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Chemical profile, *in vitro* pharmacological activity and *Satureja cuneifolia* Ten. evaluation of essential oil based on distillation time

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ABSTRACT

The medicinal plant *Satureja cuneifolia* Ten. was widely utilized as spice, tea and traditional medicine. The objective of the current study was to examine the chemical composition and *in vitro* biological activities (LOX, MMP-1, and MMP-12 enzyme inhibition activity and cytotoxicity on A549 cell line) of *Satureja cuneifolia* extracts and essential oils. The essential oils of the flowering aerial parts were hydro-distilled at four different distillation times (5, 30, 60, and 180 min) using the Clevenger apparatus. The total essential oil and four fragments were compared in terms of the major component, yield, and distillation time. Volatile compounds of the infusion were extracted by using HS-SPME. Ethanolic extract had the strongest inhibition activity on the LOX enzyme (84.50%), while the essential oils exhibited more cytotoxic activity on the A549 cell line than the extracts. The oils and the infusion were analyzed using GC-MS and the primary chemicals identified by LC-MS/MS.

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KEYWORDS
Satureja cuneifolia; essential oil; biological activities; GC-MS/FID; LC-MS/MS

Introduction

Satureja, belonging to the Lamiaceae family, was represented by 16 species and 17 taxa in the flora of Turkiye. The endemism ratio was 31% as a species and 35% as a taxon (Celep and Dirmenci 2017; Başer and Kırımer 2018). These species were locally named as "kekik" and used as thyme in Turkiye. Many studies have been reported on the pharmacological activities of the chemical constituents of *Satureja* species worldwide. *Saturaja* species have various bioactive compounds, such as essential oils, phenolic acids, flavonoids, tannins, and inorganic elements responsible for antimicrobial, antidiabetic, anticholinesterase, antioxidant, anti-inflammatory, and cytotoxic activities (Akdeniz et al. 2021; Taslimi et al. 2020; Taskin et al. 2020; Gedik et al. 2022; Ezaouine et al. 2022; Kiziltas 2022). Studies have been done mostly on essential oils and extracts of *Satureja montana* L. and *Satureja hortensis* L. (Fierascu et al. 2018; Abou Baker et al. 2020; Gomes et al. 2020; Vilmosh et al. 2022). Although *Satureja cuneifolia* Ten. is consumed in abundance among the public with other species, there is not enough study about the plant (Taslimi et al. 2020). Besides, *S. cuneifolia* was one

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of the natural exported products in Turkiye and was utilised as a folk remedy against respiratory tract diseases and colds, especially in the form of tea. In addition to the production of plant essential oil, it is generally used in the food industry as a spice, flavoring agent, and herbal tea (Carikci et al. 2020). The main components of the EO of *S. cuneifolia* were carvacrol and *γ*-terpinene (Gedik et al. 2022), and its EO had important antimicrobial activity (Khoury et al. 2016). Due to the presence of phenolic acids and flavonoids, *S. cuneifolia* exhibited a number of biological properties, including those that were antibacterial, antidiabetic, anti-inflammatory, antiurease, anticholinesterase, and anti-inflammatory (Taskin et al. 2020; Taslimi et al. 2020).

Expression and functions changes of lipoxygenases (LOX) and their pro-inflammatory products played a role in the formation various cancers and in vascular, myocardial, peritoneal, liver, and urological disorders, and in inflammatory diseases such as Crohns' disease (Laczko and Csiszar 2020). Matrix metalloproteinases (MMP) have an important role regulating the biological processes such as trophoblast implantation, embryogenesis, bone growth, wound healing and tissue regeneration. Because of MMPs's multifunction, they are determinant in the formation of many different pathophysiological disorders including inflammatory and fibrotic diseases, arthritis, cardiovascular disorders, cancer (prostate, osteosarcoma, colorectal) (Tokuhara et al. 2019; Geervliet and Bansal, 2020). MMP-1 was detected in many tissues with both physiological and pathological remodeling. MMP-1 enzyme supports the re-epithelialization of wounds (Keskin et al. 2021). MMP-12 was mainly produced by macrophages and was associated with inflammatory skin diseases, atherosclerosis, aneurysms, and cancers. MMP-12 also plays a role in inflammatory respiratory diseases such as asthma and chronic obstructive pulmonary diseases, and neurological diseases such as spinal cord injury, multiple sclerosis, intracerebral hemorrhage, and ischemic stroke (Abd-Elaziz et al. 2021).

Lung cancer had an increasing incidence worldwide. It was known that lung cancer cannot be diagnosed until the advanced phase of the disease. Various risk factors caused lung cancer formation, such as demographic factors (age, gender, ethnicity), behavioral factors (tobacco and smoking), environmental factors (radon, especially for miners, asbestos, pollution, air quality, infection), and genetic factors (1.7-fold increase) (de Groot et al. 2018). Change in the extracellular matrix of cancer cells was a marker in cancer progression. MMP, proteolytic enzymes of the matrix metalloproteinase, were important biomarkers for lung cancer. MMP-12 overexpressed in lung cancer tissue and metastases. MMP have been reported to induce epithelial-mesenchymal transition (EMT) in the A549 cell line (human lung adenocarcinoma cell line), but the function of the MMP-12 was not fully understood in non-small cell lung cancer EMT (Hung et al. 2021).

Considering the important health benefits of *S. cuneifolia* and its use in food, it was thought that this plant should be considered in terms of its different pharmacological effects. Hence, the present study aimed at investigating the effect of the distillation time on the yield and composition of the essential oil. The compositions of the obtained total essential oil and essential oil fractions, as well as the volatile components obtained from the infusion with the headspace solid-phase microextraction (HS-SPME) method, were analyzed using gas chromatography/mass spectrometry (GC/MS). The phenolic composition of the ethanol extract and infusion prepared from the plant was determined by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS). The cytotoxic impact of the oil samples and extracts on the A549 cell line as well as their ability to inhibit the LOX, MMP-1, and MMP-12 enzymes were assessed.

Material and method

Materials

S. cuneifolia was collected during the flowering season on 27 August 2018 from an altitude of about 1864 m in Tota plateau, Sütçüler/Isparta, Turkey. The material was identified by Prof. Dr. Yavuz Bülent Köse (Anadolu University, Faculty of Pharmacy, Department of

Pharmaceutical Botany, 26470, Eskişehir-Turkiye). The material was dried in a well-ventilated room and preserved at room temperature until analysis. Air-dried flowering aerial parts of *S. cuneifolia* were studied in all experiments and analysis. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy of the Anadolu University, Eskişehir, Turkiye (Herbarium number of ESSE: 15510). Experimental materials (chemicals and solvents) were provided by Merck (Germany), Sigma (Germany), and Fluka (Germany). All experiments were performed with ultrapure water.

Isolation of the essential oil

The essential oils from flowering aerial parts of *S. cuneifolia* were isolated by hydrodistillation using a Clevenger apparatus. The oils were obtained at the given four different distillation times (5, 30, 60, and 180 min.) and no essential oil was obtained after 180 min. 180 min was determined as the total essential oil (total EO) distillation time. Essential oil fractions (Fr 1, 2, 3, and 4) were collected after boiling. After 5 min from the boiling, the first fraction was taken, and then the second fraction was taken for 25 mi later from the first fraction. The third and fourth fractions were obtained by applying the same method, at 60 and 180 min, respectively, during the distillation. All fractions were collected from the same Clevenger apparatus. The essential oils were kept at 4°C until further analysis.

Preparation of ethanol extract and infusion

20 g of flowering aerial parts of *S. cuneifollia* were broken into small pieces and macerated of 200 mL 70% ethanol (EtOH) using a shaker two times for 48 h and filtered into a beaker. The filtrate was concentrated by a rotary evaporator. The remaining aqueous residue was lyophilized using a lyophilizer.

In order to prepare the infusion of the plant, 5 g of broken plant material was weighed in a beaker and 100 mL of boiling distilled water was added to it and left for 10 min. After this period, the infusion was filtered into a new beaker. Volatile components were extracted by a SPME device as soon as the infusion was filtered. Filtrate was lyophilized using a lyophilizer. The dried infusion and ETOH extracts were kept at – 20°C.

HS-SPME method

The manual SPME device (Supelco, Bellafonte, PA, USA) with a fiber precoated of a 65-µm-thick layer of dimethylsiloxane/divinylbenzene (PDMS/DVB-blue) was used for the extraction process of volatile components of the infusion. The analysis procedure was carried out as described in detail by Demirci et al. (2005). This method was applied in triplicate.

Essential oil and infusion analysis with GC and GC-MS

The GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. The GC analysis was carried out using an Agilent 6890N GC system. To obtain the same elution order with GC-MS, the simultaneous auto-injection was done on a duplicate of the same column applying the same operating conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms GC and GC-MS analysis conditions were done as described in detail by Demirci et al. (2022). Identification of the essential oil and the infusion volatile components were compared to retention indexes and the process was carried out using the Baser Essential Oil Components Library, Wiley, Adams, and MassFinder 4 Library Scanning Software (McLafferty and Stauffer 1989; Hochmuth 2008). The analysis results are given in Table 1.

Table 1. Compounds of the total essential oil, the oil fractions, and volatile compounds of the infusion of *S. cuneifolia*.

RRI[a]	RRI[b]	Compound	Inf.	Total EO	Fr-1	Fr-2	Fr-3	Fr-4	IM
1014	(998–1029)[c]	Tricyclene	-	-	-	tr	tr	-	MS
1018		Methyl 2-methyl-butyrate	-	0.1	0.1	-	-	-	MS
1032	(1008–1039)[c]	*α*-Pinene	0.6	0.8	0.7	0.6	1.5	0.7	RRI, MS
1035	(1012–1039)[c]	*α*-Thujene	-	1.0	0.8	0.6	1.3	0.5	MS
1076	(1043–1086)[c]	Camphene	-	0.5	0.7	0.4	0.6	0.2	RRI, MS
1118	(1085–1130)[c]	*β*-Pinene	0.1	0.2	0.2	0.1	0.2	0.1	RRI, MS
1132	(1098–1140)[c]	Sabinene	0.3	tr	tr	tr	-	-	RRI, MS
1174	(1140–1175)[c]	Myrcene	**3.5**	1.4	1.1	1.0	1.6	0.9	RRI, MS
1176	(1148–1186)[c]	*α*-Phellandrene	-	0.1	0.1	0.1	0.2	0.1	MS
1188	(1154–1195)[c]	*α*-Terpinene	0.7	1.2	1.1	0.8	1.4	0.9	RRI, MS
1203	(1178–1219)[c]	Limonene	-	0.5	0.5	0.4	0.6	0.4	RRI, MS
1218	(1188–1233)[c]	*β*- Phellandrene	-	-	-	0.2	0.2	tr	MS
1213	(1186–1231)[c]	1,8-Cineole	**3.7**	0.8	1.1	-	-	-	RRI, MS
1246	(1211–1251)[c]	(*Z*)-*β*-Ocimene	0.1	1.1	0.8	0.8	1.5	1.0	MS
1255	(1222–1266)[c]	*γ*-Terpinene	**3.2**	**8.6**	**6.7**	**6.4**	**11.0**	**7.7**	RRI, MS
1266	(1232–1267)[c]	(*E*)-*β*-Ocimene	0.6	0.5	0.4	0.4	0.6	0.4	MS
1280	(1246–1291)[c]	*p*-Cymene	**16.3**	**11.9**	**19.2**	**7.9**	**5.3**	2.3	RRI, MS
1290	(1261–1300)[c]	Terpinolene	0.2	0.1	0.1	0.1	0.1	0.2	RRI, MS
1348	(1317–1357)[c]	6-Methyl-5-hepten-2-one	0.1	-	-	-	-	-	MS
1382	(1334–1394)[c]	*cis*-Allo-ocimene	0.4	-	-		-	-	MS
1393	(1372–1408)[c]	3-Octanol	0.7	0.3	0.5	-	-	-	MS
1409		*trans*-Alloocimene	0.3	-	-	-	-	-	MS
1452	(1411–1465)[c]	1-Octen-3-ol	0.3	0.3	0.4	-	-	-	MS
1452	(1412–1457)[c]	α, *p*-Dimethylstyrene	0.3	-	-	-	-	-	MS
1474		*trans*-Sabinene hydrate	0.7	0.9	1.6	0.1	-	-	MS
1497	(1462–1522)[c]	*α*-Copaene	-	-	-	-	0.2	0.5	MS
1535	(1496–1546)[c]	*β*-Bourbonene	0.7	-	-	tr	0.2	0.6	MS
1553	(1507–1564)[c]	Linalool	1.6	0.2	0.3	0.1	0.2	0.2	RRI, MS
1589	(1547–1589)[c]	*β*-Ylangene	-	-	-	-	0.1	0.3	MS
1597	(1550–1603)[c]	*β*- Copaene	-	-	-	-	0.1	0.3	MS
1556		*cis*-Sabinene hydrate	-	0.3	0.5	0.1	-	-	MS
1571	(1557–1625)[c]	*trans*-*p*-Menth-2-en-1-ol	0.2	-	0.1	tr	-	-	MS
1612	(1569–1632)[c]	*β*-Caryophyllene	-	0.7	0.2	0.4	**3.4**	**7.5**	RRI, MS
1614	(1576–1614)[c]	Carvacrol methyl ether	**3.7**	1.4	1.9	1.1	0.8	0.6	RRI, MS
1611	(1564–1630)[c]	Terpinen-4-ol	1.5	0.7	1.0	0.4	0.2	tr	RRI, MS
1628	(1583–1668)[c]	Aromadendrene	-	-	-	-	0.4	0.9	MS
1687	(1637–1689)[c]	*α*-Humulene	-	-	-	-	0.2	0.3	RRI, MS
1624	(1600–650)[c]	*trans*-Dihydrocarvone	0.8	0.1	0.2	tr	-	-	MS
1638	(1555–1645)[c]	*cis*-*p*-Menth-2-en-1-ol	-	-	tr	tr	-	-	MS
1645		*cis*-Isodihydrocarvone	0.6	tr	0.2	tr	-	-	MS
1683	(1665–1691)[c]	*trans*-Verbenol	-	-	0.1	-	-	-	MS
1694	(1641–1706)[c]	Neral	**3.3**	1.2	1.8	0.7	tr	-	RRI, MS
1704	(1655–1714)[c]	γ-Muurolene	-	-	-	-	0.7	1.4	MS
1708	(1658–1712)[c]	Ledene	-	-	-	-	0.3	0.9	MS
1706	(1659–1724)[c]	*α*-Terpineol	0.7	0.3	0.3	0.2	tr	-	RRI, MS
1719	(1653–1728)[c]	Borneol	**3.6**	1.5	**3.0**	1.3	0.3	-	RRI, MS
1726	(1676–1726)[c]	Germacrene D	-	0.3	-	0.1	1.0	2.1	MS
1755	(1692–1757)[c]	Bicyclogermacrene	-	0.5	-	-	0.9	2.1	MS
1741	(1698–1748)[c]	*β*-Bisabolene	-	-	0.3	0.4	3.2	**9.2**	MS
1740	(1680–1750)[c]	Geranial	**4.6**	1.6	2.4	1.1	0.3	-	RRI, MS
1751	(1699–1751)[c]	Carvone	0.2	-	-	-	-	-	RRI, MS
1773	(1722–1774)[c]	*δ*-Cadinene	-	0.1	-	0.1	0.6	1.6	MS
1776	(1735–1782)[c]	γ-Cadinene	-	0.1	-	-	0.3	0.8	MS
1784	(1763–1786)[c]	(*E*)-*α*-Bisabolene	-	-	-	-	0.2	0.4	MS
1765	(1764–1810)[c]	Cadina-1,4-diene	-	-	-	-	-	0.2	MS
1740	(1686–1753)[c]	*α*-Muurolene	-	-	-	-	-	0.1	MS
1802	(1747–1805)[c]	Cumin aldehyde	0.2	-	-	-	-	-	RRI, MS
1808	(1752–1832)[c]	Nerol	1.2	0.5	0.5	0.4	0.1	-	RRI, MS
1857	(1795–1865)[c]	Geraniol	**7.7**	**8.4**	**10.1**	**9.1**	**3.0**	2.3	RRI, MS
1890	(1868–1890)[c]	Carvacryl acetate	tr	-	-	-	-	-	RRI, MS
1940		4-Isopropyl salicylaldehyde	0.3	-	-	-	-	-	MS
2006		8,9-Dehydrothymol	tr	-	-	-	-	-	RRI, MS
2008	(1936–2023)[c]	Caryophyllene oxide	-	0.3	0.1	0.5	1.1	0.8	RRI, MS

(*Continued*)

Table 1. (Continued).

RRI[a]	RRI[b]	Compound	Inf.	Total EO	Fr-1	Fr-2	Fr-3	Fr-4	IM
2029	(1963–2029)[c]	Perilla alcohol	tr	-	-	-	-	-	MS
2113	(2070–2114)c	Cumin alcohol	0.1	-	-	-	-	-	RRI, MS
2144	(2074–2150)[c]	Spathulenol	0.1	0.4	0.2	0.5	0.8	0.9	MS
2181		Isothymol	0.2	tr	tr	tr	tr	-	MS
2198	(2100–2205)[c]	Thymol	**5.1**	2.3	0.8	1.1	0.9	0.7	RRI, MS
2221		Isocarvacrol	0.1	tr	tr	tr	tr	-	MS
2239	(2140–2246)[c]	Carvacrol	**25.0**	**48.1**	**38.9**	**61.8**	**54.1**	**48.9**	RRI, MS
		Total	**93.6**	**99.3**	**99.0**	**99.3**	**99.7**	**99.0**	

Inf.: Infusion; Total EO: Total essential oil; Fr-1:5 min; Fr-2:30 min; Fr-3:60 min; Fr-4: 180 min; RRI[a]: Relative retention indices calculated against *n*-alkanes; RRI[b]: literature on the polar column [c] (Babushok et al. 2011); %: calculated from FID data; tr: Trace (<0.1%); -: not detected.

Extract analysis with LC-MS/MS systems

AbSciex 3200 MS/MS detector was used for LC-MS/MS analysis. A negative ionization mode was preferred for ionization. Chromatographic separations were made with GL Science Intertsil ODS 150 × 4.6 mm, 3 µm column using Shimadzu 20A HPLC. For mass scanning, a mass range of 100–1000 amu was chosen. LC-MS/MS analysis of phenolic components was detailed by Karatoprak et al. (2022).

Enzyme inhibition activity

LOX enzyme assay

Samples were studied using the procedure outlined by Baylac and Racine's (2003) method. LOX enzyme was supplied type I-B; Glycine max, Sigma Aldrich Co., USA. The change in absorbance was taken at 234 nm for 10 min. Nordihydroguaiaretic acid (NDGA) was used as a positive control. Samples and NDGA have been studied at a concentration of 100 µg/mL. A 96 quartz microplate was used for the kinetic measurements in the experiment. All measurements were repeated three times and the results were expressed as Mean±Standard error.

MMP enzyme assay

The assays were processed as described in the manufacturer's instructions. MMP-1 (Catalog no: BML-AK404) and MMP-12 (Catalog no: BML-AK402) colorimetric kits were provided by Enzo Life Sciences Inc. (Farmingdale, NY, USA). The UV absorbance was read at 412 nm using a microplate reader (BioTek, PowerWave, Gen5 software, Winooski, VT, USA). The assays were carried out in a convenient 96-well microplate. N-hydroxy-2-[[(4-methoxyphenyl)sulfonyl] (2-methyl propyl)amino]-acetamide (NNGH) was used as a control inhibitor at a concentration of 0.04 µg/mL. The samples and NNGH were tested in triplicate. Data were expressed as Mean ±Standard error.

Cell culture and cell viability assay

Cell culture

In this study, the A549 (ATCC CCL-185, Human Lung Cancer Cell Series) cell line was grown in Roswell Park Memorial Institute Medium (RPMI) with 1% penicillin, streptomycin mix solution (Gibco-Invitrogen, Grand Island, NY, USA), and 10% fetal bovine serum. Cultures were maintained at 37°C in 5% CO_2 and 95% air.

Cell viability assay

The cytotoxicity of the extracts and essential oil samples on A549 cells was determined by 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) colorimetric method. Cells were

inoculated into 96-well microtiter plates (1×10^4 cell/well in 100 µL). Extracts were prepared at 4 mg/mL concentration in a cell culture medium containing 1% dimethyl sulfoxide (DMSO). DMSO alone was added to another set of cells as a solvent control (DMSO<0.5%). At the end of 24 hr, the supernatant of the cells was discarded. 3.25; 7.5; 15; 31.25; 62.5; 125; 250; 500 and 1000 µg/mL concentrated extracts were added as 100 µL to the wells. Essential oil samples were studied on a different plate and added to the wells in a volume of 100 µL. After 24 hr of incubation, the wells were emptied. MTT solution was dissolved in PBS (0.5 mg/mL) and prepared by transfer to a flask with sterile filtration. Then, MTT solution was diluted with 1/10 of the culture medium and added 100 µL to the wells. After 3 hr of incubation, the wells were emptied and 100 µL DMSO was added. Finally, the absorbance was read by using ELISA (Bio-Rad Laboratories Inc., USA) at 540 nm wavelength. The experiments were repeated three times and the results were given as Mean ±Standard error.

The absorbance values obtained from the control wells were averaged and this value was accepted as 100% viable cells. The absorbance values obtained from the wells treated with solvent and extract were proportioned to the control absorbance value and were considered as percent viability.

Statistical analysis

The variance homogeneity was tested by the Levene test. One-way analysis of variance was used in the comparison between multiple groups. Dunnett test and Tukey test were applied for multiple comparison tests at a level of $p < 0.05$ (SPSS for Windows, 12.0, SPSS Inc. Chicago, IL, USA).

Results and Discussion

Essential oil analysis

Flowering aerial parts of *S. cuneifolia* were hydrodistillated for 180 min. by using a Clevenger apparatus. Distilled oil was named total oil (total EO). Four different fractions (Fr 1–4) were obtained using the same procedure according to four different distillation times (5, 30, 60, and 180 min.) and analyzed by GC-FID and GC-MS systems, separately. The composition of the total EO and all oil fractions are given in Table 1 and their chromatograms were presented in Supplementary Material.

The total EO has been found to contain 42 components and the yield of the total EO was calculated as 2.7%. Total EO was taken, respectively, according to distillation time. 42 Compounds were identified in the Fr-1 (5 min) and the Fr-2 (30 min) fractions. The yield of the Fr-1 was found as 0.72%, while the essential oil yield was calculated as 0.74% in the Fr-2. 45 components were determined in the Fr-3 (60 min) and the yield of the Fr-3 was found as 0.16%. The last fraction (Fr-4) (180 min) has been found to contain 39 compounds and the yield of the Fr-4 was determined as 0.06%. Depending on the distillation time on the essential oil yield and the ratio in the obtained total EO is shown in Table 2.

The major compound was determined as carvacrol in all samples. This compound varied between 38.9% and 61.8% in all fractions (Fr-1–4) depending on distillation times, while it was found to be 48.1% in total EO. The other major components were p-cymene 2.3–19.2%, γ-terpinene 6.4–11.0%, and geraniol 2.3–10.1% of all fractions (Table 1).

The distillation times of the major component level (carvacrol, *p*-cymene, γ-terpinene, and geraniol) are shown in Figure 1, while the effect of the distillation time on essential oil yield is shown in Figure 2.

The results showed that amount of essential oil and its compositions were affected by distillation times. Compared to the other fractions, essential oil content taken in the 5 min (Fr-1) was found to have the most similar content with total EO content. According to current research, 30 min for

Table 2. LC-MS/MS analysis of *S. cuneifolia* EtOH extract and infusion.

No	T_R, [min.]	MW	[M-H]- [*m/z*]	MS/MS (-) [*m/z*]	Tentatively identification	EtOH Extract/ Infusion	References
1	17.1	595	594	107, 125, 135, 151, 161, 175, 193, 287, 459	Eriocitrin	EtOH, Inf.	Li et al. (2019)
2	18.4	609	608	301	Quercetin rutinoside	EtOH, Inf.	Jancheva et al. (2017)
3	19.7	579	578	119, 151, 175, 193, 227, 270, 295, 313, 325	Naringenin rutinoside	EtOH, Inf.	Sánchez-rabaneda et al. (2004)
4	20.3	594	593	285, 327, 447	Luteolin rutinoside	EtOH, Inf.	López-Cobo et al. (2015)
5	20.8	448	447	133, 151, 175, 199, 227, 241, 255, 285, 297 327	Luteolin glucoside	EtOH, Inf.	Taskin et al. (2020)
6	21.1	610	609	134, 199, 241, 283, 301, 325, 343, 355	Hesperidin rutinoside/ Quercetin rutinoside	EtOH, Inf.	Taamalli et al. (2015)
7	21.4	462	461	285, 327, 357	Luteolin glucuronide	EtOH, Inf.	Karatoprak et al. (2022)
8	22.1	360	359	135, 161, 179, 197, 223	Rosmarinic acid	EtOH, Inf.	Taslimi et al. (2020)
9	22.5	578	577	269	Apigenin rutinoside	EtOH, Inf.	Gopčević et al. (2019)
10	22.5	718	717	295, 321, 339, 519	Salvianolic acid B	EtOH, Inf.	Gomes et al. (2020)
11	22.8	494	493	135, 151, 179, 197, 251, 295, 359	Isosalvianolic acid	EtOH, Inf.	Kapp et al. (2020)
12	24.0	445	444	113, 175, 269	Apigenin glucuronide	EtOH, Inf.	Karatoprak et al. (2022)
13	24.4	288	287	107, 125, 135, 151, 161, 169, 269	Eriodictyol	EtOH, Inf.	Choulitoudi et al. (2021)
14	24.7	535	534	177, 359	Rosmarinic acid glucuronide	EtOH, Inf.	Miao et al. (2016)
15	25.7	695	694	161, 179, 197, 359	Rosmarinic acid derivative	Inf.	Taslimi et al. (2020)
16	26.3	494	493	161, 179, 223, 295, 313, 359	Salvianolic acid A	Inf.	Gopčević et al. (2019)

(*Continued*)

Table 2. (Continued).

17	26.6	462	461	113, 133, 175, 199, 217, 255, 285, 287	Luteolin glucuronide	EtOH, Inf.	Karatoprak et al. (2022)
18	27.6	272	271	119, 125, 151, 165, 177, 271	Naringenin	EtOH, Inf.	Choulitoudi et al. (2021)
19	27.6	302	301	121, 151, 179, 229, 45, 273	Quercetin/Hesperidin	EtOH, Inf.	Kiziltas (2022)
20	29.1	286	287	121, 133, 151, 175, 198	Luteolin	EtOH, Inf.	Taslimi et al. (2020)

Inf: infusion extract, EtOH: 70% Ethanol extract.

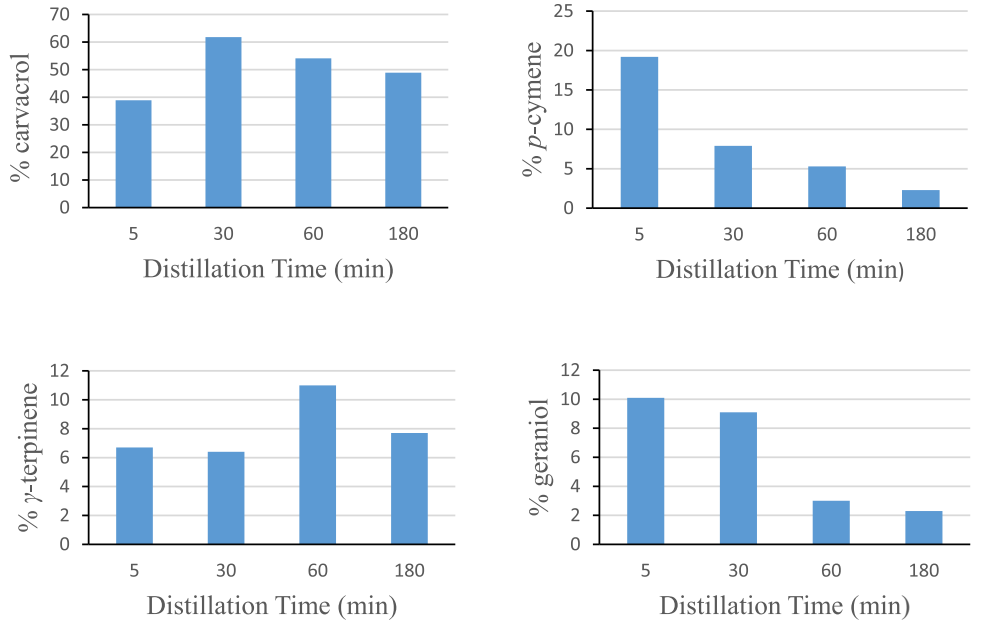

Figure 1. Effect of the distillation time on the main components of the fractions.

distillation duration was the best for increased carvacrol content and 60 min was the best for essential oil yield.

Koşar et al. (2008) compared the essential oil of *S. cuneifolia* in terms of content and yields during pre-flowering, flowering, and post-flowering periods and found that the main compounds in the oil were thymol (44.53-45.20-42.47%), *p*-cymene (24.07-19.43-24.30%), carvacrol (9.23-13.20-8.50%), *γ*-terpinene (4.57-4.20-2.50%), and borneol (2.80-2.97-3.57%) all in the three stages of the plant. The oil yields were calculated within the range of 1.1–1.3%. In the same analysis, monoterpene hydrocarbons (36.15%) were more abundant in the pre-flowering stage, while oxygenated monoterpenes (66.43%) were more abundant in the flowering stage. El Beyrouthy et al. (2015) examined the essential oils of *S.cuneifolia*

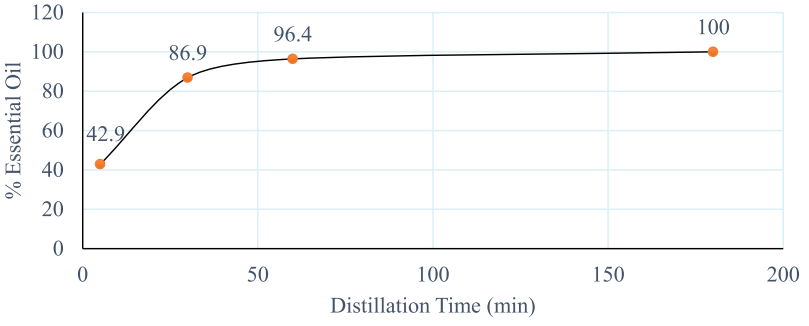

Figure 2. Effect of the distillation time on the yield of essential oil.

collected in three different seasons (before flowering, flowering, and maturing fruit) and collected from two different regions (Ehden and Faraya), in Lebanon. 35 compounds were detected in the oils and the major compounds were determined as carvacrol (20.4–52.1%), *p*-cymene (9.1–30.2%) and *γ*-terpinene (5.9–23.9%) in the oil. The oil yields of the fresh samples were 0.2-0-4% (before the flowering stage), 0.8–1.1% (flowering stage), and 0.4–03% (maturing fruit). The highest rate of carvacrol (52.1%) was found in the flowering period, while the sample with the highest rate of thymol in essential oil (18.7%) was collected in the Ehden region. The essential oil of the plant collected from the Dalmatia region in three different seasons (before, during, and after flowering) was investigated. 32 components were determined and linalool (18.2–17.2%), carvacrol (16.0–5.0%), *p*-cymene (14.8–4.0%), *α*-pinene (12.0–5.8%), and limonene (11.0–1.8%) were reported to be main compounds in the oils (Mirjana et al. 2004). These studies show that secondary metabolite synthesis in the plant varies depending on the different vegetation periods and localities of the plant.

In a different study, *S. cuneifolia* was collected from different localities (Kastamonu, Eskişehir, Manisa, İzmir, Burdur, Muğla, Antalya, and İçel) from Turkiye, and carvacrol (3–72%), *p*-cymene (7–31%), *γ*-terpinene (0.8–19%) were determined as the main components of the essential oil. Some of the samples have been reported to contain thymol (0.1–58%) as a major component in the same study (Tumen et al. 1998). Related studies in the literature had similar results to our study, according to the composition and the main component of the essential oil (Eminagaoglu et al. 2007; Oke et al. 2009; Yayli et al. 2014). However, there were some differences in essential oil composition between some analyses, particularly about minor compounds (for example, camphor, *β*-selinene, *α*-cadinol) (Kan et al. 2006; Ćavar et al. 2013; Kordali et al. 2022). In other studies, it was reported that thymol was the predominant compound by 35.8%–65.5% of the oil of the plant collected at different vegetation times from Konya (Akgül et al. 1999) and Çanakkale (Altun and Goren 2007). On the other hand, plant samples collected from Croatia were analyzed and the main components were characterized as *β*-cubebene (8.7%), limonene (8.3%), *α*-pinene (6.9%), spathulenol (5.3%), *β*-caryophyllene (5.2%). Interestingly, lower percentages of thymol and carvacrol were found (Skočibušić and Bezić 2004; Bezić et al. 2005). These differences in analyses may be due to the chemotypes of *S. cuneifolia*. The essential oil obtained from the above-ground part of *S. montana* was compared in terms of content using three different parameters: distillation times (2, 3, and 4 hr), different apparatus (Deryng apparatus and Clevenger apparatus), and two harvest years (2014 and 2015). The difference in distillation time from these parameters did not affect the ratios of the main components in the essential oil for both apparatus types, but changes occurred in the oil content with this parameter. The amount of essential oil and its chemical composition obtained with the Deryng apparatus was found to be higher than that obtained with the Clevenger apparatus. The type and relative percent of compounds synthesized by the plant varied in the two years (Wesołowska et al. 2017).

In the sum of the above results, environmental factors, distillation duration, distillation method, storage conditions, harvesting time, region, and year might all have an impact on the composition of essential oils. Distillation time has a significant impact on essential oil production and quality. The yield and quality of the essential oil have economic value for standardization of the essential oil (Azizi et al. 2009; Khorshidi et al. 2009; Toker et al. 2017).

Forty-three volatile components were detected in the infusion based on the analysis results. The abundant compounds were determined as carvacrol (25.0%), *p*-cymene (16.3%), geraniol (7.7%), and thymol (5.1%). Besides the difference in the number of compounds, the ratio of the main compounds in the infusion was found to be less than the total EO. When the infusion and the oils were compared in terms of their major contents, it was evaluated that the ratio of thymol (5.1%) in the infusion was higher than the total EO and the oil fractions (0.7–2.3%), while the ratio of *γ*-terpinene in the infusion was found to be lesser (3.2%) than the total EO and the oil fractions (6.7–11.0%). Yayli et al. (2014), on the other hand, used SPME-GC/MS to study *S. cuneifolia* collected in Antalya (Turkiye) and discovered that carvacrol (32.6%), *p*-cymene (22.2%), *γ*-terpinene (15.1%), myrcene (5.5%), and caryophyllene (3.3%) were the major components. Aside from the first two major compounds, it was established that there was a discrepancy between the volatile compounds of the plant studied by Yayli et al. (2014) and the findings of our investigation.

Enzyme inhibition assays

The samples were studied at 100 µg/mL concentration. EtOH extract (84.50%) had the strongest inhibitory activity on the LOX enzyme, while the other samples had no activity. The infusion (90.82%) showed higher inhibition on the MMP-12 enzyme compared to the EtOH extract (27.65%). None of the samples were found to be effective on the MMP-1 enzyme. The activities of the oil fractions were not assessed since total EO did not exhibit activity on any enzyme (Table 3).

Cell viability assay

The assay results are given in Figure 3 and 4. Cell control group viability was considered 100% and toxicity results were given as a percentage (%). The infusion, the EtOH extract, and the total EO with the oil fractions were evaluated for cytotoxic activity on A549 cells. The results showed that, when compared to the control, the EtOH extract and infusion were $p < 0.001$ significant at 1000 µg/mL concentration and $p < 0.05$ significant at 500 µg/mL. Over the concentration range between 3.25 and 125 µg/mL, viability did not significantly decline (Figure 3). When the findings of total EO and three fractions (Fr-1–3) were analyzed, they all significantly decreased viability when compared to the control ($p < 0.001$) (Figure 4). Fr-4 could not be tested due to the small quantity.

Although *S. cuneifolia*'s effects on the enzymes LOX, MMP-2, and MMP-12 as well as the A549 cell line have not been previously examined in the literature, its effects on different enzymes and cells have been evaluated. In a previous study, different extracts of *S. cuneifolia* were examined for antiurease, anticholinesterase, anti-inflammatory, and cytotoxic activity (MCF-7 and L-929). Direct methanol and fraction methanol extracts of the plant were found to be the most effective compared

Table 3. LOX, MMP-1 and MMP-12 enzymes % inhibition results.

Sample (100 µg/ml)	LOX	MMP-1	MMP-12
Total EO	NA	NA	NA
EtOH extract	84.50±0.25[b]	NA	27.65±1.24[b]
Inf.	NA	NA	90.82±1.97[a]
NDGA (20 µg/mL)	99.00±0.05[a]	NT	NT
NNGH (0.04 µg/mL)	NT	NT	91.43±3.43

*Standard error mean (n=3); NA:Not active; NT: Not tested; Data were presented as mean values±95% confidence interval; a-b Values within a column with different superscripts differ significantly (p<0.05).

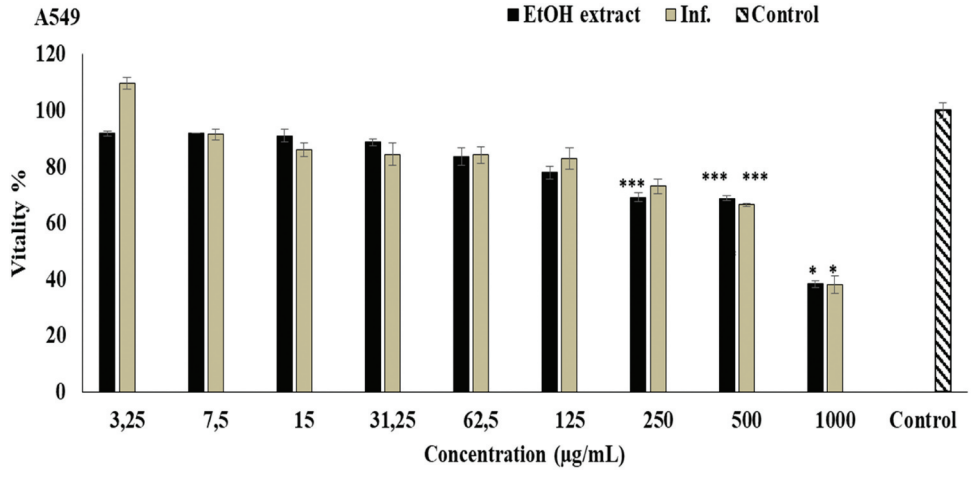

Figure 3. The cytotoxic effect of *S. cuneifolia* EtOH and infusion extracts on A549 cell line. Values were given as Mean±Standard error specified in the±95% confidence interval (*n*=3) *p<0.001 ***p<0.05

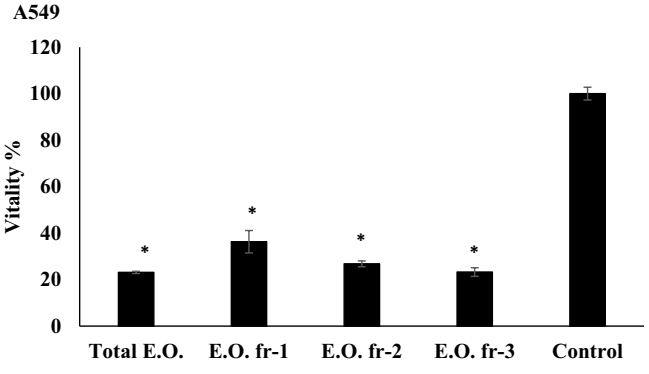

Figure 4. The cytotoxic effect of *S. cuneifolia* essential oil and the oil fractions on the A549 cell line. Values were given as Mean ±Standard error specified in the±95% confidence interval (*n*=3). *; *p*<0.001

to other extracts, and these extracts inhibited cholinesterase with 48.96% and 69.02%, at 500 µg/mL and urease with 9.65% and 12.52%, at 12.5 µg/mL, respectively. Moreover, the methanol extracts showed the most cytotoxic effect on the MCF-7 (39.92%) and L-929 (27.61%). In the paw edema test, it was reported that direct methanol extract showed very effective and rapid anti-inflammatory activity (65.9%) in the first hr, but the fraction methanol extract (27.1%) preserved its effect for 4 hr (Taskin et al. 2020). In a different study, the aqueous extract of *S. cuneifolia* dramatically decreased the viability of the liver cancer cell line (HepG2) at dosages of 1 to 30 µg/mL, as shown by the results of the 48- and 72-hour tests. (Yücel 2018). In studies investigating the effect of the plant on enzymes, it was reported that anti-cholinesterase (AChE IC_{50} = 63.69 µg/mL; BChE IC_{50} = 23.17 µg/mL) and anti-α-glucosidase (IC_{50} = 10.66 µg/mL) effects of *S. cuneifolia* methanol extract were higher compared to water extract (Taslimi et al. 2020), while the oil had a remarkable inhibition effect on AChE (86.5%) and BChE (98.4%) (Orhan et al. 2008).

Chromatographic analysis by LC-MS/MS

When the chromatograms of the EtOH extract and the infusion were examined, it was observed that the chemical components overlapped with each other (Figure 5 and 6). However, there were some

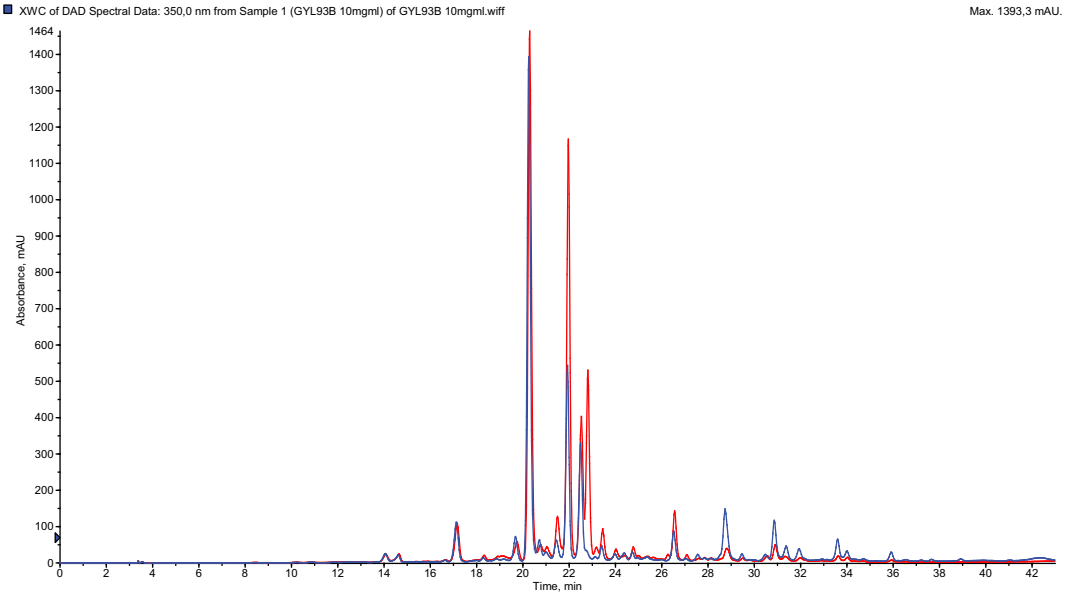

Figure 5. Chromatogram of infusion and EtOH extract, Blue color: EtOH extract, Red color: Infusion.

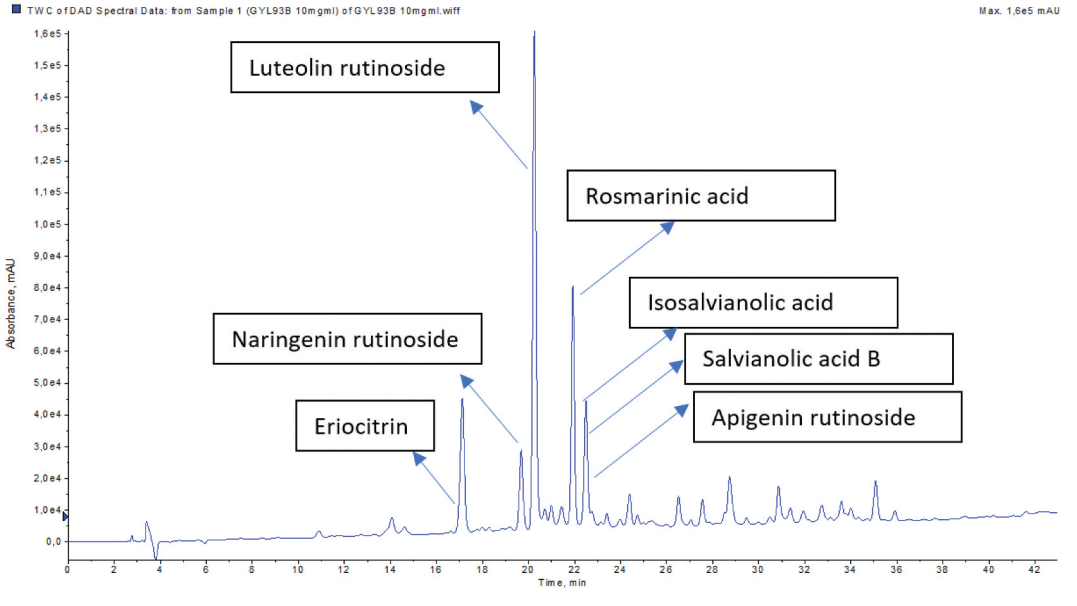

Figure 6. Chromatogram of EtOH extract.

differences in the relative % of these compounds. In the analysis of the EtOH extract and the infusion, 20 compounds were detected (Table 2) and luteolin rutinoside, rosmarinic acid, isosalvianolic acid, salvianolic acid B, apigenin rutinoside, eriocitrin, and naringenin rutinoside were found as the predominant compounds according to chromatogram Figures 5 and 6. When the same data (Figure 5 and 6) were examined, it was observed that the ratio of rosmarinic acid, isosalvianolic acid, and salvianolic acid B in the infusion was higher than in the EtOH extract. The public's use of the plant in the form of infusion demonstrates that these components will benefit more. Although

very few studies determine the chemical content of *S. cuneifolia* extracts by LC-MS/MS, one of them was carried out by Taslimi et al. (2020). According to the results of this study, cyanidin chloride, fumaric acid, and chlorogenic acid were found to be high, while the amount of luteolin derivatives and rosmarinic acid was found to be low in the methanol extract of the plant collected from the Antalya-Burdur region. When these two studies were compared, it can be considered that the difference in the dominance rate of the detected items stems from the place and time they were collected.

In the literature, the biological activities studies of *S. cuneifolia* and other *Satureja* species (*S. hotensis* L., *S. khuzestanica* Jamzad., *Satureja* macrostema (Moc. & Sessé ex Benth.), *Satureja parvifolia* (Phil.) Epling) have been carried out especially on hydroalcoholic (methanol, ethanol) extracts (Dikbas et al. 2008; Sadeghi-Nejad et al. 2011; Cabana et al. 2013; Gutierrez 2013; Taslimi et al. 2020) as well as extracts prepared with different polar solvents (hexane, petroleum ether, chloroform) (Gutierrez and Navarro 2010; Taskin et al. 2020). We also conducted our study on hydroalcoholic (EtOH) extract in accordance with the literature data results. The biggest reason for this is that hydroalcoholic extracts are rich in phenolic and flavonoid compounds which contain bioactive structures and have strong biological activity (Taslimi et al. 2020; Taskin et al. 2020).

It was known that phenolic substances were highly antioxidant compounds. For this reason, it has recently attracted attention with both *in vitro* and *in vivo* studies, that it can be effective in the prevention, formation, and progression of many diseases such as oxidative stress-related neurodegenerative diseases, cardiovascular disease, rheumatoid arthritis, cardiovascular diseases, and cancer. Moreover, phenolic compounds act as a regulator of some enzymes and cell receptors (Roleira et al. 2015). According to the literature data, there was information that the compounds we detected in our study have anti-inflammatory effects via different pathways such as arachidonic acid synthesis (Odontuya et al. 2005; Han et al. 2015; Sroka et al. 2017; Içen et al. 2021).

Lung cancer (12.7%) was one of the most frequently diagnosed cancers in the world. Although there were many elements that contribute to the development of cancer, diet was one of the most essential. Plant-derived foods rich in phenolic compounds as well as their extracts were associated with anticancer activity (Roleira et al. 2015). As a result of their accessibility and nutritional value, plants were advantageous for human health. In this regard, the literature was supported by our findings.

Conclusion

In terms of analyzing the characteristics of essential oil extraction and figuring out the chemical composition of oil and extracts, this study was the first one on *S. cuneifolia*, a plant that was frequently consumed by people as food and tea. Based on the essential oil study, it can be concluded that the best distillation period for achieving the maximum carvacrol content was 30 min, whereas the best period for achieving the largest yield was 60 min. Additionally, the biological activities of *S. cuneifolia*, were linked in this study to its phenolic profile and the essential oil it contains. It was determined that the total EO contained 42 components and the infusion had 43 volatile components. The research showed that the main metabolites of the extracts were flavonoids and phenolic acids. The EtOH extract, the infusion, the total EO, and the oil fractions of *S. cuneifolia* were examined in this work for the first time to determine the LOX, MMP-1, and MMP-12 enzyme inhibitory activities as well as the cytotoxic impact on the A549 cell line. The infusion and EtOH extract were found to successfully reduce inflammatory indicators. Essential oils exhibited more cytotoxic activity on the A549 cell line than the EtOH extract and the infusion. Until now no study has been available about the volatile and phenolic components of *S. cuneifolia* infusion. This research will help us understand the phytochemical composition and pharmacological effects of *S. cuneifolia*, which was frequently utilized.

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