

Phlomis species protects the cells from oxidative stress by upregulating catalase enzyme expression

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Recently, interest in the pharmacological effects of plants has increased and studies on bioactive compounds have intensified. In this study, we aimed at evaluating the antioxidant and neuroprotective potential of Lamiaceae plants including *Stachys lavandulifolia*, *Salvia verticillata*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana* in both cell-free systems and the cell culture model. Methanol extracts of the aerial parts of these plants, cultivated in Elazig, Turkey were carried out. Antioxidant activity assays (ABTS and DPPH) and total phenolic and flavonoid contents of the extracts were measured. Next, the effects of methanol extracts on cell viability and catalase protein levels were investigated in hydrogen peroxide induced oxidative stress model. DPPH radical scavenging activities were determined with the IC₅₀ value of 31.36±0.25, 30.44±0.19, 30.16±0.08 and 56.54±0.93 for *Stachys lavandulifolia*, *Salvia verticillata*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana*, respectively. ABTS radical scavenging activities were determined as 6.38±1.48, 16.93±0.68, 5.97±4.04 and 2.28±0.51. The highest cellular protection and catalase expression were determined in *Phlomis*-treated cells against H₂O₂ (p<0.05). On the other hand, no statistically significant change in cell viability was observed when the extracts were compared with each other. Our findings support the neuroprotective and antioxidant potential of these four Lamiaceae plants, particularly *Phlomis* species.

Keywords: *Phlomis*, *Salvia*, *Stachys*, antioxidant, cell culture.

INTRODUCTION

The Lamiaceae family consisting of herbs which produce and release the aromatic smell is stated as the mint family. There are more than 3.000 species in the Lamiaceae plant family. *Salvia*, *Scutellaria* and *Stachys* are the largest genera of Lamiaceae [1].

The genus *Salvia* comes about more than a thousand plant species distributed worldwide [2, 3]. The name of the genus derives from the Latin word “salvare” which means “to save, to heal” [4]. *Salvia* species are aromatic herbs used as food spices in cosmetic industries and in traditional medicine due to their bioactive effects [5]. *Salvia verticillata* is known as ‘dadirak’ in Turkey. The genus *Stachys*, contains more than 300 species, is also known as mountain tea. *Stachys* species have generally been used in traditional medicine as herbal teas for the treatment of various diseases in different countries [6]. An ethnobotanical study has showed a similar pattern of *Stachys* species consumption in different regions, from Europe to Asia. In these regions, they are used as medicinal herbal teas with a pleasant smell. *Stachys* species have wide utilisation as anti-spasmodic, antitussive, wound healing, anti-asthmatic, antiseptic, anticancer, and emmenagogue [7, 8]. Besides recent studies have revealed the antibacterial, antifungal, anti-anxiolytic, and cytotoxicity

activities of their essential oils [9, 10]. *Stachys lavandulifolia* is known as 'tüylü çay' in Turkey.

The genus *Phlomis* that includes perennial plant species, is a large genus of the Lamiaceae family with over 100 species mainly distributed in Asia, Europe and North Africa [11]. According to literature, a wide range of *Phlomis* species throughout the world have the same manner of use qua herbal tea to heal several disorders such as diabetes, gastric ulcer, hemorrhoids, inflammation, and wounds. Phytochemical studies on various species of genus *Phlomis* have resulted in the isolation of terpenoids, phenylethanoid glycosides, flavonoids and lignans that are responsible for the pharmacological properties [12, 13]. In Turkey, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana* are known as 'silvanok' and 'kuduz adaçayı', respectively.

Considering our limited knowledge on the main causes of some chronic diseases such as Alzheimer's, Huntington's, Parkinson's, epilepsy and diabetes, there is strong evidence that oxidative stress is one of the mechanisms that play a role in the progression of the disease [14]. For the treatment of central nervous system diseases (CNS), there are limited treatment alternatives. Therefore, approaches that treat or prevent the progression of CNS diseases or improve its function are important. Thus, interest in the use of plant-derived compounds for CNS diseases is increasing day by day.

Previous studies have reported high antioxidant capacities of plants belonging to the Lamiaceae family. In this study, we aimed to evaluate four species of Lamiaceae family namely *Stachys lavandulifolia*, *Salvia verticillata*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana* in terms of regulation of antioxidant mechanisms in a cellular model of oxidative stress.

In the literature fatty acid composition determination study was carried out on *Phlomis herba venti* sp. *pungens* collected from Iran and germacrene D (39.2%), α -pinene (9.3%) and 2-pentadecanone were found as the main components [15]. In another study on the volatiles from *Phlomis* included *Phlomis olivieri*, *Phlomis fruticosa*, *Phlomis lanata* and *Phlomis younghusbandii*. In *P. olivieri*, various main components have been found such as hexahydrofarnesylacetone, spathulenol, germacrene D, β -caryophyllene and caryophyllene oxide. This oil was characterised by a high content of sesquiterpenes and trace amounts of monoterpenes [16]. In another report on the essential oil of the aerial parts of *P. olivieri*, the main compounds were germacrene D, β -caryophyllene, α -pinene and β -selinene [17]. The flowers of *P. fruticosa* collected in Greece yielded an essential oil rich in germacrene D, g-bisabolene, α -pinene and β -caryophyllene [18]. In a different study the main chemicals identified in the essential oil of the aerial parts of *P. lanata* were found as α -pinene, limonene and β -caryophyllene and antimicrobial activity of the plant was detected [19].

The essential oil of *P. younghusbandii* contained

non-terpenic derivatives such as eugenol, hexadecanoic acid, 9,12-octadecadienoic acid methyl ester and guaial as principal constituents [20]. Among non-volatile derivatives, neolignan glucosides were characterised from *P. chimerae* [21]. Furthermore, the anti-ulcerogenic activity of *P. grandiflorais* reported by the Turkish folk medicine [22]. This is the first report about the composition of the essential oil of these three species of *Phlomis*.

In another study water-distilled essential oil of *Salvia pillifera* Montbret et Aucher ex Bentham was previously analysed using GC and GC-MS and α -Thujone (39.8%) and α -pinene (11.2%) were found to be the main components in the oil (20). In another study of *S. pillifera*, α -thujene (36.1%) and α -pinene (13.8%) were also determined as the major compounds. Water-distilled essential oil of *Salvia viscosa* Jacq. growing in Lebanon was previously analysed using GC and GC-MS and caryophyllene oxide (12.7%) and β -copaen-4- α -ol (5.4%) were found to be the main components in the oil [23, 24]. In addition, the use of essential oils obtained from the species included in this study in oils and fats has also been stated in the literature [15, 25]. In the studies carried out, the extracts obtained from other species in the mentioned families are used in vegetable oils [16-22].

MATERIALS AND METHODS

PLANT MATERIALS

The plants *Stachys lavandulifolia*, *Salvia verticillata*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana* were collected from Harput, Elazığ, Turkey in 2019, during the flowering stage. Voucher specimen has been deposited at the Herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Turkey. The aerial parts of the plants were air-dried and powdered under appropriate conditions. Then plants were macerated with methanol at the room temperature for 24 hours three times. The extracts were filtered, and the solvents were entirely removed using the rotary evaporator under the low pressure for obtaining the dry methanol extracts for experiments. Dark green-coloured extracts were obtained. The extraction yields of methanol extracts of *Stachys lavandulifolia*, *Salvia verticillata*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana* were calculated as 12.46%, 14.71%, 10.87%, and 14.26%, respectively.

DPPH

The DPPH free radical scavenging activity measurement was carried out according to the method of Fukumoto and Mazza [26] with some modifications. The measurement was carried out on a 96 well microplate. Microdilution series of samples (1 mg/mL, dissolved in HPLC grade MeOH) were prepared starting with 150 μ L. To each well 50 μ L of DPPH reagent

(100 μ M, made with HPLC grade MeOH) was added to gain 200 μ L working volume. The microplate was stored at room temperature in dark conditions. The absorbance was measured after 30 minutes at 550 nm using a BMG Labtech FluoStar Optima plate reader. For the blank control HPLC grade MeOH was used instead of the sample. As a standard solution, ascorbic acid (0.01 mg/mL, in HPLC grade MeOH) was used. The EC₅₀ values were calculated with GraphPad Prism 6.05.

ABTS

For ABTS [2,2'-azinobis (3-ethylbenzotiazolyn-6-sulfonic acid) diammonium salt] assay, ABTS solution was prepared and diluted with ethanol until giving 0.750 absorbance in 734 nm [27]. 0.1 mL extract and 10 μ L α -tocopherol were added to 1 mL ABTS⁺ solution and absorbance changing was observed at 734 nm for 6 minutes. For the standard solution, α -tocopherol was used. The ABTS scavenging activity was calculated using the equation:

$$\% \text{ ABTS} = [\text{Abs}_1 - \text{Abs}_2] / \text{Abs}_1 \times 100$$

Abs₁: Absorbance value obtained from the first measurement,

Abs₂: Absorbance value obtained from the second measurement

TOTAL PHENOLIC CONTENTS

2.8 mL of deionised water, 2% Na₂CO₃ and 50 mL of Folin-Ciocalteu reagent were added to the 100 μ L test solution in accordance with the Folin-Ciocalteu method [28]. Thereafter, an absorbance was measured against the blank solution at 750 nm. Calibration curves were prepared with gallic acid and antioxidant activity was determined according to the equation.

TOTAL FLAVONOID CONTENTS

In the total flavonoid content, the determination method was applied according to the aluminium chloride method, 95% ethanol, aluminium chloride solution and water are added to the extracts [29]. They were then allowed to incubate at room temperature for a period of up to 40 min and measure the absorbance against ethanol at a wavelength of 415 nm. To draw the curve of the standard, quercetin, prepared at different concentrations as per standard, was used.

CELL CULTURES AND TREATMENTS

The human neuroblastoma cell line (SH-SY5Y) used in this study was purchased from the American Type Culture Collection (ATCC, Catalogue #CRL-2266). Cells were suspended in complete Dulbecco's modified Eagle Medium (DMEM) (Life Technologies, Gibco BRL, Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Hyclone), 1% penicillin and streptomycin (100 U/ml, Invitrogen) and plated

in cell culture dishes. The cultures were maintained at 37°C in 5% CO₂ 95% humidified atmosphere. After reaching 85% confluence, cells were transferred to 6- or 96-well plates. Cells were seeded into 96-well cell culture plates at a density of 5x10³ cells per well and allowed to adhere for 24 h. The culture medium was then replaced with fresh medium containing extracts (1-100 μ g/ml) or hydrogen peroxide (H₂O₂, 250 μ M) and incubated in 5% CO₂ incubator. Meanwhile, extracts were added and incubated at 37°C in 5% CO₂ for cell proliferation assay for 24 hours. The stock solutions of extracts were prepared in sterile DMSO and these stocks were then appropriately diluted with the complete culture medium and DMSO levels were maintained below 1% in the test concentrations. WST-1 was obtained from Takara Bio USA (Mountain View, CA, USA).

CELL VIABILITY ASSAY AGAINST H₂O₂-INDUCED TOXICITY

Cells were seeded at a density of 5x10³ cells/well into 96 well plates and incubated for 24 h. After 24 h, the cells were treated with extracts (1, 10 and 100 μ g/ml) for 2 h. After 2 h, the medium was aspirated, and the cells were washed twice with sterile PBS and then treated with 250 μ M of H₂O₂ in sterile PBS (with Ca²⁺ and Mg²⁺) for 1 h. After 1 h, the H₂O₂ solution in the well was replaced with the warm medium and incubated for 17 h. After the 18 h exposure, the cell viability was determined using the WST-1 assay.

WST-1 ASSAY

The cell viability was performed according to the standard protocol of manufacturer using the WST-1(2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) assay kit (Takara Bio Inc., Shiga, Japan). Briefly, following exposures, 10 μ L Premix WST-1 was added to each well, and plates were incubated for 2 h at 37°C in 5% CO₂ humidified incubator. Then the absorbance was measured at 440 nm using a microplate reader (VersaMax, Molecular Devices, USA). Percent survival was plotted relative to vehicle control cells, which were normalised at 100% survival.

PROTEIN ANALYSIS

Following the incubations, the cells were washed with PBS and lysed in lysis buffer. Following centrifugation at 10.000 g for 10 min at 4°C, BCA protein assay was used to determine protein concentration. Western Blot assay were done by using 30 μ g of protein and densitometric analyses of bands were performed as described previously [30].

STATISTICAL ANALYSIS

Data were expressed as means \pm standard deviation (SD) for five independent experiments in triplicate. Comparisons of means between groups were performed by one-way analysis of variance (ANOVA)

followed by Tukey's post hoc test. P value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

In the current study, antioxidant potential of the species of Lamiaceae family were studied. Previous studies have shown that methanolic extracts are effective in terms of recovery of polyphenols and antioxidant potential of the plants [31]. In our study, active components of plants were extracted using methanol to examine their therapeutic potential. DPPH and ABTS were chosen to determine the antioxidant capacities of these extracts. The results of the antioxidant assays including DPPH and ABTS, and total phenolic/total flavonoid contents were shown in Table I. It was determined as *Salvia verticillata*, *Stachys lavandulifolia*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana*, respectively as the most effective extracts in antioxidant activity. The levels of total phenolic content and total flavonoid content supported these findings.

The presence of phenolic and flavonoid substances increases the antioxidant capacity of plants [32-36]. When previous studies with different species belonging to the same genus are compared, the results of antioxidant activity tests and total phenol/flavonoid content values are found consistent with our results [37-43]. In studies conducted with *Salvia verticillata* species, phenolic compound contents, especially rosmarinic acid and caffeic acid, were determined [44-46]. In the literature studies conducted with *Stachys lavandulifolia* species collected from different regions, it was seen that the phenolic components of the plant extracts were determined by HPLC and LC MS methods. Major compounds were identified as chlorogenic acid, quinic acid and rosmarinic acid [47, 48]. When the literature was examined, it was seen that the components of many *Phlomis* species,

including the species in this study, were determined by different methods. It has been determined that they contain many phenolic compounds, especially verbascoside, and it has been determined that the extracts support their antioxidant activities [49 - 51]. Studies conducted with these species have also supported that antioxidant activity is strengthened by the richness of phenolic compounds. Considering previous studies showing that the antioxidant capacity of these plants may exert protective effects, cellular protection by these species were evaluated in a cell culture model of oxidative stress to verify their antioxidant potential. Exposure to hydrogen peroxide is a widely used method to induce oxidative damage / stress in cell models [52]. In this study, various concentrations of methanol extracts were exposed to hydrogen peroxide-treated cells. The results of cell culture assays were given in Table II. Cell viability was found 44.42% in H₂O₂-treated cells (p<0.05). After extraction treatments at three concentrations (1-100 µg/mL), cell viability significantly increased compared to the H₂O₂ group regardless of the selected dose (Tab. II). The highest protection was determined after the treatment of the *Phlomis sieheana* extract ranging from 54.25% to 55.84%. On the other hand, when the plants were compared, no significant difference was found (p> 0.05). Based on these results, the lowest concentrations were chosen for treating cells in further experiments. There are only few studies on cellular effects of selected species of Lamiaceae family. A study reported that alcoholic extract of *Phlomis herba venti* sp. *pungens* exerts anti-inflammatory and antioxidant effects in isolated rat colon [53]. Thus, our study may lead to understand the molecular mechanisms of protection of these plants.

In the light of cell viability data, we hypothesised that the cellular protection of methanol extracts may related to the regulatory roles of active compounds in ex-

Table I - Total phenolic contents (TPC) and total flavonoid contents (TFC) and antioxidant capacities of plant extracts in DPPH and ABTS⁺ scavenging reactions.

Species/assays	DPPH EC ₅₀ (µg/ml)	ABTS (µmol/g)	TPC (mg GAE/g)	TFC (mg KE/g)
<i>Phlomis herba venti</i> sp. <i>pungens</i>	30.16±0.08 ^a	5.98±4.04	47.96±2.45	20.96±3.36
<i>Phlomis sieheana</i>	56.54±0.93	2.28±0.51	29.63±0.56	11.45±1.43
<i>Stachys lavandulifolia</i>	31.36±0.25	6.38±1.48	50.65±1.42	28.36±3.01
<i>Salvia verticillata</i>	30.44±0.19	16.93±0.68	53.04±3.56	29.78±1.75

^aAll the values were represented as Mean± SD (n = 3).

Table II - The effect of plant extracts applied in different concentrations on cell viability in H₂O₂ mediated damage. % change compared to the control group. Mean ± S.D

Species/concentration	1 µg/mL	10 µg/mL	100 µg/mL
<i>Phlomis herba venti</i> sp. <i>pungens</i>	52.01±1.88	50.61±4.14	50.80±0.90
<i>Phlomis sieheana</i>	54.25±2.95	55.45±2.11	55.84±3.36
<i>Stachys lavandulifolia</i>	52.16±4.67	52.26±1.42	51.96±1.13
<i>Salvia verticillata</i>	51.61±2.94	52.71±1.51	52.56±0.97

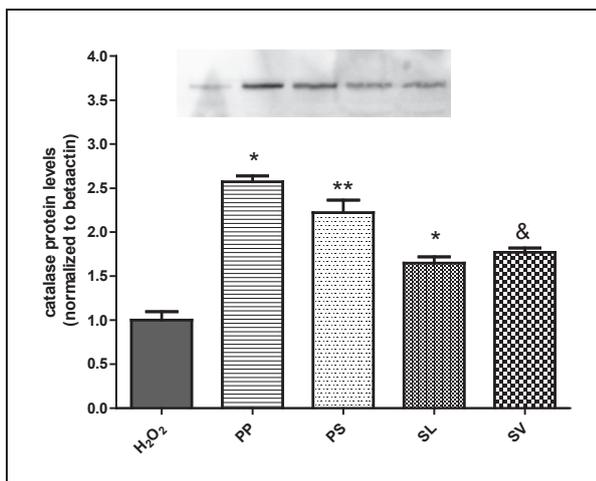


Figure 1 - Changes in catalase protein levels in extract-treated (1 μg / mL) cells against H₂O₂. *p=0,0002, **p=0,0014, & p=0,0006.

tracted samples. Catalase is an antioxidant enzyme responsible for removing H₂O₂ in the cell. Low concentrations of extracts were not significantly different from higher concentrations in terms of cellular protection. Thus, cells were treated with 1 $\mu\text{g}/\text{mL}$ of extracts for catalase protein analysis against H₂O₂ (Fig. 1). As shown in Figure 1, catalase protein levels were found lower in H₂O₂ treated cells. Catalase protein levels following the extract treatment was increased by 2.57- ($p = 0.0002$), 2.22- ($p = 0.0014$), 1.64- ($p = 0.0002$) and 1.67-fold ($p = 0.0006$) in the *Phlomis herba venti* sp. *pungens*, *Phlomis sieheana*, *Stachys lavandulifolia*, *Salvia verticillata*-treated cells, respectively (Fig. 1). The major components were determined by examining the previous fatty acid analysis studies with the plants included in the study [54 - 62]. Accordingly, the contribution of the detected sesquiterpene compounds and aliphatic compounds to the antioxidant activity is obvious. Many studies have revealed that the antioxidant capacity of extracts is directly proportionate to being rich in these compounds [63, 64]. It is known that compounds capable of fighting free radicals that play an important role in the formation of various diseases (including cancer, aging and DNA damage) have an inhibitory or therapeutic effect on the progression of these diseases [65]. This situation leads us to having a preliminary idea on the ingredients of the plants in this study.

CONCLUSIONS

It can be suggested that the selected plants stimulate catalase expression in the cell and activates antioxidant systems to remove H₂O₂. Previously, aerial parts of *Phlomis anisodonta* methanolic extract were shown to increase antioxidant enzyme activities including catalase in streptozotocin-induced diabetic rats [66]. As mentioned above, there are a few pharmacological and biological studies with Lamiaceae family on

cell culture and in vivo models. Our results suggest that treatment with selected plants may control free radicals inside the cell and protect macromolecules. Besides since the DPPH and ABTS antioxidant activity assays are performed on different mechanisms and different oxidant radicals, the obtained activity results are not exactly the same. However, both antioxidant activities applied to different plant extracts gave results in the same order. It was determined as *Salvia verticillata*, *Stachys lavandulifolia*, *Phlomis herba venti* sp. *pungens* and *Phlomis sieheana*, respectively as the most effective extracts in antioxidant activity assays.

The medical importance of four different plants, *Salvia lavandulifolia*, *Salvia verticillata*, *Phlomis sieheana* and *Phlomis herba venti* sp. *pungens*, which we studied within the scope of our study, has been proven by the data we obtained. This is the first comprehensive study on these plants, including cell culture and antioxidant activity tests. Our study confirmed the efficacy of these plants in exerting protective effects in oxidative stress conditions, possibly due to upregulated antioxidant enzymes such as catalase. However, the lack of previous studies in cellular responses following these extracts limits the interpretation of the obtained data.

Conflicts of Interest

The authors have declared that there are no conflicts of interest.

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