Phenolic screening and anticancer potential of various wild savory extracts

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ABSTRACT
Cancer is one of the leading causes of death in our age. In addition to the treatments used in cancer, plants are frequently used in complementary or alternative approaches. Plants of the genus Satureja are used worldwide for various purposes, primarily as complementary therapies. For this purpose, in this study, the antioxidant potential and phenolic content of the Satureja cuneifolia plant, which is frequently consumed as a spice and tea among the public, as well as its cytotoxic and apoptosis-inducing effects on cancer cells were tried to be revealed. The cytotoxic effect of the extracts prepared with three different solvents (methanol, ethanol and water) was determined by the MTT test in colorectal cancer (DLD1) and promyelocytic leukaemia cell lines (HL60), and then the expression levels of five apoptotic gene regions (apaf-1, bax, bcl2, casp3 and tp53) were evaluated with Real Time PCR. The antioxidant potential was determined via DPPH test and HPLC was used to screen for phenolic substances. As a result, it was determined that the methanolic extract had more radical scavenging effect (IC_{50} = 105.02 ± 0.034 µg/ml). In conclusion, the cytotoxic and apoptosis-inducing potentials of the extracts obtained from the S. cuneifolia plant were revealed the first time upon DLD1 and HL60 cell lines in this study, and this study is a pioneer for future studies in cancer therapy.

Keywords: Apoptosis; qRT-PCR; Spice; Turkey; Wild savory

INTRODUCTION
Phyotherapy has become a very important strategy for many diseases, especially cancer, in recent years, thanks to the advantages it provides in the treatment and prevention of diseases (Pan et al., 2010; Song et al., 2018). In general, the use of plants in the treatment of cancer has a long history, and for this reason, plants have been the main source of effective traditional and complementary therapies in cancer treatment (Sewell and Rafieian-Kopaei, 2014). About 20% of known plants have been used in pharmaceutical studies, affecting the health system in positive ways, such as in the treatment of cancer and harmful diseases (Naczk and Shaidi, 2006). The absence of effective therapeutic strategies for cancer control yet is the most important factor causing high mortality rates (Siegel et al., 2016; Geziçi and Şekeroğlu, 2019). A large number of plant species used in cancer treatment have been reported since ancient times, and today, there is an increasing interest in studies evaluating the effects of plants and phytochemicals obtained from plants. More than half of the anticancer drugs used today are derived from natural sources such as sea creatures, plants and microorganisms (Cragg and Newman, 2005).

The genus Satureja grows in Western Asia and the Eastern Mediterranean region, spreading in vast geographies such as Anatolia, Turkmenistan, Caucasus, Iraq and Iran. In Turkey, the genus represented by 15 species (Güner et al., 2012). Satureja taxa are medicinal plants that are notable for the existence of secondary metabolites such as flavonoids, terpenoids and tannins. Due to these crucial compounds, there are many studies carried out to determine its essential oil content, chemical content, ethno medical effects as well as pharmacological activities and antimicrobial activities (Lamaison et al., 1991; Mueller-Riebau et al., 1995). Moreover, they have long been known for their healing properties and have been used as traditional folk remedies to treat various illnesses such as nausea, indigestion, diarrhoea, cramps, muscle aches, and infectious diseases (Bezić et al., 2009).

More recently, interest in herbs and spices has increased not only for their spice and aroma properties but also for their...
biological potential (Albano and Miguel, 2011). *Satureja cuneifolia* is a perennial shrub and sprouts every spring with new branches full of leaves. *S. cuneifolia* Ten. is known as “Dağ Kekiği” in Turkey and commonly used as a spice (Kan et al., 2006). It is used to produce essential oils and aromatic water, in the mountainous regions in Turkey’s Aegean and Mediterranean regions is a well-known and aromatic plant widely used. The essential oil of this plant is applied to flavouring spices, soups, flavours, canned meats, spicy table sauce and sausages. The composition and antimicrobial effects of oils, is depending on the local conditions and growth stages of plant species (Bezić et al., 1999; Milos et al., 2001). As far as we know, there is broad literature information on various biological activities of *S. cuneifolia* (Milos et al., 2001; Taslimi et al., 2020; Taskin et al., 2020; Eminagaoglu et al., 2007; Tepe and Cilkiz, 2016; Oke et al., 2009; Bezić et al., 2005). Moreover, although there are many studies on the content of this plant, the bioactive properties and chemical composition of the plant can vary significantly from one region to another, depending on the changing habitat and environmental conditions (Ganamé et al., 2021; Le et al., 2020). According to the present literature, there are reported many studies that reveal the volatile components related to the species and as a result, there is too much data about that subject. As a different approach to gain new information, therefore, it is aimed to work on mostly phenolic compounds in this study. *S. cuneifolia* has been previously reported to exhibit *in vitro* anti-Alzheimer, antidiabetic, antimicrobial, antioxidant, antirelease anticholinesterase and cytotoxic potential (Taslimi et al., 2020; Taskin et al., 2020; Eminagaoglu et al., 2007; Tepe and Cilkiz, 2016), but there is no study on its cytotoxic and apoptosis-inducing effects on colorectal cancer and leukaemia cells. The main objective of this study is to reveal the phytotherapeutic potential of the extracts prepared using different solvents from *S. cuneifolia*, which is frequently used as a tea and spice. This study aims to show the phenolic contents, antioxidant capacities and cytotoxic and apoptosis-inducing effects of extracts obtained from the plant. This article is the first effort to understand the cytotoxic and apoptosis-inducing potential of different *S. cuneifolia* extracts.

**MATERIALS AND METHODS**

**Plant material and preparation of plant extracts**

Plant specimen was collected from its natural habitat and a sample material was stored in the herbarium of Selçuk University. The locality information of the plant specimen is Afyon, Emirdağ, around Karakuyu village, 1000 m, 38°05’22”N, 30°12’58”E and collected in 2018. The plant specimen was collected and identified by Dr Tuna UYSAL. The collection number of the plant specimen is TU-3750. After the collection, the aerial parts of the sample were dried in a sun-free environment and made ready for analysis. The sample was pulverized with herb grinder under sterile conditions and extracted with absolute methanol and ethanol for 6-8 hours using a Soxhlet apparatus (Raaman, 2006). The aqueous extract was prepared via maceration method. Obtained total extracts were evaporated at 40 °C. Extracts were coded as, methanolic extract SCUmet; ethanolic extract SCUet and the aqueous extract was SCUaq, respectively.

**HPLC analyses**

For the determination of phenolic compounds in the content of the extract, 100mg of each extract sample was dissolved in methanol (% 100) and the final volume of the solution was adjusted to 10 ml. After the mixture was filtered through a 0.22µM sterile filter, HPLC analyses were performed at Selçuk University Research and Development Center. Analyses were carried out via Shimadzu LC-20AD device equipped with UV/VIS (SPD-20A) detector and with using the INERTSIL ODS-3V (5µm; 4.6 x 250 mm) column. The method used by Wen et al., 2005 in the analyses was used with some minor modifications (Wen et al., 2005). In our study, 6 phenolic substances were scanned and quantified. The used standards are; phenolic acids (protocatechuic, caffeic, syringic and trans-coumaric), flavonoids (rutin trihydrate and naringenin). HPLC analyses were performed with at least three parallel tests and mean values were taken into consideration.

**Antioxidant assay**

DPPH analyses to reveal the antioxidant capacity were performed by following the Chu method with minor amendments (Chu et al., 2000). Absorption of the extracts and standards were measured at 490 nm using an Elisa reader.

**Cytotoxicity assay**

*Satureja* extracts prepared at different concentrations (0.09-0.75 mg/ml) were applied on the cells and incubated in two time periods (24-48 hours). Cell lines DLD1 (colorectal cancer) and HL60 (leukemia) used in cytotoxicity determination were kindly provided by Dr Ali Uğur URAL and Dr Zerrin CANTURK. Cells were routinely cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin and grown under conditions of 37 °C and 5 % CO₂. Cell proliferation experiments were performed via MTT (Sigma-Aldrich GmbH, Sterenheim, Germany) assay. The optical density of the plates was measured at 570 nm. Each experiment was performed in triplicate with at least three wells in the plate. Vinblastine was used as a positive control. IC50 concentrations were calculated from dose-response curves.
**PBMC Isolation and cytotoxicity assay**
Peripheral blood mononuclear cells (PMBCs) were isolated from fresh human blood with Ficoll reagent. The isolated cells cultured with DMEM medium and were grown under conditions at 5% CO$_2$ and 37 °C for 24h. Then the extracts applied for 24-48h. Cell proliferation experiments were performed via XTT (XTT Cell Proliferation Kit, Biological Industries) assay. The optical density of the plates was measured at 450-500 nm. Each experiment was performed in three replicates, at least two wells in the plate.

**RNA isolation and RT-PCR**
Total RNA was isolated with the AxyPrep Multisource Total RNA Miniprep Kit according to the manufacturer's recommendations, and the concentration and quality of the RNA samples were estimated with Nanodrop 2000 (Wilmington, DE, USA). Total RNA (0.5-1 µg) was converted to cDNA via the First strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Gene expression experiments were performed following the previously reported procedure (Şimşek Sezer & Uysal, 2021). The data were analysed comparatively with the CT method and the fold change was calculated with the formula $2^{-\Delta\Delta CT}$ (Livak et al., 2013).

**Statistical analysis**
All of the results were presented as mean values. Untreated cells were used as control both MTT assay and RT-PCR.

The statistical analysis was conducted using one-way analysis of variance (ANOVA), Dunnett and Games Howell tests were used as post hoc tests. Statistical analysis was performed using SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA) and p less than 0.05 was selected as the level of significance. Pearson correlation analysis was performed to determine the relationship between antioxidant effect and cytotoxic effect.

**RESULTS**

**HPLC analyses**
In the present study, 6 phenolic substances in the extract composition of *S. cuneifolia*; protocatechuic, caffeic, trans-p-coumaric and syringic acid, rutin trihydrate and naringenin were screened by HPLC and their amounts were determined. When the results were evaluated, different phenolic substances were determined in different amounts depending on the solvent (Fig. 1). Rutin trihydrate was common for methanol and ethanol extract, while naringenin was detected only in the ethanolic extract. On the other hand, four phenolic; protocatechuic, caffeic, trans-p coumaric and syringic acid were detected only in aqueous extract. The HPLC chromatogram of the *S. cuneifolia* extracts is shown in Fig. 1, together with analytical standards. The phenolic components and amounts were given in Table 1.

![HPLC chromatograms of studied samples](image_url)
Antioxidant potential of extracts
The antioxidant potentials of plants and plant-derived compounds can be defined as the level of prevention or inhibition of the oxidation of various biomolecules in the environment (Gülçin, 2012). Antioxidant, or free radical scavenging, activities of the *S. cuneifolia* extracts were determined using DPPH assay. DPPH radical scavenging activity of extracts was compared with ascorbic acid. The results are displayed in Table 2. When we evaluated the antioxidant activity results in general, it was found that the extracts had antioxidant potential but they have a lower antioxidant effect than ascorbic acid, and SCU-MET extract has the most antioxidant effect. When we list the antioxidant capacities of the extracts, it is SCU-MET>SCU-ET>SCU-AQ. Although there are various studies in the literature revealing the antioxidant properties of *S. cuneifolia*, the plant localities, and extraction methods used in these studies are different and the essential oil of the plant was generally studied. The antioxidant capacity of *S. cuneifolia* essential oil and methanolic extracts were evaluated and it was reported that methanol extracts of *S. cuneifolia* exhibited greater antioxidant activity (Taslimi et al., 2020; Eminagaoglu et al., 2007; Oke et al., 2009). Our results consistent with previous studies.

Cytotoxic effect of extracts
In order to reveal the cytotoxic effect of *S. cuneifolia* extracts on cancer cells, two cancer cell lines (DLD1 and HL60) were used. MTT assay was used for cytotoxicity assignment. In order to determine the cytotoxic effects, they were applied over 4 different doses (0.09-0.75 mg/ml) on cell lines and two different time intervals (24 and 48 h). The MTT assay results were given as a % viability graph in proportion to the control group. It was determined that all extracts showed a cytotoxic effect on DLD1 cells in a dose and time-dependent manner. (Fig. 2). When we evaluate the cytotoxic effects of the extracts on the HL60 cell line in general we can say that the extracts have merely a dose-dependent cytotoxic effect (Fig. 3). The IC_{50} values of the extracts were given in Table 2. According to the MTT results, the DLD1 cell line was more sensitive than the HL60 cell line to all extracts treated Therefore, DLD1 cells were selected for further study. On the other hand, no cytotoxic effect was observed on PBMC's (Fig. 3). Especially this finding is very important because of showing the selective effects of the *S. cuneifolia* extracts.

**Effects of extracts on apoptotic gene expressions**
In addition to the fact that a plant extract performs cell death, it is desirable to have this cell death by apoptotic pathways. In order to reveal the potential anticancer mechanism at the molecular level of *S. cuneifolia* extracts qRT-PCR analysis was used to detect the gene expression levels of *apaf-1*, *bax*, *bcl2*, *card4*, *casp3* and *tp53* genes in DLD1 cells. In gene expression determination studies, β-actin was used as an internal control and relative expression graphs were created. Real-Time PCR results were evaluated in general, it is seen that the extracts have variable effects on different apoptosis-related gene regions. When the expression levels of apoptotic gene regions were evaluated, it was found that methanolic and ethanolic extracts caused an increase in *apaf-1*, *bax/bcl2*, *card4*, *casp3* and *tp53* gene expressions that trigger apoptosis. On the other hand, there is a different situation in aqueous extract than others, it caused an increase in *apaf-1*, *bax* and *card4* expressions, while it did not cause a significant difference in other gene regions. The relative expression graphs of studied gene regions were given in Fig. 4.

**DISCUSSION**
Today, chemotherapeutic drugs are used in a wide range in the treatment of many diseases such as cancer, and new treatments continue in the field due to their side effects and damage to the immune system. Plants are a well-known potential source of many bioactive compounds,
and plant-derived compounds are preferred over others (Atanasov et al., 2021). Recently, it is very popular to reveal the various biological properties of plants consumed as food and the many components derived from them. The present study was conducted to reveal the potential anticancer activity of different extracts of *S. cuneifolia*, a well-known and aromatic plant. The cytotoxic effect of *S. cuneifolia* extracts against liver (HepG2) and breast cancer (MCF-7) has been reported in previous studies (Taskin et al., 2020; Yücel, 2018). As previous studies were limited to two cancer cell lines, the efficacy of the plant extract against other cancer cell lines is still lacking and further study of more cell lines is required. Therefore, this study is the first report on the anticancer potential of different extracts of *S. cuneifolia* on DLD1 and HL60 cell lines.

According to the literature survey, the antioxidant and free radical scavenging potential of *S. cuneifolia* is well known. Dorman, et al. (Dorman et al., 2004) reported the antioxidant properties of aqueous extracts from some Lamiaceae species grown in Turkey, including *S. cuneifolia*. Particularly *S. cuneifolia* extract, has been reported to be having usable in vitro antioxidant properties. In another study, the in vitro antioxidative properties of the essential oils and methanol extracts of *S. cuneifolia* were investigated and reported that they exerted antioxidant activities and especially methanolic extract showed greater activity in the DPPH system (IC$_{50}$: 68.0 ± 1.76 µg/ml) (Eminağaoğlu et al., 2007). Oke et al., (2009), also determined DPPH radical scavenging activity of the methanolic extract (IC$_{50}$ = 26.0 ± 1.2 µg/ml) and the essential oil...
(IC$_{50}$ = 65.1 ± 2.2 µg/ml) of *S. cuneifolia*. In another study, the composition and antioxidant capacities of volatile components of *S. montana* and *S. cuneifolia* essential oils were investigated in Croatia, and according to the DPPH results, these two species were reported to have very high reducing power (IC$_{50}$: 7.04±0.55- 28.90±1.14 mg/ml), which may be interesting for pharmaceutical and food engineering studies (Ćavar et al., 2008). Taskin et al., (2020), investigated various biological effects of *S. cuneifolia* fractions, including antioxidant effect, and reported that chloroform fraction and methanol fraction showed high antioxidant effect (IC$_{50}$: 0.03- 0.07 mg/ml), but petroleum ether fraction had low efficacy in the DPPH test. In a recent study on the antioxidant capacity of the plant, the antioxidant capacity of the methanolic and water extracts were investigated by various tests and it was reported that the methanolic extract had a stronger radical scavenging effect than the water extract (IC$_{50}$: 26.03- 30.63 µg/ml) (Taslimi et al., 2020). In this study, we found that the methanolic extract had the highest antioxidant effect. Although IC$_{50}$ values differ, our results are consistent with previous reports. There are many studies on the antioxidant potential and the chemical composition of the plant, and it should not be ignored that locality, environmental conditions and especially preferred extraction methods can change the chemical composition and radical scavenging effect of the extract or essential oil (Ganamé et al., 2021; Le et al., 2020).

Considering the use of a plant for medicinal purposes, it is very important to reveal its various biological properties and content. In particular, the antioxidant and cytotoxic effect is thought to be closely related to the phenolic content of plants. There are many factors that play a critical role in successfully isolating phenolic compounds from plant materials. Among these, the solvent selection is the most important (Bhebhe et al., 2016). In the present study, 6 phenolic substances in the extract composition of *S. cuneifolia*, protocatechuic, caffeic, trans-p-coumaric and syringic acid, rutin trihydrate and naringenin were screened by HPLC and their amounts were determined. Protocatechuic Acid (PCA), one of the phenolic substances identified in this study, is a simple phenolic acid and is recommended as a potential chemotherapeutic agent with its antioxidative properties and pro-apoptotic effects (Lin et al., 2011; Semaming et al., 2015; Abotaleb et al., 2020; Quinn et al., 2017; Gani et al., 2019). Another compound, caffeic acid, has anti-inflammatory and antiproliferative properties and has been reported to have antioxidant properties in normal cells and pro-oxidant properties in cancer cells (Kanimozhi and Prasad, 2015; Pelinson et al., 2019). Many *in vitro* and *in vivo* studies have documented the beneficial role of syringic acids in various cancers and non-communicable diseases (Boutayeb and Boutayeb, 2005; Kumar and Pandey, 2013; Kowalczyk et al., 2019; Gheena and Ezhilarasan, 2019). On the other hand, many beneficial effects of routine, especially anti-inflammatory and antioxidant effects, in the content of screened extracts have been described (Chen et al., 2000; Jung et al., 2007). In addition, the routine has been previously described to cause *in vitro* cytotoxic effects on cancer cell lines, including human colon cancer cells (Ben Sghaier et al., 2016; Guon and Chung, 2016; Kuntz et al., 1999; Menon et al., 1995).
Since the phenolic substances in the extracts have both antioxidant and cytotoxic effects, it can be thought that the antioxidant capacity and cytotoxicity observed as a result of the extract application come from the phenolic substances. According to the Pearson correlation analysis, a significant (p<0.05) positive correlation was found between the DPPH inhibitory capacities and cytotoxic effects of the extracts (methanol and ethanol) (Correlation coefficient DLD1- DPPH: Scuet 24-48h: 0.914 and 0.902; Scuet 24-48h: 0.893 and 0.930; HL60 -DPPH; Scuet 24-48h: 0.934 and 0.942; Scuet 24-48h: 0.902 and 0.907 respectively). On the other hand, the antioxidant and cytotoxic activity of Scuaq had a positive relationship (correlation coefficient DLD1- DPPH: 24-48h 0.761 and 0.857; HL60 -DPPH: 24-48h: 0.791 and 0.675 respectively), but that was not significant (p>0.05). Apoptosis, a programmed cell death that does not affect surrounding cells in terms of its mode of action, is considered a good target for anticancer therapy (Pfeffer and Singh, 2018). We determined by qRT-PCR that S. cuneifolia extracts showed apoptosis-triggering effects on pro-apoptotic and anti-apoptotic gene expressions in DLD1 cells. In the present study, apaf-1, tp53, card4 and Casp-3 genes were found to be upregulated in cancer cells after exposure to these extracts; these proteins play an important role in tumour suppression, cell cycle arrest and application phase of cell apoptosis (Delbridge et al., 2012; Kao et al., 2015; Kobayashi et al., 2000; Mattson and Bazan, 2012; Umetani et al., 2004; Carneiro and El-Deiry, 2020; Yu et al., 2019). On the other hand, the increase in the interrelated expressions of card4 and apaf-1 in the apoptotic pathway with the administration of aqueous extract may indicate that the aqueous extract causes apoptosis, but the pathway may be different. Representative CARD-containing proteins in apoptosis signalling are associated with apoptotic protease activating factor-1 (apaf-1) and receptor-interactive serine/threonine-protein kinase (RIP) (Rodriguez and Lazebnik, 1999; Shakeri et al., 2017). Based on the data we have obtained; we can clearly say that more studies are needed to fully elucidate the apoptotic pathways followed by the extracts. Our results show that treatment of cancer cells with S. cuneifolia extracts leads to changes in the expression pattern of apoptosis-related genes. Although apoptosis-inducing effects of S. cuneifolia have not been previously reported, we have found reports of similar effects from extracts from other species of the genus Satureja. Asadipour, et al. (Asadipour et al., 2020) reported the anti-leukemic effects of dichloromethane and hexane extracts of S. bachtiarica due to induction of apoptosis on K562 and Jurkat cells. It was previously reported that S. subspicata and S. loriensis induce apoptosis in human lymphocyte cell culture in mice by regulating pro-apoptotic and anti-apoptotic gene expressions in vitro and in vivo (Čakar et al., 2018). In addition, in another study, it was reported that S. khuzestanica extract induced apoptosis in human breast cancer cells (MCF-7) (Esmaeili-Mahani et al., 2018).

CONCLUSIONS

This study once again showed that herbal treatment methods have an increasing potential and importance in the fight against cancer. It is of particular importance that this effect originates from a plant that is frequently consumed as a spice in meals. Such studies, which try to determine active ingredients from extracts or drugs obtained from wild plant sources and to reveal their pharmaceutical potential, are very important for more specific studies to be conducted in the future. This study once again revealed the potential effects of Satureja species. The use of extracts and/or active substances obtained from Satureja species in the treatment of cancer may be promising. While some of the results obtained in this study are reported for the first time, other findings are in agreement with previous studies. And this supports the need for further investigation of the molecular action pathways of the extracts. Among the studies planned to be done in the future is the discovery of the active ingredient(s) that may be found in the extracts. In the next step, it will be determined whether there is a single substance responsible for the effect or if there is more than one substance with a synergistic effect. In addition, it will be tried to determine at the molecular level which pathways are triggered in apoptotic cell death by using different markers.

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Authors’ contribution
Simsek Sezer, EN designed the study, carried out the experiments and wrote the manuscript.

REFERENCES


