples were then analyzed with a spectrophotometer. Gallic acid was employed as a reference point. Each analysis was repeated three times.

Determination of phenolic compounds by LC-MS-MS

Samples for phytochemical analysis of SAME were prepared by dissolving samples in methanol and then filtrated with 0.22 µM filter. The LC-MS-MS apparatus of Nexera UHPLC (Shimadzu) with LC-20AD two pumps, DGU-20A3R degasser, CTO-10ASVP column furnace and SIL-20AC autosampler was used for the study. C18 Intersil ODS-4 analytical colon (3.0mm × 100mm, 2µm) was used. The injection volume was 2 µL and flow rate 0.3 mL/min. Mobile phase A (water and 0.1% formic acid) and mobile phase B (methanol and 0.1% formic acid) were used in a linear gradient flow and column temperature was set at 40°C initially.

Anticancer activities

Determination of anti-proliferation activity of SAME

After preserving lung cancer cell cultures in DMEM for 24 h, 96 well plates containing 70–80% confluent of lung cancer cell cultures (A549) were treated for 24 h with various concentrations of SAME (25, 50, 100, 200 µg/mL). MTT was utilized to evaluate cell viability (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). The culture media was replaced with a DMEM medium containing 1 mg/mL MTT (Sigma) and incubated at 37°C for 15 min. Cells were then treated with MTT solution and dimethyl sulfoxide (DMSO, Sigma). The optic density (OD) of cells were measured at 550 nm with a colourimetric reader.

Determination of Caspase-3 level in A549 cells treated with S. aramiensis

To assess apoptosis in lung cancer cells, after 24 h of incubation following the application of SAME to the lung cancer cells, level of caspase-3 (ALGEN) was determined according to manufacturer's instructions.

Determination of pro-inflammatory cytokines IL6 and IL8 levels

After 24 h of incubation following the treatment of SAME to the lung cancer cells, the medium (DMEM, SIGMA D6429) in which the cells were cultured was collected. Next, interleukin 6 (IL6: ALGEN) and interleukin 8 (IL8, ALGEN) levels were analyzed or estimated according to the manufacturer's instructions.

Antioxidant tests

Determination of DPPH free radical scavenging activity

Free DPPH radical scavenging activity of SAME was performed according to the method of Gaulejac *et al.*[15]. Separate test tubes were filled with 0.1 mL of control and extract solutions and 2.9 mL of 6×10^{-5}. The produced solution was then measured with a spectrophotometer at 517 nm after 60 min in the dark at room temperature. Three repeated measurements were made for each experiment.

Determination of metal chelating activity

The chelating activity of SAME was measured following the ferrozine method with minor modifications[16]. The reaction mixture contained 500 µL of SAME and 50 µL of $FeCl_2$ (2 mmol/L). After 5 min, the reaction was initiated by the addition of 5 mmol/L ferrozine (100 µL) and the total volume was adjusted to 3 mL with 80% acetone solution. Then, the mixture was shaken vigorously and incubated at room temperature for 10 min. Absorbances of the control and extract solutions were measured at 562 nm. The final results were expressed as a percentage of inhibition.

Statistical analysis

Statistical analyses were performed using the Prism 7.01. The normal distributions of antioxidant test results (DPPH and metal chelating), MTT, IL6, IL8 and caspase-3 were evaluated by one way analysis of variance (ANOVA). Dunnett test were used to compare the doses applied with controls. Results were given as mean ± SD. The level of significance was accepted to be at least $P<0.05$.

Results and Discussion

Determination of phenolic compounds of SAME by LC-MS-MS

In this study, the total phenolic content of the SAME was determined to be 214.7 mg/kg GAE. In addition, the phenolic compounds of the extract were evaluated using LC-MS-MS. In the analysis, luteolin was the main compound (325.36 µg/mL). Salicylic acid (65.16 µg/mL), hydroxybenzoic acid (21.47 µg/mL), fumaric acid (250.12 µg/mL) and acetohydroxamic acid (200.46 µg/mL) were also detected in SAME.

Antioxidant activities

DPPH free radical scavenging activity

DPPH scavenging activity of SAME was determined by DPPH method and the results were given in Fig. 1. As shown in Fig. 1, none of the 25, 50, 100 or 200 µg/mL concentrations of SAME exhibited DPPH scavenging activity. On the other hand, the 100 and 200 µg/mL concentrations of ascorbic acid, which use as a positive control, exhibited significant DPPH radical-scavenging activity.

Metal chelating activity

The chelating activity of SAME were shown in Fig. 2. None of the 25, 50, 100 and 200 concentrations µg/mL of SAME exhibited the metal chelating activity. 50, 100, and 200 µg/mL concentrations of the positive control EDTA exhibited significant metal chelating activity.

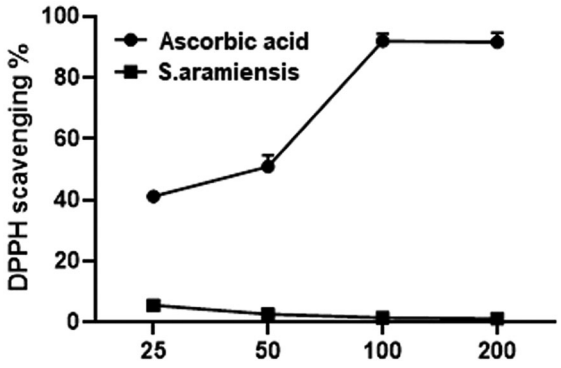

Fig. 1 — Percentage of DPPH scavenging activity of SAME.

Anticancer activities

Effect of SAME on viability of lung cancer cell line A549

In this study, the antiproliferative effect of SAME on A549 cancer cells was investigated, and the results were presented in Fig. 3. 25 and 50 µg/mL concentrations of SAME had no effect on the viability of A549 cells when compared to the DMSO control. Moreover, the 100 ($P<0.01$) and 200 ($P<0.001$) µg/mL concentrations of SAME significantly decreased the viability and proliferation of A549 cells.

Determination of the levels of pro-inflammatory cytokines IL6 and IL8 in A549 cells treated with SAME

The levels of IL6 and IL8 were measured in lung cells treated with SAME. As shown in Fig. 4, no change in the level of IL6 was observed ($P>0.05$). Although the level of IL8 was increased at 25, 50, and 100 µg/mL concentrations of SAME ($P<0.001$ for 100 µg/mL concentration), it was decreased rapidly at 200 µg/mL concentration of SAME. That decrease was not statistically significant when compared to the DMSO control ($P>0.05$).

Determination of level of Caspase-3 in A549 cells treated with SAME

Fig. 5 shows the effects of SAME on the level of caspase-3 in lung cancer cells. When compared to the DMSO control, the caspase-3 level was decreased at

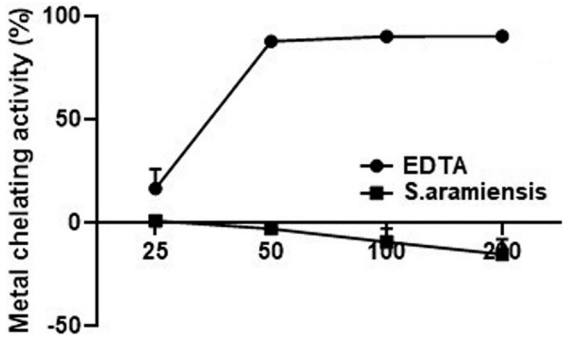

Fig. 2 — Percentage of metal chelating activity of SAME.

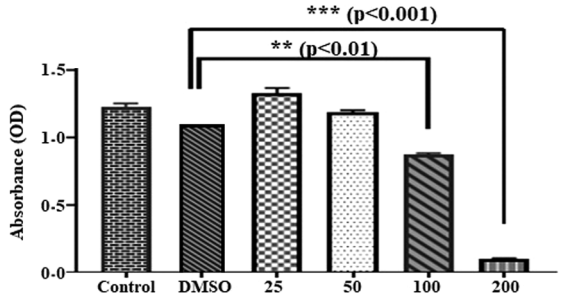

Fig. 3 — Cytotoxic effect of SAME on viability of lung cancer cells.

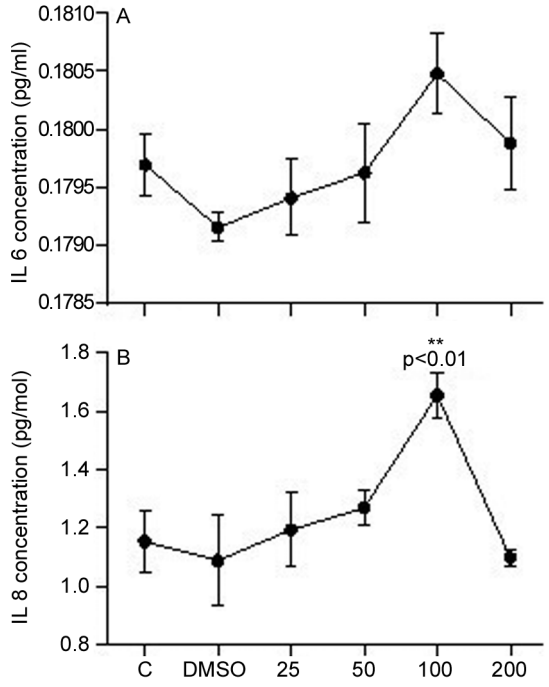

Fig. 4 — IL6 and IL8 levels in A549 cells exposed to SAME. C; Control, DMSO; Solvent control.

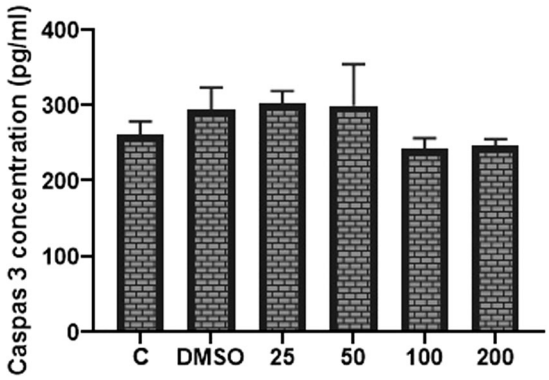

Fig. 5 — Caspase-3 levels in lung cancer cells exposed to SAME. C; Control, DMSO; Solvent control.

100 and 200 µg/mL concentrations of SAME. That decrease, was statistically not significant. Also, caspase-3 levels remained unchanged at 25 and 50 µg/mL concentrations.

Phenolic compounds are among the most abundant phytochemicals in plants and can exhibit a wide variety of bioactivities[17]. Phenolic compounds have subtypes such as flavonoids, phenylpropanoids, phenolic acids, quinone, tannin and lignin.[18]. These compounds generally neutralize free radicals by transferring an electron[19]. In the present study, luteolin was identified as the main phenolic compound of SAME. Luteolin is an important phenolic compound from the flavonoid group of phenolic compounds. It has many activities, especially antioxidant activity. Regarding luteolin in the literature, anticancer[20,21], antioxidant, anti-inflammatory and antiallergenic[22], neuroprotective[23,24], antidiabetic[25], antibacterial[26]. In this study, the number of phenolic compounds detected in SAME was lower than the number of phenolic compounds detected in other studies. It has been considered that differences in the number and amount of phenolic compounds are due to factors such as extraction, harvesting time, and geographical features.

Antiproliferative activity assays demonstrate the cytotoxic effects of extracts or compounds on cell viability. Previous research demonstrated that the application of plant extracts inhibited the viability of numerous cancer cells[27-30]. In the present study, it was determined that SAME has a potent cytotoxic effect on A549 lung cancer cells in a dose-dependent manner. Although there are studies demonstrating the cytotoxic effects of *Salvia* species on cancer cells[27], no studies have been found on the antiproliferation activity of SAME. Therefore, the present study has been considered to be the first report showing the antiproliferative effect of SAME on lung cancer. On the other hand, the antiproliferation assays are insufficient for determining the anticancer activity alone and should be augmented by further molecular research. Therefore, the present study determined the levels of various molecular markers, including interleukins IL6 and IL8, which promote the proliferation of lung cancer cells, and caspase3, which is an apoptotic marker.

The levels of IL6 and IL8 are known to be elevated in cancer cells. In a previous studies, the serum levels of IL6 were found to be elevated in patients with lung cancer[31]. Pine *et al.*[32] demonstrated that IL6 and IL8 levels were elevated in the serum of patients with lung cancer. An increase in IL8 levels several years before diagnosis increases the risk of lung cancer. In this context, suppressing lung cancer cells can be ensured by preventing the rise of IL6 and IL8 levels. For this purpose, the levels of IL6 and IL8 in lung cells treated with SAME were measured. Because high concentrations of SAME can reduce IL8 levels in particular, it is believed that it may have a protective effect against lung cancer.

The induction of apoptosis in cancer cells has been targeted in numerous anticancer research using synthetic or natural substances. The controlled cell death process known as apoptosis is suppressed in

cancer cells[33]. In cells, the balance between apoptotic and antiapoptotic protein is maintained. Apoptotic protein levels are elevated, particularly when a mutation occurs. Intracellular mechanisms trigger the intrinsic pathway, whereas the extrinsic pathway is driven by external stimuli such as TNF-α and FASL[34]. Caspase-3 turns on in both ways and activates the CAD enzyme, one of the apoptosis indicators, causing DNA fragmentation. The present study evaluated the effects of SAME on the levels of caspase-3 in lung cancer cells. Caspas3 levels decreased at high concentrations, but this decrease was not statistically significant. Therefore, it is believed that the cytotoxic effect of SAME on lung cells may be associated with a caspase-independent apoptotic or non-apoptotic death pathways such as ferroptosis, necroptosis, *etc*.[34]. Therefore, it is recommended that future research investigate various cell death pathways or caspase-independent apoptotic system markers in order to elucidate the mechanism underlying anticancer activity of SAME.

In addition to anticancer activity, antioxidant activity of SAME was also evaluated in this study. DDPH scavenging and metal chelating are two of the most common antioxidant systems. In both systems, the concentrations of SAME exhibited modest antioxidant activity. In previous studies, the antioxidant activity of *S. aramiensis* in different test systems was evaluated. Kelen & Tepe[35] reported that *S. aramiensis* had a strong antioxidant activity in the DPPH system. Contrary to the findings in the scientific literature, in the present study, the weak antioxidant activity of SAME can be attributed to factors such as the low concentrations used, the different period of collection, and the extraction method.

Conclusion

In the present study, SAME was found to inhibit the viability and proliferation of lung cancer cells and reduce the level of IL8. High concentrations of SAME have been shown to reduce the level of Caspase-3 and therefore lung cancer cells are thought to die via a caspase-independent death pathway. Limitations of our study include the fact that SAME was applied to cells for only 24 h. In future studies, in order to better understand the effect of SAME on caspase, 48 and 72 h of treatment and concentrations should be increased.

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Conflict of interest

The authors declare no conflict of interest.

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