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Research Article

Biological Activities of Two *Stacys* Species: *Stachys thirkei* K. Koch and *Stachys macrantha* (K. Kock) Stearn

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Abstract

The study aimed to investigate the antioxidant activities of methanol extracts from aerial parts of *Stachys thirkei* and *Stachys macrantha* growing in Eastern Anatolia using the DPPH (1,1- Diphenyl-2-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethyl-benzothiazoline-6- sulphonic acid)) methods, as well as their cytotoxic effects on BPH, DU-145, PC-3, and LNCaP cell lines by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Radical scavenging activities were determined spectrophotometrically, revealing that both species exhibited high antioxidant capacity; however, *S. thirkei* demonstrated relatively higher activity. In the preliminary investigation of potential treatments for prostate cancer, neither species affected the viability of the BPH, DU-145, or PC-3 cell lines. Only the *S. thirkei* extract exhibited a cytotoxic effect on LNCaP cell lines, with an IC_{50} value of 19.25 µg/mL.

Keywords: *Stachys thirkei*, *Stachys macrantha*, antioxidant activity, cytotoxicity

İki *Stacys* Türü Üzerinde Fitokimyasal Çalışmalar: *Stachys thirkei* K. Koch ve *Stachys macrantha* (K. Kock) Stearn

Öz

Çalışmanın amacı, Doğu Anadolu'da yetişen *Stachys thirkei* ve *Stachys macrantha* bitkilerinin toprak üstü kısımlarının metanol ekstrelerinin antioksidan aktivitelerini DPPH (1,1- Difenil-2-pikrilhidrazil) ve ABTS (2,2'-azino-bis(3-etill-benzotiyazolin-6- sülfonik asit)) yöntemleri kullanarak, BPH, DU-145, PC-3 ve LNCaP hücre hatları üzerindeki sitotoksik etkilerini de MTT (3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromit) analizi ile araştırmaktır. Radikal süpürücü aktiviteler spektrofotometrik olarak belirlenmiş ve her iki türün de yüksek antioksidan kapasiteye sahip olduğu ortaya çıkmıştır; ancak *S. thirkei* örneği nispeten daha yüksek aktivite göstermiştir. Prostat kanseri için potansiyel tedavilerin ön araştırmasında, iki tür de BPH, DU-145 veya PC-3 hücre hatlarının canlılığını etkilememiştir. Sadece *S. thirkei* ekstresinin LNCaP hücre hatları üzerinde IC_{50}: 19,25 µg/mL değeriyle sitotoksisik etkiye sahip olduğu görülmüştür.

Anahtar Kelimeler: *Stachys thirkei*, *Stachys macrantha*, antioksidan aktivite, sitotoksisite

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1. Introduction

The genus *Stachys* L. is part of the tribe Lamioideae and the subfamily Stachydeae Dumort, which is the second largest subfamily within the Lamiaceae family. Globally, there are 362 species and 435 taxa within this genus [1], with Türkiye hosting 96 species and 123 taxa. Notably, 67 of these taxa are endemic to Türkiye giving the country an impressive endemism rate of 54% [2]. As such, Türkiye is recognized as one of the most significant gene centers for this genus. *Stachys* species are traditionally utilized as herbal tea and this usage has been known for treating extensive disorders comprising common colds, asthma, stomach diseases, and skin inflammations [3]. Regarding the ethnopharmacological usage of *Stachys*, several researchs have been conducted about their biological effects such as anti-inflammatory activity, cytotoxic, antioxidant, analgesic, and antibacterial [4-7]. The physiological and pharmacological activities described in the literature are closely associated with the rich diversity of secondary metabolite groups found in *Stachys*, which include volatile oils, phenolic acids, flavonoids, lignans, iridoids, diterpenes, and triterpenes [8-11]. Among the phytochemical studies, limited reports have been about *Stachys thirkei* K. Koch and *Stachys macrantha* (K. Kock) Stearn. The antimutagenic effect of ethanolic extracts of *S. thirkei* was tested using the AMES assay [12]. Other research pointed that antioxidant and antimicrobial activities of different extracts of *S. thirkei* [13]. The chemical composition of *S. thirkei* was investigated by high performance liquid chromatography (HPLC) and acteoside, lavandulifolioside, isoscutellarein and chlorogenic acid were detected [14]. As for *S. macrantha*, macranthoside, harpagide, allobetonicoside, ajugol, 8-*O*-acetyl-harpagide, ajugoside and reptoside were isolated from iridoids, lavandulifolioside, verbascoside, leucosceptoside A and martynoside were isolated from phenylpropanoids [15]. In another study, the main iridoids of *S. macrantha* growing in Hungary, harpagoside, harpagide and acetylharpagide, were determined by thin layer chromatography (TLC)-densitometric method. [16]. Moreover, volatile compounds of *S. macrantha* from Türkiye were analyzed by solid phase microextraction (SPME) method coupled with gas chromatography-flame ionization detector (GC-FID) and gas chromatography- mass spectrometry (GC-MS), *α*-pinene, *p*-cymene and carvacrol were the main components [17].

According to data obtained from Cancer Fact&Figures by the American Cancer Society, prostate cancer (PCa) is placed on top of the estimated new cases list of cancer statistics with a rate of 29 %. Strikingly, it is the second leading cause of cancer (after lung cancer with 20 %) with 11 % based on a list of estimated deaths amongst men. Patients with localized PCa have high recovery rates after treatment with prostatectomy and radiotherapy. Androgen deprivation therapy (ADT) is still considered the gold standard therapy for the treatment of locally advanced and metastatic PCa [18]. For patients with metastatic PCa, ADT treatment initially works well but for most patients, treatment resistance occurs inevitably due to the progression of the disease to the more aggressive and lethal stage which is known as castration-resistant PCa [19]. Although overall survival of patients has been improved by the development of new therapeutic options, metastatic castration-resistant PCa is still not curable and unfortunately, patients die within 2-3 years [18, 20-22]. Therefore, it is crucial to develop new therapeutic strategies for

the treatment of the disease and to preserve the patient's quality of life. Interest in phytotherapeutics has increased recently due to their safety and fewer side effects, even though many chemotherapeutic drugs used today are derived from plants. While the cytotoxic effects of *Stachys* species are recognized, particularly owing to their diterpenes and phenolics, research on prostate cancer using PC-3 cell lines has focused solely on the species *S. parviflora* [23] and *S. obtusicrena* [24]. In addition, studies conducted on Iranian *Stachys* species, *S. byzantina*, *S. inflata*, *S.setifera*, *S.persica*, *S.laxa*, *S. trinervis*, *S. subaphylla*, and *S. turcomanica*, evaluated their extracts for cytotoxic effects on various cell lines, including HT-29 (colon carcinoma), CaCo-2 (colorectal adenocarcinoma), T-47D (breast ductal carcinoma), and NIH-3T3 (Swiss Mouse embriyo fibroblast), with the exception of prostate cancer cell lines. The chloroform extract of *S. setifera* demonstrated pronounced cytotoxicity across all tested cell lines [25]. Conversely, the chloroform and ethyl acetate fractions of *S. laxa* and *S. turcomanica* exhibited notable cytotoxic effects specifically on HT-29 and T-47D cell lines [26]. Additionally, the HepG2 (hepatocellular carcinoma) cell line was also investigated, and the hydroalcoholic extract of *S. pilifera* [27] and the dichloromethane extract of *S. circinata* [28] were found to be significantly cytotoxic against this cell line. Polyphenols, found commonly in the *Stachys* genus, play a unique dual role as both antioxidants and pro-oxidants. Their antioxidant properties help reduce the imbalance caused by oxidative stress, while their pro-oxidant effects can induce cytotoxicity in cancer cells. Furthermore, polyphenols can inhibit several neoplastic processes, including cell proliferation, invasion, metastasis, and angiogenesis [29,30]. For that reason, we aimed to search antioxidant and cytotoxic effects of *S. thirkei* and *S. macrantha* from Eastern Anatolia. This is the first report of these two *Stachys* species on BPH (primary prostate epithelial cells), DU145 (prostate epithelial cells), PC3 (prostate adenocarcinoma cells), and LNCaP (human prostate adenocarcinoma cells) cell lines.

2. Materials and Methods

2.1. Chemicals and Reagents

DPPH (1,1- Diphenyl-2-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid)), trolox, methanol, potassium persulfate, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma Aldrich (USA).

2.2. Plant Materials and Extraction

S. thirkei was collected from Pötürge, Malatya on 13.07.2024, and *S. macrantha* was collected from Çıldır, Ardahan on 21.07.2024. Both of them were identified by Prof. Dr. Hasan Yıldırım. Voucher specimens (No: H.Yıldırım 10984, H.Yıldırım 11020, respectively) have been deposited in the Herbarium of the EGE University. The methanol extracts were prepared from the aerial parts of *Stachys* species. Five grams of air-dried, powdered plant material were macerated in 100 mL of methanol three times. The solvent was then removed under reduced pressure using a rotary evaporator (Büchi, Switzerland). The extraction yields were assessed 2.708 for *S. thirkei* and 13.82 for *S. macrantha*, relatively (%, w/w).

2.3. Antioxidant Activity

2.3.1. 1,1- Diphenyl-2-picrylhydrazyl (DPPH) Assay

According to the Blois method [31], methanol extract concentrations ranging from 1 to 1000 ppm were used. After incubating the sample and DPPH solution in the dark for 30 minutes, the absorbance change was measured at 517 nm. Trolox served as the positive control. The IC_{50} values of the extracts were then calculated with three parallels. A UV-visible spectrophotometer (Optima SP-3000 Nano, Japan) was utilized to obtain the absorbance readings.

2.3.2. 2,2'-Azino-bis(3-ethyl-benzothiazoline-6- sulphonic acid) (ABTS) Assay

The blue-green ABTS+· radical was generated by mixing an ABTS solution (7 mM) with a potassium persulfate solution (2.45 mM) and incubating the mixture at room temperature for 16 hours [32]. After the incubation, the ABTS+· solution was diluted with ethanol, and methanol extract was added to this mixture. The combined solution was then incubated at 25°C for 2 hours, after which the absorbance was measured at 734 nm. Trolox was used as the positive control.

2.4. Cell Culture

BPH (Primary Prostate Epithelial Cell line), DU145 (Prostate Epithelial Cells cell line), PC3 (prostatic adenocarcinom cell line), and LNCaP (human prostate adenocarcinoma cell line) were obtained from ATCC and are preserved in liquid nitrogen in our inventory. For cytotoxicity assays, the cells were grown in 100 mm dishes and subcultured until they reached approximately 70 % confluence. The cultures were kept in a humidified incubator at 37°C with 5 % carbon dioxide.

2.5. Cytotoxicity Activity

To assess the IC_{50} values extracts were prepared in DMSO at concentrations of 10, 25, 50, 100, 200 and 400 µg/ml. Cells were first seeded in 96-well culture plates, and cell viability was measured after 24 hours of incubation. After a 48-hour exposure to the extracts, an MTT assay was performed. A 0.5 mg/mL MTT solution was introduced into the wells of the 96-well plate and incubated for 4 hours. Following the incubation, the medium was removed, and 200 µL of DMSO was added to dissolve the formazone crystals. Absorbance was recorded using a microplate reader (Varioskan, Thermo Fisher Scientific, USA) at wavelengths between 570-690 nm, with blank wells (without cells) used to correct for any background absorbance. The IC_{50} values were calculated using regression analysis in GraphPad Prism version 10. The MTT results presented here were obtained from five replicate wells for each condition

2.6. Statistical Analysis

The IC_{50} results were presented as means ± standard errors. ANOVA (One-way analysis ofvariance) followed by Tukey's test was applied to determine differences among. Analyses with *p* values less than 0.05 were considered statistically significant.

3. Results and Discussion

The antioxidant activity results of methanol extracts were given in Table 1. In both DPPH and ABTS analyses, the IC_{50} values of *S. thirkei* are lower than those of *S. macrantha*, indicating that the antioxidant capacity of *S. thirkei* extract surpasses that of *S. macrantha* extract. In the literature, a study, along with *S. thirkei* from Düzce, presented DPPH and ABTS activities for hydrolyzable and extractable phenolics of plant extract. The phenolics' DPPH activity values ranged from 39.97±0.88 to 172.16±9.78 µmol Trolox/g, while ABTS results were calculated between 78.52±1.99 to 198.87±1.25 µmol Trolox/g [33]. In another study, the DPPH activity was investigated in three different, chloroform, n-hexane, and methanol extracts, of the *S. thirkei* plant collected from Tekirdağ, and methanol extract was found to be most active with a value of 0.039118±0.005585 mg ascorbic acid/mg extract [13]. As for *S. macrantha*, researchers collected samples from Gümüşhane, and among the diverse samples, the methanol extract showed potent activity with 23.62±0.1 mg trolox/ g extract for DPPH; 49.99±1.6 mg trolox/ g extract for ABTS [34]. The findings from this study were inconsistent with the results obtained from the abovementioned *Stachys* species due to differences in collection location and timing. Numerous studies have shown that geographical variations can lead to changes in the composition of phytochemicals.

Table 1. Antioxidant activities of methanol extracts of *S. thirkei* and *S. macrantha*

Methanol extracts	DPPH (IC_{50} mg/mL)	ABTS (IC_{50} mg/mL)
S. thirkei	0.675 ± 0.48^{a}	0.893 ± 0.68^{a}
S. macrantha	1.052 ± 0.92^{a}	1.570 ± 0.57^{a}
Trolox*	0.035 ± 0.07^{b}	0.028 ± 0.03^{b}

Superscripts indicate significant differences in the studied extracts ($P<0.05$), * positive control for antioxidant tests.

This study investigated the cytotoxic effects of two *Stachys* species on four cancer cell lines: BPH, DU-145, PC-3, and LNCaP for prostate cancer. The methanol extracts from *S. thirkei* and *S*. macrantha showed no activity against the BPH, DU-145, and PC-3 cell lines. However, S. *thirkei* exhibited cytotoxic effects on the LNCaP cell line, with an IC_{50} value of 19.25 µg/mL across varying concentrations in Figure 1. This situation may correlate with not only the stronger antioxidant activity of *S. thirkei* but also with its iridoid and phenylpropanoid contents. It has been reported that acteoside, verbascoside common phenylpropanoid glycoside, has inhibitory effect on prostate specific antigen level [35]. Previously, Serbetci et al. reported that

verbascoside was found to major compound in *S. thirkei* by HPLC-ESI/MS [14]. Additionally, different species *S. parviflora* has been studied for isolation of tanshinones and their cytotoxicity on MCF-7 and PC-3 cells. The results pointed that 1-hydroxy-tanshinone IIA induced DNA fragmentation in PC-3 cells [23]. Also, the endemic *S. obtusicrena* from Iran was analyzed for its antitumor activity in PC-3 cells. The methanol extract of this plant reduced cell viability by 98.3 ± 21.0 % at a concentration of 100 µg/mL [24].

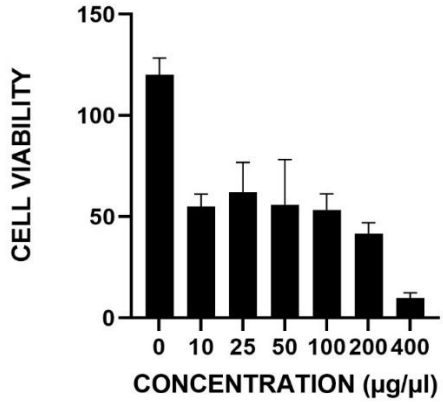

Figure 1. Cell viability of LNCaP against *S. thirkei* extract

4. Conclusion

In the current study, the antioxidant activities of two *Stachys* species growing in Eastern Anatolia were investigated. Both methanol extracts of species exhibited antioxidant activity, with *S. thirkei* showing relatively stronger effects and several studies have reported that oxidative stress are related to prostate cancer strictly. Furthermore, cytotoxic activity experiments conducted on prostate cancer cells revealed that the *S. thirkei* species collected from Malatya had cytotoxic effects on the LNCaP cell line. Consequently, this research data contributes to the limited number of researches on these two species and prostate cancer treatment at the same time, however further isolation and mechanisms of biological activity investigations are needed.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Gökçe YILDIRIM BUHARALIOĞLU-Recep İLHAN: Cytotoxicity assay, Hasan YILDIRIM: Plant collection and identification, Ceren EMİR: analyzing experiments, writing and editing manuscript.

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