Investigation of Antimicrobial, Antiproliferative and Antioxidant Properties of *Satureja boissieri* Essential Oil

Satureja boissieri Uçucu Yağının Antimikrobiyal, Antiproliferatif ve Antioksidan Özelliklerinin Araştırılması

Research Article

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ABSTRACT

The aim of this study was to investigate the biological activities of *Satureja boissieri* Hausskn. ex Boiss. (Lamiaceae) essential oil. Antimicrobial effects of the major components from the essential oil were determined by agar well and disc diffusion methods. Antiproliferative effect of the essential oil against human colorectal adenocarcinoma (HT-29) cell line was evaluated by Real Time Cell Analyzer xCELLigence method. In addition, total phenolic content and radical scavenging activity of the essential oil were determined. The results show that thymol, thymol 40%:carvacrol 60%, and terpinene-4-ol 25%:carvacrol 75% had significant antimicrobial activity against *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 strains. Also, the essential oil exhibited the high inhibitory effect on the growth of HT-29 cell line (IC_{50}<10 μg/mL).

Keywords

Satureja boissieri, antimicrobial, antiproliferative, HT-29.

ÖZ

Bu çalışmanın amacı *Satureja boisiseri* Hausskn. ex Boiss.(Lamiaceae) uçucu yağının biyolojik aktivitelerinin araştırılmasıdır. Uçucu yağın büyük bileşiklerinin antimikrobiyal aktivitesi agar kuyu ve disk difüzyon yöntemleri ile belirlenmiştir. Uçucu yağın, insan kolorektal adenokarsinom (HT-29) hücre hattına karşı antiproliferatif etkisi Gerçek Zamanlı Hücre Analizörü xCELLigence yöntemi ile değerlendirilmiştir. Ayrıca, uçucu yağın toplam fenolik miktarı ve radikal giderim aktivitesi de tespit edilmiştir. Sonuçlar göstermektedir ki; timol, %40 timol : %60 karvakrol, %25 terpinen-4-ol: %75 karvakrol S. *aureus* ATCC 29213 suşlarına karşı önemli bir antimikrobiyal aktiviteye sahiptir. Bununla birlikte, uçucu yağ HT-29 hücre hattının büyümesi üzerine yüksek bir inhibisyon etki göstermiştir (IC_{50}<10 μg/mL).

Anahtar Kelimeler

Satureja boissieri, antimikrobiyal, antiproliferatif, HT-29.

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INTRODUCTION

Antimicrobial activities of essential oils have been extensively investigated due to their potential applications in the food and pharmacy industries. This is of particular relevance due to the increased resistance of bacteria to antibiotic agents, and to the more common microbiological agents used in food preservation [1]. *Staphylococcus aureus* is an important pathogen that causes a wide range of infections and rapidly develops resistance to some antimicrobial agents. In the USA, approximately 35 hospital strains of *S. aureus* are resistant to methicillin (or other penicillin antibiotics) and in recent years the emergence of vancomycin-resistant *S. aureus* has caused additional concern [2].

Colon cancer is one of the greatest cancer incidences and death in the world [3]. Colorectal cancer (CRC) is among the top three most commonly diagnosed cancers in the world, accounting for 8% of all cancer-related deaths annually[4] . Recently, colon cancer rates are increasing due to the changing of dietary pattern. Diet is an important factor in both development and prevention of carcinogenesis[5]. Reports on the use of herbs are as old as humanity and have demonstrated that plant-derived essential oils exert better therapeutic activity than their isolated major compounds. In addition, the essential oils are the products of extraction of a plant species, so they are more concentrated and may exhibit higher toxicity than the original plant [6].

Satureja L. is a genus of the well-known aromatic plant of the family *Lamiaceae*. The aerial parts of these species have distinctive tastes and can be added to stuffing, meat, chicken, pies and sausages as a seasoning [7]. *Satureja boissieri* is known as Catri/Kekik and used as a condiment and herbal tea in the kitchen of local people in Turkey [8]. The main objectives of this study were i) to determine the antimicrobial activity of the major components and the essential oil against reference strains of *Staphylococcus aureus* by using disc diffusion and agar well diffusion methods ii) to evaluate the antiproliferative effect of the essential oil against HT-29 cell line and iii) to determine total phenolic content and radical scavenging activity of the essential oil.

MATERIALS and METHODS

Chemicals

Mueller Hinton Broth, Mueller Hinton Agar and sodium chloride were purchased from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), 2,6-di-*tert*-butyl-4-methylphenol (BHT), fetal bovine serum (FBS), penicillin-streptomycin, Dulbecco's Modified Eagles Medium-High Glucose (DMEM-HG) and dimethylsulphoxide (DMSO) were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Tetracycline was purchased from Oxoid (Basingstoke, United Kingdom). All the chemicals and solvents used in this study were analytical grade.

Plant Material

Satureja boissieri was collected at the flowering stage in Ortaköy-Bingöl (Turkey) village in July 2012. The samples were identified and stored at the Herbarium in Biology Department of Bingol University.

Essential Oil and Isolated Compounds

2 kg of dried aerial parts of the plant were shredded and combined with liquid nitrogen in a clean metal container. Then, the plant materials were processed for approximately 3 h in a Clevenger hydro-distillation apparatus. 28 mL (~27.3 g) of the essential oil was obtained. Anhydrous sodium sulfate was added to eliminate residual water in the oil. Finally, filtration was performed using blue band filter paper. The oil was stored in amber glass bottles at +4 °C. We have recently reported the isolation of pure compounds from S. boissieri essential oil [9]. Column chromatography was performed using analytical grade solvents and with loading 7.68 g (8 mL) of the crude essential oil. The pure chemical structures were determined by GC-MS (Agilent Technology 7890A GC system coupled to a 5975C inert MSD with Triple-Axis Detector (Agilent Technologies)) USA) [9].

Test Bacteria

Lyophilized ATCC bacterial strains were purchased from Mecconti (Italy). Lyophilized cultures were revived by breaking the valve, squeezing cap until all fluid moistens the lyophilized pellet in the bottom of tube, transferring the hydrated culture to Mueller Hinton Agar, and incubating inoculated media at 37°C. Following the incubation, representative well-isolated colonies were selected. Bacterial cultures were cultivated on Mueller Hinton Agar at 37°C for 24 h. Then, the bacteria were suspended in sterile saline solution 0.875% (w/v). The suspension was adjusted to 0.5 McFarland scale to obtain standardized suspension equivalents of 10^8 CFU/mL.

Inhibitory Effect by Agar Well Diffusion Method

The determination of the inhibitory effect of the essential oil and the components on test bacteria was carried out by agar well diffusion method [10]. The media (Mueller Hinton Agar) along with the inoculum (10^8 CFU/mL) was poured into the petri plate. The wells were prepared in 7.0 mm plates. 20 μL of the test compound (diluted in 10% DMSO to test concentration 1:5) was introduced into each well. DMSO (10%) was used as a negative control. The inoculated plates were incubated for 24 h at 37°C. After incubation, the diameter of the inhibition zone was measured with digital calipers. The measurements were done basically from the edge of the zone to the edge of the well.

Inhibitory Effect by Disc Diffusion Method

The disc diffusion method was employed for the determination of the antimicrobial activity of the essential oil and the components [11]. The media (Mueller Hinton Agar) along with the inoculum (10^8 CFU/mL) was poured into the petri plate. The filter paper discs (6 mm in diameter; Whatmann no: 1) were saturated with 6 μL of the test compounds, allowed to dry, and introduced on the upper layer of the inoculated agar plates. The inoculated plates were incubated for 24 h at 37°C. To determine the microbial growth, the diameter of the inhibition zone was measured using digital calipers. An antibiotic disc of tetracycline (30 μg/disc) was also used as a positive control.

xCELLigence Assay

The antiproliferative effect of the essential oil against the HT-29 cell line was determined by using the xCELLigence Real-Time Cell Analyzer–Single Plate (RTCA-SP) instrument (Roche Applied Science, Basel, Switzerland) according to method of Koldas, Demirtas, Ozen, Demirci and Behcet [12]. DMEM with 10% fetal bovine serum and 2% penicillin-streptomycin was used as the cell culture medium during the assessments. First, 50 μL of medium was added to each well of E-plate 96 and the plate was left in the hood for 15 min and then in the incubator for 15 min to let both the E-plate's golden electrode well bottoms and the medium reach a thermal equilibrium. Then, the E-plate was inserted into the RTCA station in the incubator and a background measurement was performed. After ejection of the Eplate from the station, 100 μL HT-29 cell suspensions were added to the wells to obtain a $2.5x10^4$ cell/well concentration in each well except for three of the wells. The cell concentration (cells/well) was analysed by using fully automated Cedex Hires Analyzer system (Roche Diagnostic Ltd, Rotkreuz, Switzerland) based on the manual Trypan Blue Exclusion Method. Three wells were left without cells to check if there would be an increase in cell index (CI) originating from the medium. 100 μL of medium was added to these wells instead of the cell suspension. After leaving the E-plate in the hood for 30 min, it was inserted to the RTCA station and the second step measurement was initiated for 80 min. During this period, the cells adapted to the bottom of the wells and entered into a growth and division phase. After this step, the E-plate was ejected from the station and solutions of the essential oil that was prepared with DMSO (final concentration of DMSO was less than 1% in each of the wells) and medium were added to the wells to obtain final concentrations of 100, 50, and 10 in each well, respectively. Then final volume of the wells was completed to 200 μL by adding medium. After this step, the E-plate was inserted into the station and the main measurement period of 48 hours was initiated.

DPPH Radical Scavenging Assay

The free radical scavenging activity of the

essential oil was determined by the method of Blois [13], with some modifications [14]. The solution of DPPH in methanol (0.004%) was prepared fresh daily and 1 ml of this solution in methanol was mixed with 1 ml of sample solution of varying concentrations. Each mixture was kept in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a blank on a UV visible light spectrophotometer (Rayleigh, UV-2601). BHT and BHA were used as positive controls. The activity was calculated using the following formula:

$$\text{Scavenging\%} = [(A_{control} - A_{sample}) / A_{control}] \times 100.$$

Determination of Total Phenolic Contents

The total phenolic content of the essential oil was determined according to the Folin–Ciocalteu method Singleton and Rossi [15], with some modifications [16]. Briefly, 0.1 mL of sample solution, 0.2 mLof 50% Folin-Ciocalteu's reagent and 1 mL of 2% Na_2CO_3 was mixed in a tube. Then, the mixture was incubated at room temperature for 45 min. The absorbance of the each mixture was measured at 760 nm. The same procedure was repeated for the standard solutions of gallic acid. Total phenolic contents of the sample was expressed in terms of gallic acid equivalent (μg of gallic acid/mg of essential oil).

Statistical Analysis

All experiments were done in triplicate. The results were expressed as means±standard deviations (SD). Statistical analyses were performed using the SPSS 11.5 (SPSS, Chicago, IL). Differences among means were done by analysis of variance (ANOVA), and averages were compared using the Tukey test. The level of statistical significance was taken at $p<0.01$.

RESULTS and DISCUSSION

Antimicrobial Activities of the Components against *S. aureus*

The inhibition zones of disc diffusion values of the essential oil and its isolated components against *S. aureus* strains were in the range of 11.84±0.68 - 27.08±0.22 mm, respectively (Table 1). Thymol and thymol 40% : carvacrol 60% components exhibited strong antimicrobial activity against *S. aureus*. Also, Thymol (3%): Carvacrol (97%) and terpinene-4-ol 25% : carvacrol 75% components showed remarkable antibacterial activity. Among the components, thymol was the most active against *S. aureus* ATCC 25923 (25.41±0.12 mm) and *S. aureus* ATCC 29213 (27.08±0.22 mm) by disc diffusion method. Tetracycline (standard antibiotic) exhibited a lower inhibitory effect against tested bacteria than thymol and thymol : carvacrol components. As expected, 10% DMSO (negative control) had no inhibitory effect.

The inhibition zones of agar well diffusion values of the essential oil and its isolated components (Figure 1) against *S. aureus* strainswere in the range of 3.62±0.16 - 9.35±0.02 mm (Table 2). Among the components, thymol was the most active against *S. aureus* ATCC 25923 (9.24±0.01mm) and *S.*

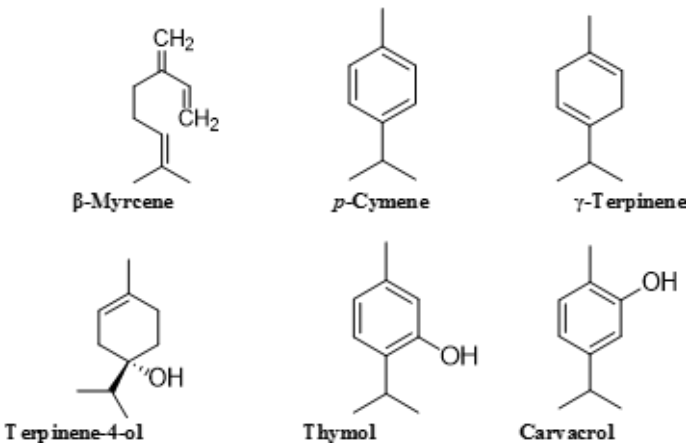

Figure 1. Isolated volatile compounds used in the antimicrobial activity test.

Table 1. Antimicrobial acitivities of the components from *S. boissieri* by disc diffusion assay*.

Material	Disc diffusion (mm)	
	S. aureus ATCC 25923	*S. aureus* ATCC 29213
The essential oil	14.23±0.20^{e}	13.78±0.04^{e}
β-mycrene 5%: *p*-cymene 80%:γ-terpinene 15%	14.05±0.72^{e}	11.84±0.68^{f}
Thymol (100%)	25.41±0.12^{a}	27.08±0.22^{a}
Thymol (40%): Carvacrol (60%)	23.35±0.01^{b}	26.43±0.49^{a}
Thymol (3%): Carvacrol (97%)	22.15±0.49^{c}	21.57±0.20^{b}
Terpinen-4-ol (25%): Carvacrol (75%)	17.64±0.23^{d}	18.12±0.43^{d}
Tetracycline	23.31±0.06^{b}	20.51±0.04^{c}

*Values represent averages ± standard deviations for triplicate experiments. Values in the same column with different superscripts are significantly ($p < 0.01$) different.

Table 2. Antimicrobial acitivities of the components from *S. boissieri* by agar well diffusion assay*.

Material	Agar well diffusion (mm)**	
	S. aureus ATCC 25923	*S. aureus* ATCC 29213
The essential oil	4.82±0.09^{e}	3.62±0.16^{e}
β-mycrene 5%: *p*-cymene 80%:γ-terpinene 15%	5.35±0.11^{e}	5.12±0.10^{d}
Thymol (100%)	9.24±0.01^{a}	9.35±0.02^{a}
Thymol (40%): Carvacrol (60%)	8.25±0.04^{b}	8.65±0.08^{a}
Thymol (3%): Carvacrol (97%)	7.44±0.03^{c}	7.34±0.03^{b}
Terpinen-4-ol (25%): Carvacrol (75%)	6.14±0.09^{d}	6.09±0.11^{c}

*Values represent averages ± standard deviations for triplicate experiments. Values in the same column with different superscripts are significantly ($p < 0.01$) different. **Materials were diluted in 10% DMSO due to the test concentration (1/5).

aureus ATCC 29213 (9.35±0.02 mm).

Essential oils rich in phenolic compounds such as thymol and carvacrol are reported to possess high antimicrobial activity against *S. aureus* [7,17,18]. Xu, Zhou, Ji, Pei and Xu [19] reported that the mechanism of action of thymol and carvacrol is disrupting to the cytoplasmic membrane; it increases its permeability and depolarizes its potential. Souza, Oliveira, Stamford, Conceição and Gomes Neto [20] determined that carvacrol and thymol suppress enzymatic activities in *S. aureus* and inhibit its production of staphylococcal enterotoxins. The reductions in the enzymatic activities of the cells and in the synthesis of enterotoxins most likely occurred due to a prevention of protein secretion, which could have been a consequence of changes in the physical nature of the staphylococcal cytoplasmic membrane [21].

In this study, the data of inhibition zones have indicated that the inhibitory effect of the whole essential oil was lower than those of the components. This effect may be due to the presence of components in various concentrations that can have additive, synergistic or antagonistic interactions. Different inhibitory effects were observed for combinations of carvacrol and thymol. The tested components which have high thymol percentage were more effective than the others. Thymol was the most effective antimicrobial substance against tested bacteria, which was consistent with other studies [22].

In this study, β-myrcene 5% :*p*-cymene 80% :γ-terpinene 15% exhibited the lowest inhibitory effect against *S. aureus*. Bagamboula, Uyttendaele and Debevere [23] reported components containing only an aromatic ring with alkyl substituents as in *p*-cymene have low antimicrobial activity. Galluci

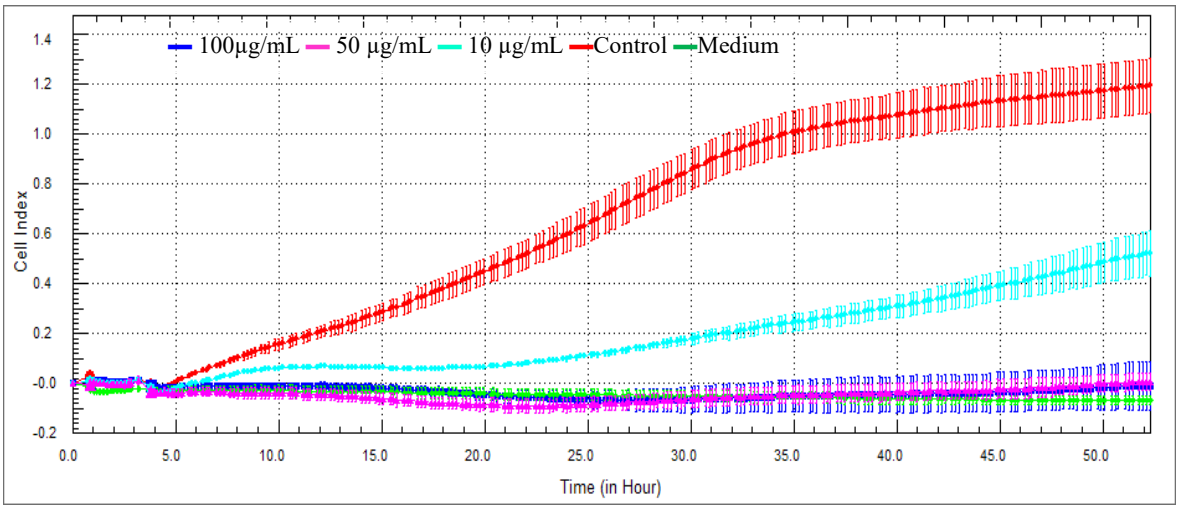

Figure 2. Real-time cell monitoring of the proliferation of HT-29 cells treated with the essential oil for 48 h period.

et al. [18] reported that the myrcene did not show antimicrobial activity against slime producing and non-producing *Staphylococci*. In addition, no antimicrobial activity has been reported for γ-terpinene [24].

S. aureus is a cause of local infections (wound infections, skin infections, deep infections such as myositis, osteomyelitis, endocarditis, pericarditis, pneumonia) which can develop into systemic infections such as bacteremia and sepsis. The second group of infections caused by *S. aureus* are toxin-mediated diseases [25]. Staphylococcal food poisoning (SFP) is an intoxication that results from the consumption of foods (meat and meat products, egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings) containing sufficient amounts of one (or more) preformed enterotoxin [26]. Therefore, controlling the numbers and growth of *S. aureus* remains an important objective for sectors of the food industry. *S. boissieri* is frequently used as a condiment and found as herbal tea. It may be used as a natural preservative against *S. aureus* for the food production industry. Morever, *S. boissieri* may be considered as natural and effective alternative agent to antibiotics due to the increasing antibiotic resistance of *S. aureus*.

Antiproliferative Effect by Real Time Cell Analyzer xCELLigence

The antiproliferative effect of the essential oil was examined on HT-29 cell line at the concentrations of 10, 50 and 100 µg/mL by using real time cell analyzer xCELLigence method. The system measures impedance differences in order to derive cell index values at time points whose intervals can be set by the operator. These impedance differences and thus the cell index values depend on the cell activity at the bottom of the wells [27]. Cell Index (CI) is a dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows us that the cancer cells are dying. Our data showed that the essential oil displayed significant antiproliferative effect against HT-29 cell line (IC_{50}< 10 µg/mL) (Figures 2 and 3).

Sadeghi, Yousefzadi, Behmanesh, Sharifi and Moradi [28] reported the cytotoxic effect of the essential oil from *S. boissieri* on KYSE30 and 5637 cells and they observed the oil significantly reduced cell viability of the cells in a dose-dependent manner starting 39 to 1000 µg/ml with IC_{50} 156 µg/ml. Studies are reported the antiproliferative effect of the essential oil from *Satureja* species against a variety of human cancer cell lines. Lampronti, Saab and Gambari [29] determined the antiproliferative effects of essential oils from different plants belonging to the Magnoliophyta division on human leukemic K562 cells and found that the essential oil derived from *Satureja montana* showed the most interesting activity in inhibiting the cell growth of the K562 cell line.

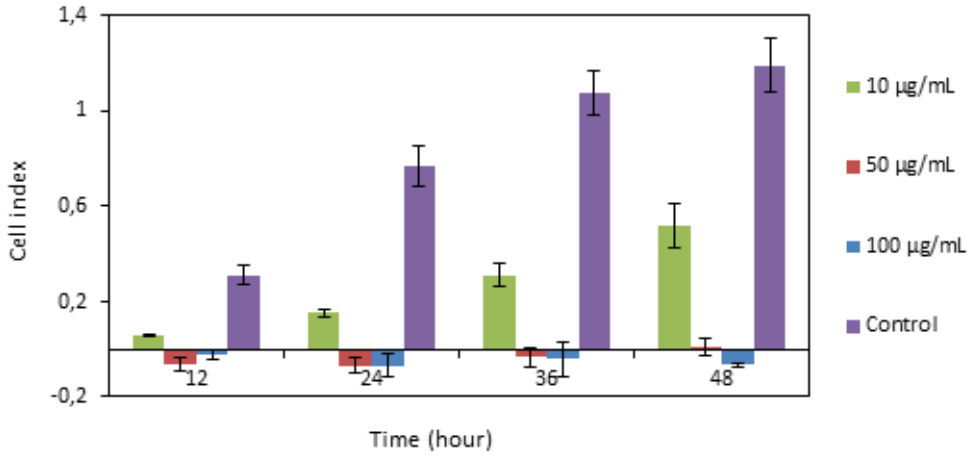

Figure 3. Cell index values for 12h, 24h, 36 h, and 48h after the treatment of the essential oil at the concentrations of 10, 50 and 100 µg/mL. Cell Index is a dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows us that the cancer cells are dying.

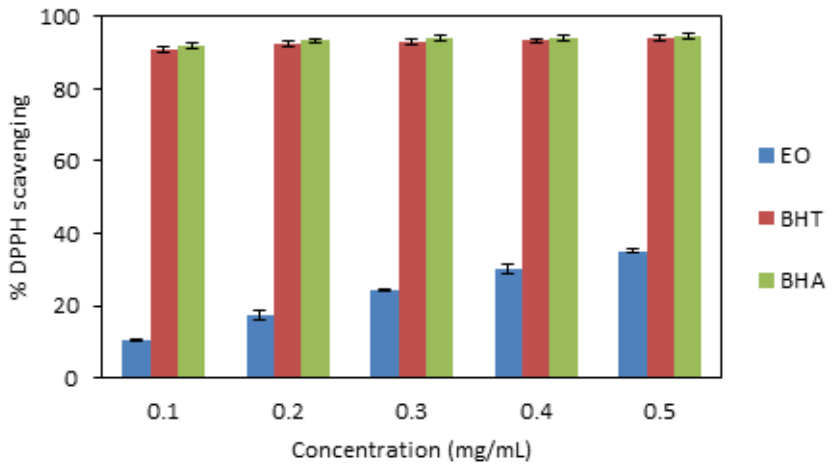

Figure 4. DPPH scavenging activity of the essential oil and synthetic antioxidants at tested concentrations (EO: The essential oil; BHT: Buthylated hydroxytoluene; BHA: Butylated hydroxyanisole).

Miladi et al. [30] observed that a concentration- and time-dependent inhibitory effect *S. montana* essential oil on human respiratory epithelial cell line (A549).

In our previous study, we reported the inhibitory effect of the essential oil and the isolated pure or mixture components from S. boissieri against human cervical carcinoma cell line (HeLa). In that study, the components containing the high concentrations of p-cymene, thymol and carvacrol effectively inhibited the growth of HeLa cells [9]. Koparal and Zeytinoglu [31] demonstrated the carvacrol was very potent cell growth inhibitor of A549 cell line. Therefore, in our study, the highest antiproliferative effect of the essential oil against HT-29 cells attributed the including active compounds p-cymene, thymol and carvacrol.

Antioxidant Activity of the Essential Oil

Free radical scavenging activity of essential oil from *S. boissieri* was measured by DPPH assay. Free radical scavenging increased by increasing the essential oil concentration (Figure 4). The IC_{50} value of the essential oil was determined as 0.760±0.019 mg/mL. The radical scavenging effect was low compared to synthetic antioxidants. Gulluce et al was determined the radical scavenging effect of *Satureja hortenis* essential oil with a IC_{50} value of 350.00±5.00 µg/mL. [32]. The total phenols content, which were measured

Table 3. Antioxidant activities of the essential oil from *S. boissieri*

Material	DPPH IC_{50} (mg/mL)	Total phenolic content (μg GAE/mg)
The essential oil	0.760±0.019[a]	214.46±6.74
BHT	0.022±0.001[b]	NS
BHA	0.003±0.001[c]	NS

[a]Values represent averages ± standard deviations for triplicate experiments. Values in the same column with different superscripts are significantly ($p < 0.05$) different. NS: not

by Folin-Ciocalteu reagent in terms of gallic acid equivalent, was 214.46±6.74 μg /mg, presented as gallic acid equivalent in μg /mg essential oil.

CONCLUSIONS

In this study, the most of the isolated components exhibited significant antimicrobial activity. The synergistic and antagonistic effects were found in combinations of the components. It was also established that the components containing the high concentrations of thymol effectively inhibited the growth of *S. aureus* strains. The essential oil exhibited the excellent inhibitory effect on the growth of HT-29 cell line. According to the results of this study, *S. boissieri* and its active components may be suggested as a new potential source of natural agent for pharmacy and food industries.

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