RESEARCH ARTICLE

The Investigation of Cytotoxic, Antioxidant, Antimicrobial, and Apoptotic Effects of *Satureja Cilicica* P.H. Davis Methanolic Extracts

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ABSTRACT

Species belonging to the genus *Satureja* have been used traditionally in the treatment of many diseases. *Satureja cilicica*, an endemic plant, is known as thyme or henna in Turkey, and it grows in Konya, Niğde, Adana, and Hatay. Also, the plant has antimicrobial and antifungal effects. In this study, the antioxidant and antimicrobial potential of methanolic extracts obtained from *S.cilicica* and the cytotoxic and apoptotic effects on colon cancer cell line were investigated. 2,2-diphenyl-1-picrylhydrazyl (DPPH) test was used to evaluate the antioxidant potential of extracts, minimum inhibitory concentration (MIC) test for antimicrobial effect and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test to determine cytotoxic effects. Real-Time PCR (RT-PCR) evaluated expression levels of two pro-apoptotic gene regions. As a result, it was found that methanolic extract had antioxidant and antimicrobial potential, showed a cytotoxic effect on colorectal cancer cell line and increased expression levels of pro-apoptotic gene regions studied.

Keywords: Anti-proliferative, DAPK1, DAPK2, MIC, Thyme.

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INTRODUCTION

The *Satureja* (thyme) plant is a member of the Lamiaceae, which is a subfamily of the Nepetoideae, and grows mainly in the Mediterranean region. It is known that many species of the *Satureja* plant have aromatic and medicinal properties. On the other hand, many cancer patients are turning to alternative and/or complementary treatment methods due to the serious side effects of chemotherapy and radiation therapy, the high mortality rate seen experienced in cancer disease.[1,2] *Satureja* species are generally subjected to antibacterial, antifungal, antioxidant, cytotoxicity, antidiabetic, anti-HIV, anti-hyperlipidemic, reproductive stimulant, expectorant, and vasodilatory activity studies.[3] The process of programmed cell death, or apoptosis, is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. The suicide program of inner cell protects the organism and maintains proper development by preserving tissue homeostasis and getting rid of damaged or infected cells that may interfere with normal functions.[4] It is known that apoptosis, also called programmed cell death, is a complex mechanism that causes its destruction to control the cell proliferation process or occurs in response to DNA damage.[5] The term apoptosis is a process that was used to describe the morphological processes leading to cellular self-destruction.[6] The study aims to determine the cytotoxic and apoptosis-inducing effects of extracts derived from Satureja cilicica (*thyme*), colloquially used as a spice, and to reveal their antimicrobial and antioxidant potentials.

MATERIALS AND METHODS

Plant

Satureja cilicica plants used in the study were collected from their natural habitats and brought to our laboratory. Prof. Dr. Tuna Uysal collected and identified the plant specimens.

Preparation of Extract

The Satureja cilicica plant's surface sections were dried in a suitable environment without sun exposure and then made into powder and prepared for extraction. After this process, plant powders were weighed and placed in the cartridge and extracted with methanol through the soxhlet apparatus.[7] It was evaporated in the rotary evaporator at 40ºC to remove the extracted solvent from the extraction. The crude extract obtained was stored at -20ºC.

Preparation of Cell Culture

The cells were cultured in a 25 cm^2 flask in a gas medium with 95% humidity and 5% CO_2 and 37°C CO_2 incubators. The density of the DLD-1 cells in the flask was controlled

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from time to time by an inverted microscope, and the dense cell passaging from flasks and cell proliferation was provided.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test

After counting the cells, cell culture was provided on 96-well plates. Cells were incubated at 37°C for 24 hours after distribution. Fresh media containing the desired final concentrations of extracts (0.09–0.75 mg/mL) were added to the cells after 24 hours and incubated for 24 and 48 hours. Extract concentrations were prepared by serial dilution. 5mg/mL^{-1} MTT solution was added to the cells treated with extracts for 24 and 48 hours at the end of the incubation period and then left for incubation in the incubator for 4 hours. At the end, 100 µL of isopropanol was added to each well to dissolve the formazan crystals formed by emptying the contents of the wells. After waiting for 4 hours, plates were read at a wavelength of 570nm in an enzyme-linked immunosorbent assay (ELISA) reader.[6] Wells to which extracts were applied have been compared to the control group, and viability % was determined. The analyses were performed in triplicate, and the average values were considered

Ribonucleic Acid (RNA) isolation and RT-PCR

For each extract and control group, the same number of cells were cultured into 12-well plates and incubated for 24 hours by adding IC_{50} concentration of the extract, and only the amount of Dimethylsulfoxide (DMSO) used in the preparation of the extracts was added to the control group and incubated for 24 hours. Then, all flask contents were discarded, and cells were removed and deposited by the centrifuge. Total RNA isolation was performed via the Axygen RNA isolation Kit. The RNAs obtained were stored at -86°C. The concentrations and purity of total RNAs were measured by using the Nanodrop 2000 instrument. Total RNA at an equal concentration (0.5–1 micrograms) was converted to cDNA through

Fermentas First Strand cDNA kit. Then, amplification of DAPK1, DAPK2 and B-Actin gene regions was performed by RT-PCR.

Antimicrobial Analysis

It was shown that many test samples of the Broth microdilution method were a potentially useful method for determining the Minimum Inhibitory Concentration (MIC). In microbiology, MIC is the lowest concentration of antimicrobial agents that will inhibit the visible growth of microorganisms after overnight incubation. For antimicrobial tests, sterile U-shaped 96-well microtiter plates were used. After adding 100mL of each prepared Muleller Hinton Broth (MHB) medium to each well of the plates, 100mL of the extracts diluted at 25 mg/mL were added to the wells. The extracts were diluted according to the Log2 base (12.5 mg/mL–6.1 µg/mL) by bringing them 100mL level each with an 8-channel automatic pipette and transporting them to the next wells.

RESULTS

MTT Test Results

After applying methanolic extract from Satureja cilicica on the DLD-1 cell line, the% viability plots obtained by the absorbance values of MTT test measured at the end of the 24 and 48 hour incubation period, at the end of 24 hours of application, when the activity on the plates started with an equal number of cells was evaluated as% viability, and the applied extract was 10% at 93 µg/mL, 30% at 187.5 µg/mL, 61% at 375 µg/mL, 81% at 750 µg/mL caused cell death. When we evaluate the results of 48 hours of extract application, it can be said that the cytotoxic effect increases depending on time. According to the measurements performed at the end of 48 hours, the extract produced 17% at 93 µg/mL dose, 44% at 187.5 µg/mL dose, 73% at 375 µg/mL dose, 85% at 750 µg/mL dose. When the graphs were evaluated, it was determined that the extract had dose and time-dependent cytotoxic effects in the administered dose and time interval.

Real-Time PCR Results

The results of RNA isolation from the control group after the IC_{50} dose of Satureja cilicica were applied onto the colorectal cancer cell line. cDNA synthesis was performed by using a reverse transcriptase enzyme from the obtained RNAs. Expression levels of RT-PCR and pro-apoptotic gene regions were investigated by using appropriate primers. Our study's amplification of two gene regions (DAPK1 and DAPK2) and B-Actin gene as housekeeping were performed. The values obtained after amplification were compared with B-actin, and relative expression levels were found. The graphs obtained are given below.

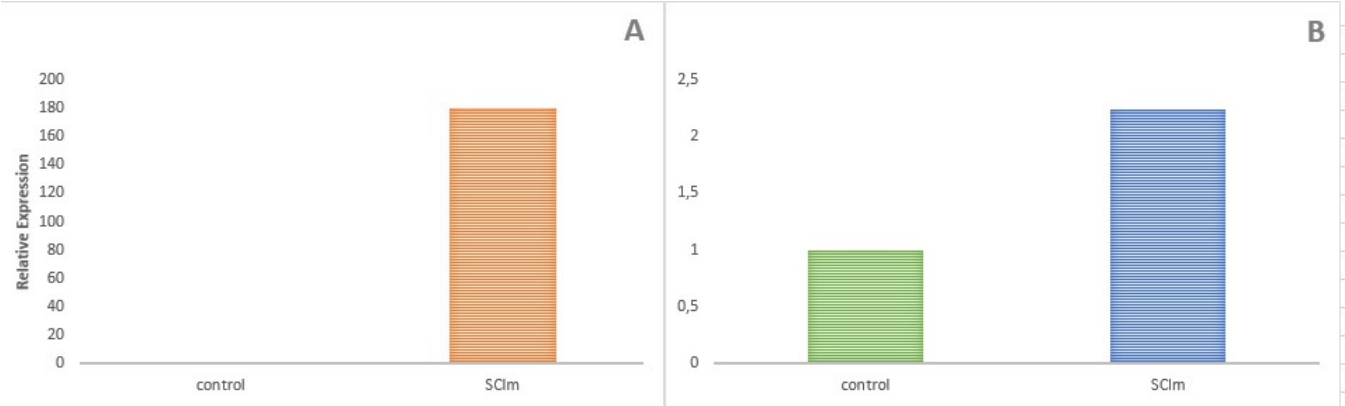

Figure 1: DAPK1 and DAPK2 gene expression levels after extract application

In our study, a significant increase in both DAPK1 and DAPK2 gene expressions was found after extract application to the DLD-1 cell line. DAPK1 gene expression increased approximately 180-fold compared to the control group, while DAPK2 gene expression increased approximately 2.5-fold. The applied extracts increased the expression levels of DAPK1 and DAPK2 genes in the apoptotic cell death pathway, which is generally inactive and/or lost function in cancer cells. This increase in the expression of pro-apoptotic gene regions following extract administration is an indication that cell death occurs by apoptosis.

Antimicrobial Analysis Results

Minimum Inhibitor Concentration (MIC) is the lowest concentration of an antimicrobial component or bacteriostatic agent. MICs are used to evaluate the effect of decreasing antibiotic and antiseptic concentrations over some time to inhibit microbial population growth. As a result of inoculating various concentrations of compounds with culture bacteria, the level at which the MIK point reacts is measured using agar dilution or broth dilution (macro or micro). The susceptibility test is typically performed using organisms that contribute to an infectious process that guarantees antimicrobial chemotherapy. Commonly used bacteria are ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and Enterobacter).

In our study, the microdilution method, which is the method we used for antimicrobial activity examination, determined minimally inhibitory concentration obtained by diluting test substances in certain proportions and decreasing doses regularly. The advantage of this method is that MIC value is determined with a small amount of substance to be tested. In the data obtained from our studies and examinations, no antimicrobial effect was observed in all test doses of Esherichia coli, Klebsilla pneumonia, Salmonella enteritidis, and Candida albicans microorganisms. Besides, Sarcina lutea, Bacillus cereus, Listeria monocytogenes, Candida parasilopsis microorganisms in the plate, 4 wells antimicrobial effect (0.781 mg/mL) was found. Antibacterial activity was determined in 2 wells (3.125 mg/mL) in Pseudomonas aeroginosa and methicillin-resistant S. aureus. Antimicrobial activity inhibition against Proteus mirabilis occurs in 3 wells (1.562 mg/mL).

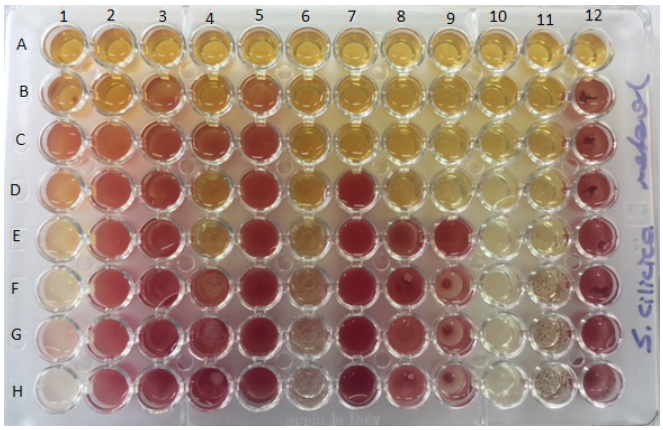

Figure 2: Plate view which is applied Satureja cilicica methanol extract; 1) *Esherichia coli,* 2) *Pseudomonas aeroginosa* 3) *Klebsiella pneumonia*, 4) Methicillin-resistant *S.aureus*, 5) *Salmonella enteritidis* 6) *Sarcina lotea* 7) *Proteus mirabilis* 8) *Bacillus cereus* 9) *Listeria monocytogenes*, 11) *Candida parasilopsis* 12) *Candida parasilopsis albicans*

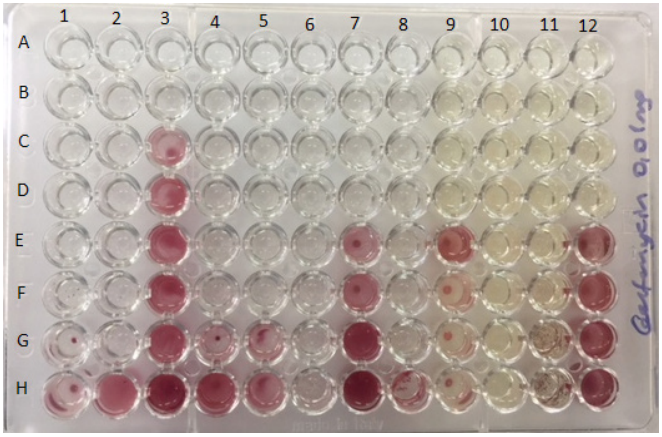

Figure 3: Plate view which is applied Gentamicin. 1) *Esherichia coli,* **2)** *Pseudomonas aeroginosa* **3)** *Klebsiella pneumonia,* 4) *Metisilin dirençli S.aureus, 5) Salmonella enteritidis* 6) *Sarcina lotea* 7) *Proteus mirabilis* 8) *Bacillus cereus* 9) *Listeria monocytogenes,* 11) *Candida parasilopsis 12*) *Candida albicans*

Table 1: The determined mic values of the studied plant extracts against the standard strains

Microorganisms		*Satureja cilicica (mg/mL)*	*Gentamicin (mg/mL)*
1	*Esherichia coli*-ATCC-25922	–	0.0001
2	*Pseudomonas aeroginosa*-ATCC-27853	3.125	0.0001
3	*Klebsiella pneumonia*-ATCC-70603	–	0.0012
4	*Metisilin direncili S. aureus-(MRSA)*ATCC-43300	3.125	0.0001
5	*Salmonella enteritidis*-ATCC 13076	–	0.0001
6	*Sarcina lutea*-ATCC 9341	0.781	0.00003
7	*Proteus Mirabilis*-ATCC 25933	1.562	0.0003
8	*Bacillus cereus*-ATCC 11778	0.781	0.00003
9	*Listeria monocytogenes*-NRRL-B-33314	0.781	0.0003
11	*Candida parasilopsis*	0.781	0.0001
12	*Candida albicans*-ATCC 26555	–	0.0003

DISCUSSION

Today, due to limitations such as cost and side effects of modern methods, treatments using herbs, widely used in the treatment of various diseases in every society and age group.[8-9] In this direction, the increasing interest in herbal medicine; can be counted as easy accessibility, cheapness, and harmlessness.[10] Species belonging to the genus Satureja are very popular plants in traditional medicine. There are many articles in the literature, including the antibacterial and antifungal activities of Satureja genus. Antimicrobial activities of *Satureja spp* were first reported in the 1950s, and the inhibitory effect has been shown to result from thymol and carvacrol content. The study reported the antibacterial effect of the methanolic extract obtained from S. cilicica has been identified that extract has a particularly strong antibacterial effect on P. vulgaris and S. aureus (MIC value: 1.6 mg/mL).[11] In our study, the highest antibacterial effect was observed on Sarcina lutea, Bacillus cereus, and Listeria monocytogenes (MIC value: 0.781 mg/mL). In our study, the MIC value of S. aureus was calculated as 3.125 mg/mL. In the study conducted by Arabacı et al., the cytotoxic activity of S. cilicica essential oil was determined by MTT test after 24 hours of application in MCF-7 cell line. The increased inhibition rate was reported due to increased dose, and IC50 value was determined (268 µg/mL).[12] Colorectal cancer cell line was used in our study. Depending on the different cell lines used naturally, cytotoxic results may also differ. In our study, the optimal IC50 dose of 48 hours was found to be approximately 0.225 mg/mL. There are no reports related to the cytotoxic effect of Satureja cilica, but there are studies on the use of various plants and plant extracts in colon cancer cell lines. The cytotoxic activity of plant extracts on the HT-29 cell line was investigated by MTT test to study ethanolic extract of Reissantia indica plant. According to MTT test results of Reissantia indica, the highest cytotoxicity of this extract against the HT-29 cell line was reported as 78.079% with a concentration of 1000 µg/mL with inhibition of cell growth. It has been reported to inhibit the HT-29 cell growth with the increase of extract dose applied in the study.[13] When the cells were examined microscopically, 24 hours after the addition of extracts, significant cell morphology, and density changes were reported. When the *P. auriculata* fractions were evaluated for cytotoxic effect, it was found that the dichloromethane fraction (CF) had the strongest cytotoxic effect. Even different extract concentrations of extract[14] were applied over three cell lines for 24 and 48 hours to evaluate the cytotoxicity of extracts obtained from Tribulus terrestris plant, and an MTT test was used to determine cytotoxic activity. As a result, Tribulus terrestris has been more effective on prostate cancer cell lines than colon and fibroblast cells. Although the cytotoxicity results and IC50 values obtained in various studies show differ, the dose and the time-dependent cytotoxic effect observed in our study is consistent with other studies conducted with Satureja species. It is necessary to know how cell death occurs rather than the cell death of the plant or herbal preparation to be used in cancer treatment. In addition to its cytotoxic effect, the extract must have the capacity to induce apoptosis. Apoptosis-mediated cell death is preferred since it does not produce an immune response. Reactivation of the mechanism of apoptosis lost in cancer cells can occur in two ways; these are activation of pro-apoptotic genes and inactivation of anti-apoptotic genes. One of the pro-apoptotic genes involved in apoptotic cell death is the DAPK1 gene region. The results we get in our study, applied extract causes an increase in pro-apoptotic DAPK1 expression. DAPK1 gene expression level of resveratrol has been reported to increase compared to control.[15] Activation of DAPK1 and DAPK2 indicates that apoptotic cell death has occurred in cancer cells. Although the method of preparation of the extract in this study is different from the method we use, our results are consistent in terms of antibacterial effect. In a study conducted with *Satureja Montana L*, antimicrobial activity and potency against microorganisms examined were characterized by inhibition zone diameter, and MIC values were measured. According to the study results, it was reported that essential oil obtained from *S. Montana L* has antibacterial activity on nine bacteria and ethanolic extract on eight bacteria.[16] Although the plant species used differ, the results are similar in terms of antibacterial effect. In our study, the extract was found to have an inhibitory effect on nine different bacteria.[5] the antimicrobial activity of *S. laxiflora's* essential oil is reported to be due to thymoline, the main component of the essential oil.[17] The effect of *S Montana* and *S. subspicata* oils have also been reported, and essential oils from *S. spicigera*, *S. biflora*, *S. masukensis* and *S. pseudosimensis* showed high inhibition against a wide range of microorganisms.[18-20] When we evaluate our antibacterial results from a general point of view, although there are limited studies with *S. cilicica*, our results are consistent with the literature regarding antibacterial and antimicrobial effects.

CONCLUSION

It was concluded from the current study that methanolic *S. cilicica* extract had antioxidant activity and antimicrobial potential activity, especially against *P. aeroginosa* and methicillin-resistant *S. aureus,* and methanolic extract showed a cytotoxic effect on colorectal cancer cell line and increased expression levels of pro-apoptotic gene regions studied.

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