Research Article

www.jrespharm.com

Investigation of total phenolic and flavonoid content of *Salvia willeana* (Holmboe) Hedge, an endemic plant of Cyprus, and screening of its antioxidant and cholinesterase inhibitory properties

Tugba ERCETIN [1*], Maryam SAFAEI [1], Hayrettin Ozan GULCAN [2]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, TR. North Cyprus, via Mersin 10, Turkey.

[2] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, TR. North Cyprus, via Mersin 10, Turkey.

* Corresponding Author. E-mail: tugba.ercetin@emu.edu.tr (T.E.); Tel: +90-392-630-24-01.

Received: 3 June 2021 / Revised: 30 December 2021 / Accepted: 31 December 2021

ABSTRACT: *Salvia willeana* (Holmboe) Hedge is an endemic plant of Cyprus island. There have been limited number of studies on the plant, beside some analytical studies on its chemical content. This study aimed to investigate total phenolic and flavonoid content of the plant concomitant to some pharmacological activity screening studies including antioxidant and cholinesterase inhibition. Following obtaining different organic solvent and aqueous extracts of the dried aerial parts of the plant, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferric-reducing antioxidant power (FRAP), iron-chelation capacity assays, and modified Ellman's method were employed for screening antioxidant and cholinesterase inhibition studies. *S. willeana* extracts displayed low to moderate activities in cholinesterase inhibition assays. The ethyl acetate extracts of the leaves and the methanol extracts of the stem part of the plant displayed the highest acetylcholinesterase inhibitory potential (i.e., 28.95 ± 0.035%, and 24.01 ± 0.007%, respectively), while the highest butyrylcholinesterase inhibitory potential was obtained in aqueous extracts of the stem at 0.5 mg/mL (i.e., 29.10 ± 0.005%). The methanol extracts of the leaves and flower of the plant provided the highest antioxidant activity in DPPH radical scavenging activity (67.94 ± 0.003%, and 45.07 ± 0.001%, respectively) at 0.5 mg/mL concentration. Ferrous iron chelating activity of the extracts were found weak in general. The plant was found rich in terms of its flavonoid and phenolic contents as shown in the ethyl acetate and methanol extracts of the leaves (i.e., 547.55 ± 3.12 mg/g, and 300.24 ± 1.23 mg/g extract, respectively).

KEYWORDS: *Salvia willeana*; antioxidant activity; cholinesterase inhibitor; Alzheimer's Disease

1. INTRODUCTION

Natural products have still been one of the most important sources to identify novel approaches for the treatment of various disease states. Regarding the nature as the basic source of natural products, it becomes particularly significant in terms of conducting novel scientific investigations on endemic plants [1,2]. This strategy becomes crucial particularly for finding alternative cures for those disease states treated with limited number of drugs. Indeed, Alzheimer's disease (AD) is within this category, and there are only a few drugs used in clinic today for its treatment (i.e., donepezil, rivastigmine, and galantamine) [3]. These drugs are mainly cholinesterase inhibitory molecules, since cholinesterase inhibition has been shown to be the most important validated target for enhancing cognitive decline seen throughout the development of the disease [4]. Unfortunately, these drugs are only available to treat the mild and moderate stages of the disease. Besides, the pharmacokinetic and pharmacodynamic variances among these drugs also become another handicap [5]. Keeping in mind that galantamine has pure natural origin from Amaryllidaceae family species including *Galanthus* species, especially *Galanthus woronowi*, *Narcissus* (daffodil) genus, *Leucojum aestivum* (snowflake), and *Lycoris radiata* (red spider lily), and other side rivastigmine has a semi-synthetic

How to cite this article: Ercetin T, Safaei M, Gulcan HO. Investigation of total phenolic and flavonoid content of Salvia willeana (Holmboe) Hedge, an endemic plant of Cyprus, and screening of its antioxidant and cholinesterase inhibitory properties. J Res Pharm. 2022; 26(2): 263-271.

© 2022 Marmara University Press

http://dx.doi.org/10.29228/jrp.124

ISSN: 2630-6344

origin of meta-aminophenol derivatives in plants, which is critical to seek for alternative plant origins offering promising cholinesterase inhibitory molecules [6].

AD is a very complex disease in terms of its pathophysiology. Indeed, the development of the disease involves many cascades including but not limited to the over-activation of amyloid-beta cascade, induced amyloid-beta and tau-protein aggregations, concomitant to high oxidative stress conditions are all linked [7]. From this point of view, as seen with other neurodegenerative central nervous system disease states, agents capable of displaying antioxidant property are also very valuable. Besides, since metals are directly involved within the generation of reactive oxygen species, it becomes also significant to find agents particularly interacting with iron species to prevent Fenton like reactions yielding out reactive oxygen species. Overall, this concept is referred to as multi-target-based approach for the treatment AD like complex diseases.

Salvia L. species have been documented to be used in folk medicine for its beneficial effects on memory disorders in European herbal medicine and have been also used as folk remedy in tea form against simple disorders in Turkey (9-12). In the continuation of our research studies on the finding of alternative approaches for the treatment of AD, within this study, we analyzed *Salvia willeana* (Holmboe) Hedge (*S. willeana*) from Labiatae family. *S. willeana* is an endemic plant for Cyprus island common in the Troodos range. Known as Troodos sage habitual to higher elevations (1050-1950 m) in the Troodos Mountain in rocky slopes, roadsides or under pines range in Cyprus [8]. The plant is hairy glandular and it has rounded or elongated leaves and its flowers generally appear starting the end of spring till the mid of autumn. Previously, there have been various types of biological activities shown for *Salvia* species [9-12]. These are mainly entire HIV, anticancer, antimicrobial, and antioxidant activities [13,14.] Employing the significance of cholinesterase inhibition and reactive oxygen species linked oxidative stress and neurodegeneration characteristics of AD, we have conducted a serious of experiments on *S. willeana* in order to determine its potential to act as both cholinesterase inhibitor and antioxidant. From this point of view, aerial parts of the plant were dried and separated to its parts (e.g., stems, leaves and flowers). Employing alternative extraction methods, each part was analyzed for their potential for the related biological activities.

2. RESULTS AND DISCUSSION

2.1. Cholinesterase inhibitory activity

The potential of plants extracts obtained through various organic solvents to inhibit cholinesterase enzymes were assessed in two different concentrations (0.5 mg/mL and 1 mg/mL). The results obtained are summarized in Table 1. Accordingly, each fraction displayed activity within the poor to moderate range. In addition, the activity was found to be concentration dependent, since the higher the concentration employed the higher the potential of the extracts to display cholinesterase inhibition. There has been no selectivity observed in terms of inhibition of cholinesterase enzymes (AChE and BChE). In general, the inhibitory potential of each extract was found to be similar for both enzymes. On the other hand, the different aerial plant parts, in general, exhibited similar activity. In other words, the activities obtained were not found specific to a certain segment of an aerial part of *S. willeana*. It is important to note that the most active fractions were found to be ethyl acetate, methanol and water extracts for both AChE and BChE inhibition assays. Under the same experimental conditions, the reference drug donepezil displayed expected results, comparable to the literature [11].

Some *Salvia* species have previously shown to be active in cholinesterase inhibition screening assays. Although it is quite difficult to make comparison among those studies on the screening of *Salvia* species activity in cholinesterase inhibitor assays, particularly regarding the dosage and enzyme source variations, it is reasonable to mention that mainly, the various extracts of *S. cryptantha*, *S. fruticosa*, *S. pomifera*, *S. russellii*, *S. trichoclada*, *S. albimaculata, S. cyanescens, S. frigida and S. migrostegia, S. candidissima ssp. occidentalis, S. ceratophylla* and *S. verticillata* species have been known to possess good cholinesterase inhibitor activity [9-12]. Terpenoids and water soluble polyphenolics are two main groups of active ingredients in *Salvia* species. Especially some terpenoid AChE inhibitor compounds found in *Salvia* species which are tanshinone I, tanshinone IIA, dihydrotanshinone, cryptotanshinone, α-pinene and 1,8-cineole. The results obtained for the *S. willeana* within this study have pointed out that *S. willeana* extracts prepared displayed low to moderate activities in cholinesterase inhibition assays.

Table 1. AChE and BChE inhibitory activities of the extracts of *S. willeana* (percentage ± S.E.M[a]).

Extracts	***E.eel* ACHE (0.5 mg/mL)**	***E.eel* ACHE (1 mg/mL)**	**Eq- BCHE (0.5 mg/mL)**	**Eq-BCHE (1 mg/mL)**
DCM[b] stem	4.63± 0.01	5.10 ± 0.03***	NA*	NA
DCM leaves	14.93± 0.06	23.11± 0.09***	7.38 ± 0.033	9.32 ±0.022***
DCM flower	9.98± 0.001	16.09± 0.005***	7.79 ± 0.054	15.85 ±0.021***
EtOAc[c] stem	14.07 ±0.023	27.35± 0.005***	14.88± 0.056	24.32 ±0.078***
EtOAc leaves	28.95± 0.035	34.31± 0.01***	15.61± 0.088	24.54 ±0.062***
EtOAc flower	15.56± 0.028	29.23± 0.16***	14.81 ±0.017	20.93 ±0.031***
MeOH[d] stem	24.01± 0.007	33.76±0.02***	23.17 ±0.027	29.76± 0.026***
MeOH leaves	12.15± 0.019	25.32± 0.009***	18.38± 0.033	28.58± 0.013***
MeOH flower	23.04± 0.006	29.06 ± 0.015***	19.75 ±0.049	32.74 ±0.032***
dH_2O[e] stem	20.06± 0.038	27.50± 0.071***	29.10± 0.005	34.87 ±0.047***
dH_2O leaves	12.90 ± 0.075	19.37 ± 0.044***	23.54 ± 0.006	29.80± 0.013***
dH_2O flower	13.13± 0.013	18.53 ± 0.009***	23.50 ±0.015	29.31 ± 0.017***
Donepezil HBr 10 µM	NT**	98.78± 0.11	NT	78.92 ± 0.022***

[a] Standart error mean, [b] Dichloromethane, [c] Ethyl acetate, [d] Methanol, [e] distilled water, * NA: No Activity, ** NT: Not tested; *** $P < 0.001$: values marked by ***which are significantly different according to the ANOVA test ($P < 0.05$)

2.2. DPPH radical scavenging activity

In order to screen the potential of plant extracts available as antioxidants, DPPH radical scavenging assay was employed. The results are shown in Table 2. Similar to the results obtained for cholinesterase inhibition, the methanol extracts displayed the highest activity. In general, it is well-known that phenols and particularly polyphenols are important sources to show antioxidant activity. Regarding that phenols and polyphenols are polar molecules, it is quite reasonable to see the highest activities in a polar solvent extract such as methanol. Indeed, both the dichloromethane and ethyl acetate fractions pointed out poor activities in comparison to results obtained for methanol extracts (within the 2-30% range). It is also important to express that the methanol extracts of the leaves and the flower parts of *S. willeana* displayed very high activity which was found comparatively poor in stem extracts including methanol.

It is noteworthy to mention that the activity results for both the 0.5 mg/mL and 1 mg/mL of the methanol extracts of the leaves and flower parts have been found to be very comparable to the results obtained for gallic acid at the same concentrations. A recent study on the chemical composition of *S. willeana* have pointed out that the plant is rich of phenols and polyphenols, particularly including gallic acid derivatives [13]. From this point of view, it is not surprise to observe high antioxidant activity for *S. willeana* that is comparable to the results obtained for the reference compound, gallic acid [15]. There is one point that might be questionable regarding the poor/no activity observed for the water extracts. Although water is also polar and it might be reasonable to see DPPH radical scavenging activity in the water extracts, the missing activity might be attributed to the sequence of extractions, wherein methanol extraction comes before water

extraction in our experimental design. Therefore, it appears that almost all phenol and polyphenol constituents including gallic acid derivatives within the leaves and flowers of *S. willeana* were extracted into the methanol phase leaving out almost no phenol constituent to the water phase extraction. This is further described within the results of total phenol content of the plant.

The extracts of *Salvia* species are known to act as antioxidant in various assay systems like ferric reducing power, superoxide anion, hydroxyl radical, iron chelation and including the DPPH radical scavenging activity. [9,11,13]. The results obtained for *S. willeana* within this study have pointed out that *S. willeana*, like other *Salvia* species, might be classified as a source of antioxidants comparable to other *Salvia* species.

Table 2. DPPH Radical Scavenging Activities of the extracts of *S. willeana* (percentage ± S.E.M).

Extracts	0.5 mg/mL	1 mg/mL
DCM stem	2.04 ± 0.001	4.01 ± 0.028***
DCM leaves	4.09 ± 0.014	6.60 ± 0.002***
DCM flower	2.67 ± 0.002	5.65 ± 0.001***
EtOAc stem	13.05 ± 0.006	21.29 ± 0.004***
EtOAc leaves	18.18 ± 0.004	27.40 ± 0.004***
EtOAc flower	11.76 ± 0.014	20.11± 0.001***
MeOH stem	12.04 ± 0.003	21.96 ± 0.005***
MeOH leaves	67.94 ± 0.003	71.30± 0.014***
MeOH flower	45.07± 0.001	64.14 ±0.003***
Water stem	NA	4.91 ± 0.004***
Water leaves	10.59± 0.003	14.99 ± 0.004***
Water flower	NA*	NA
0.5 mg/mL Gallic acid	NT**	67.12± 0.004
1 mg/mL Gallic acid	NT	90.23 ±0.005

DCM: Dichloromethane; EtOAc: Ethyl acetate; MeOH: Methanol ; *NA: No Activity; *NT: Not tested;
*** P < 0.001: values marked by ***which are significantly different according to the ANOVA test

2.3. Iron (II)-ferrozine test system for iron chelating

It is known that metals particularly iron species are important pieces of oxidative stress. Iron has been shown to be involved in various oxidative stress mechanisms particularly through its contribution in the generation of reactive oxygen species. Fenton reaction is a typical example of this situation. From this point of view, iron chelating agents are of specific interest to be identified or discovered to aid in the prevention of reactive oxygen species. Regarding natural products are good sources of antioxidants and the antioxidant capacity of *S. willeana*, the iron-chelating capacity of each extract of the plant prepared was analyzed. The results are shown in Table 3. Accordingly, almost all of the extracts displayed no capacity to chelate iron at 0.5 mg/mL concentration. At 1 mg/mL concentration varying activities were observed. None of the results was found specific to neither plant aerial parts nor extraction solvents. Particularly remembering that major chemical constituents of *S. willeana* are gallic acid derived polyphenols lower activities obtained through iron chelating assays are reasonable, since gallic acid itself has been shown to be a poor iron-chelating agent under these experimental conditions.

Table 3. Ferrous iron-chelating activities of the extracts of *S. willeana*.

Extracts	0.5 mg/ml	1 mg/ml
DCM stem	14.74 ± 0.086***	17.75± 0.049***
DCM leaves	NA*	NA*
DCM flower	7.84 ±0.011***	16.59± 0.07***
EtOAc stem	NA*	NA*
EtOAc leaves	NA*	10.54 ± 0.116***
EtOAc flower	NA*	NA*
MeOH stem	NA*	6.8± 0.05***
MeOH leaves	NA*	NA*
MeOH flower	NA*	8.78± 0.04***
Water stem	NA*	NA*
Water leaves	NA*	10.35± 0.09***
Water flower	NA*	NA*
1 mg/mL EDTA	NT**	75.05 ±0.02***

DCM: Dichloromethane; EtOAc: Ethyl acetate; MeOH: Methanol ; *NA: No Activity; *NT: Not tested;
*** $P < 0.001$: values marked by ***which are significantly different according to the ANOVA test

2.4. Total phenol and total flavonoid contents of extracts

Total flavonoid and phenol contents of *S. willeana* extracts are shown in Table 4. Accordingly, flavonoid content was found to be distributed at each extract prepared. Total flavonoid content of the extracts was obtained through the equation $y = 7.4216x - 0.2397$ ($R^2 = 0.993$) calculated according to the reference (i.e., quercetin). Mainly, in the ethyl acetate and dichloromethane extracts, flavonoid content was found to be higher. Remembering the chromen structure present in flavonoids, diversity within the flavonoid structures are related to the degree and the position of hydroxyl groups. From this point of view, both the hydrophilic and lipophilic nature of flavonoids within the structure of *S. willeana* appeared to be similar, since a comparable distribution was observed in various extracts prepared with different organic solvents with a varying polarity range. One aspect of the findings might be related to the cholinesterase inhibition potential measured. Flavonoids have been shown to be important scaffolds in both natural products and medicinal chemistry designs of cholinesterase inhibitor agents. Assuming that the measured cholinesterase inhibitor potential of *S. willeana* extracts might be at least partially related to the flavonoid present, the results were found consistent in such a way that the ethyl acetate extracts have displayed not only the higher content of flavonoid but also higher potential to inhibit cholinesterase enzymes. Overall, the flavonoid content within the extracts was found within the 187.5-547.5 mg/g range. Moreover, in comparison to other *Salvia* species, the flavonoid amounts in different extracts of *S. willeana* are all comparable [11].

Total phenol content of the extracts was obtained through the equation $y = 0.2213x + 0.0666$ ($R^2 = 0.9972$) calculated according to the reference (i.e., gallic acid). As seen in Table 4, total phenol content of *S. willeana* extracts was found to be within the 90-300 mg/g range. It is noteworthy to state that methanol extracts were found to be the richest in terms of phenol content (i.e., the leaves and flower extracts). Besides, similar studies on the extracts of other *Salvia* species pointed out a range of 50-400 mg/g range in terms of phenol content [11]. From this point of view, *S. willeana* has appeared to be one of the leading species in terms of its phenol content. Regarding the gallic acid and sugar-linked gallic acid derivative contents present in *S. willeana*, as shown previously, it has been found natural to observe the methanol extracts possessing the highest phenolic content with an optimum polarity to extract phenol constituents. From this point of view, it is also not a surprise to observe the methanol extracts displaying the highest antioxidant activity very comparable to the antioxidant activity of gallic acid.

Table 4. Total phenol and flavonoid contents ± S.E.M[a] of the extracts of *S. willeana*

Extracts	Total phenol (mg GAE/g plant extract)[b]	Total flavonoid (mg QUE/g plant extract)[c]
DCM stem	93.0 ± 3.25***	240.24 ± 2.93***
DCM leaves	90.73 ± 2.93***	490.45 ± 3.17***
DCM flower	94.31 ± 2.96***	392.23 ± 3.55***
EtOAc stem	91.95 ± 1.23***	240.84 ± 3.47***
EtOAc leaves	102.17 ± 2.76***	547.55 ± 3.12***
EtOAc flower	88.2 ± 2.10***	216.03 ± 1.36***
MeOH stem	83.76 ± 2.48***	187.54 ± 2.25***
MeOH leaves	300.24 ± 1.23***	290.08 ± 2.73***
MeOH flower	185.77 ± 1.26***	220.69 ± 3.52***
Water stem	103.01 ± 1.74***	208.23 ± 2.68***
Water leaves	99.2 ± 0.95***	270.56 ± 0.43***
Water flower	96.5 ± 2.25***	205.98 ± 0.56***

DCM: Dichloromethane; EtOAc: Ethyl acetate; MeOH: Methanol
[a] S.E.M (Standart error mean)
[b] Milligram of gallic acid equivalents/g dried extract ± S.E.M
[c] Milligram of quercetin equivalents/g of dried extract ± S.E.M
*** $P < 0.001$ values marked by ***which are significantly different according to the ANOVA test

3. CONCLUSION

Salvia species have been previously shown to have diverse biological activities, particularly including cholinesterase inhibitor and antioxidant properties with respect to their flavonoid and phenol content. Within this study, the total flavonoid and phenolic content of *S. willeana* (Holmboe) Hedge, an endemic plant of Cyprus, have been studied for the first time. Moreover, various plant extracts have been prepared and screened for their antioxidant, cholinesterase inhibitor, and iron chelating properties. Although lower activities obtained in cholinesterase inhibition and iron chelation assays, moderate activities have been obtained in antioxidants assay system employed.

4. MATERIALS AND METHODS

4.1. Chemicals

n-Hexane, methanol, ethanol, ethyl acetate, dichloromethane, sodium acetate, ferrozine, Folin-Ciocalteu reagent, gallic acid, aluminum chloride, iron (II) chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5,5-dithio-bis(2-nitrobenzoic)acid (DTNB), quercetin, acetylthiocholine chloride, butyrylthiocholine chloride, Electric eel AChE (Type-VI-S, EC 3.1.1.7) and equine serum BChE (EC 3.1.1.8) were obtained from Sigma Aldrich (Sigma, St. Louis, MO, USA). Donepezil, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), tris(hydroxymethyl)aminomethane, Bovine Serum Albumin were purchased from Sigma Aldrich (Steinheim, Germany).

4.2. Preperation Plant Material

S. willeana was collected from Promodros Forestry College in Trodos Mountain in May 2018. The plant samples were identified by Assist. Prof. Dr. Tugba Ercetin from the Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, (TR. North Cyprus). An herbarium specimen (voucher number: TE 003) has been deposited and retained in the herbarium. The whole plant parts were dried at room temperature protected from direct sun light. Following that the plant parts were separated and divided into aerial parts (i.e., stem, leaves, and flower). Employing a mechanic grinder, each part was grounded to fine powder.

4.3. Preparation of Extracts

Following the drying process of each plant aerial part, the fine powder was weighed. For the extraction process, the solvents were added in the following order: dichloromethane, ethyl acetate, methanol and distilled water. The extraction yields (w/w, %) are presented in Table 5.

Table 5. The yields of the stem, leaf and flower parts of *S. willeana* extracts (w/w, %).

Extract type	Stem	Leaf	Flower
Dichloromethane	4.76	3.74	0.013
Ethyl acetate	0.0004	0.018	0.014
Methanol	0.017	0.11	0.026
Water	0.014	0.12	0.042

4.4. Cholinesterase inhibition assay

The modified Ellman's method was employed for the measurement of the potential of plant extracts to inhibit cholinesterase enzymes (i.e., AChE, BChE) [13,16-19]. The enzymatic reactions were conducted in a 96-well microplate. Briefly, a 200 µL reaction mixture consisted of 50 mM Tris HCl buffer (pH 8,0), 6.8 mM DTNB solution in assay buffer, 2 µL sample solution in methanol, and 10 µL of substrate (i.e., either acetylthiocholine chloride or butyryl thiocholine chloride, depending on the assay) solution in distilled water. The reactions were initiated through the addition of 10 µL enzyme (either AChE or BChE, depending on the reaction) solution in assay buffer. Following the incubation time at 25°C for 15 min, the formation of 5-thio-2-nitrobenzoate was measured at 412 nm employing a 96-well microplate reader (i.e., Varioskan Flash, Thermo Scientific, USA). Each assay was performed in triplicate and the mean ± standard error mean was calculated. Percent inhibitions were calculated at both 0.5 mg/mL and 1 mg/mL concentrations of plant extracts. Each percent inhibition was calculated employing the formula (E-S)/E x 100, wherein E is the full activity in the absence of inhibitor and S is the activity in the presence of an inhibitor. For the reference studies, donepezil hydrochloride was employed (i.e., 10 µM).

4.5. Antioxidant Activity

In order to determine the potential of plant extracts as antioxidant two different assays were performed (i.e., DPPH radical scavenging activity, and iron (II)-ferrozine test system).

4.5.1. DPPH radical scavenging activity

DPPH radical scavenging activity was conducted using the UV methodology suggested by Blois (1958) [20,21]. Mainly, the samples and the reference (gallic acid) were dissolved in methanol to obtain both 0.5 mg/mL and 1 mg/mL concentrations. These solutions were added 0.15 mM DPPH solution in methanol. Following incubation at room temperature for 20 minutes, the unchanged DPPH amount was measured at 520 nm employing 96-well microplate reader (Varioskan Flash, Thermo Scientific, USA).

The percent DPPH radical scavenging activity was measured through the following formula [(A blank – A sample)/A blank] × 100 wherein the A blank is the result obtained in the absence of test sample and A sample is the result obtained in the presence of a test sample or the reference. Each assay was performed in triplicate and the mean ± standard error mean was calculated.

4.5.2. Iron (II)-ferrozine test system

The method of Chua was used to measure the capacity of the extracts to chelate ferrous ion within the iron (II)-ferrozine test system. Accordingly, into methanol, 10 µL test samples (both 0.5 mg/mL and 1 mg/mL concentrations of plant extracts) and the reference (0.5 mg/mL and 1mg/mL EDTA), and 2 mM iron (II) chloride was added in a final volume of 200 µL. Then, 5 mM ferrozine was added to the mixture. Following the incubation at room temperature for 10 min, the absorbance was measured at 562 nm. Percent activity was evaluated using the following formula [(A control – A sample)/A control] × 100 wherein the A

control is the result obtained in the absence of test sample or reference and A sample is the result obtained in the presence of a test sample or the reference. Assays were run in triplicate and the mean ± standard error mean was calculated.

4.6. Determination of total phenol and flavonoid contents

Folin-Ciocalteau's method was used to find out the total phenolic content of the plant extracts [22]. Briefly, the samples and a number of dilutions of gallic acid were obtained to prepare a calibration curve. The samples and gallic acid dilutions were mixed with distilled water, Folin-Ciocalteau's reagent and sodium carbonate. Then incubated at 40°C for 30 min. Afterward, absorption was measured at 765 nm at Varioskan Flash Microplate reader. The experiment was repeated for two concentrations (0,5 mg/mL and 1 mg/mL) for each extract.

Total flavonoid content of the extracts was calculated by aluminum chloride colorimetric method [23]. In short, a number of dilutions of quercetin were obtained to prepare a calibration curve. Then, the extracts and quercetin dilutions were mixed with methanol, aluminum chloride reagent, and sodium acetate as well as distilled water. Following incubation for 30 min at room temperature, absorbance of the reaction mixtures was measured at wavelength of 415 nm with Varioskan Flash Microplate reader.

The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg/g extract), respectively.

4.7. Statistical analysis of data

Data obtained from all experiments were expressed as mean standard error (±SEM). Statistical calculations were done using Design Expert Version 12 software and differences between the extracts were evaluated by ANOVA test. $P < 0.05$ was considered to be significant [***$P < 0.001$].

Author contributions: Concept – T.E., M.S., H.O.G; Design– T.E., H.O.G; Supervision–T.E.; Recources – T.E., M.S.; Materials – T.E., M.S., H.O.G; Data Collection and Processing – T.E.; Analysis and /or Interpretation – T.E., M.S., H.O.G; Literature Research – T.E., M.S.; Writing – T.E., M.S, H.O.G; Critical Reviews – T.E., H.O.G; M.S.

Conflict of interest statement: The authors declared no conflict of interest.

REFERENCES

[1] Cragg GM, Newman DJ. Natural product drug discovery in the next millennium. Pharm Biol. 2001; 3: 8-17. [CrossRef]

[2] Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA, Kent J. Biodiversity hotspots for conservation priorities. Nature. 2000; 403(6772): 853-858. [CrossRef]

[3] Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry. 1999; 66(2): 137-147. [CrossRef]

[4] Colović MB, Krstić DZ, Lazarević-Pašti TD, Bondžić AM, Vasić VM. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr Neuropharmacol. 2013; 11(3): 315-335. [CrossRef]

[5] Goh CW, Aw CC, Lee JH, Chen CP, Browne ER. Pharmacokinetic and pharmacodynamic properties of cholinesterase inhibitors donepezil, tacrine, and galantamine in aged and young Lister hooded rats. Drug Metab Dispos. 2011; 39(3): 402-411. [CrossRef]

[6] Mehta M, Adem A, Sabbagh M. New acetylcholinesterase inhibitors for Alzheimer's disease. Int J Alzheimers Dis. 2012: 1-8. [CrossRef]

[7] Lin H, Bhatia R, Lal R. Amyloid β protein forms ion channels: implications for Alzheimer's disease pathophysiology. FASEB J. 2001; 15(13): 2433-2444. [CrossRef]

[8] Meikle RD. Flora of Cyprus. Volume One, Kew: The Bentham-Moxon Trust Royal Botanic Gardens. 1977.

[9] Şenol FS, Orhan I, Celep F, Kahraman A, Doğan M, Yılmaz G, Şener B. Survey of 55 *Salvia* taxa for their acetylcholinesterase inhibitory and antioxidant activities. Food Chem. 2010; 120: 34-43. [CrossRef]

[10] Orhan I, Kartal M, Naz Q, Ejaz A, Yılmaz G, Kan Y, Konuklugil B, Şener B, Choudhary MI. Antioxidant and anticholinesterase evaluation of selected Turkish *Salvia* species. Food Chem. 2007; 103(4): 1247-1254.

[11] Orhan IE, Şenol FS, Erçetin T, Kahraman A, Celep F, Akaydın G, Bilge Şener, Doğan M. Assessment of anticholinesterase and antioxidant properties of selected sage (*Salvia*) species with their total phenol and flavonoid contents. Ind Crops Prod. 2013; 41: 21-30. [CrossRef]

[12] Demirezer LÖ, Gürbüz P, Kelicen Uğur EP, Bodur M, Özenver N, Uz A, Güvenalp Z. Molecular docking and ex vivo and in vitro anticholinesterase activity studies of *Salvia* sp. and highlighted rosmarinic acid. Turk J Med Sci. 2015; 45(5): 1141-1148. [CrossRef]

[13] Wu YB, Ni ZY, Shi QW, Dong M, Kiyota H, Gu YC, Cong B. Constituents from *Salvia* species and their biological activities. Chem Rev. 2012; 112(11): 5967-6026. [CrossRef]

[14] Lopresti AL. Salvia (Sage): A review of its potential cognitive-enhancing and protective effects. Drugs R D. 2017; 17(1): 53-64. [CrossRef]

[15] Yen GC, Duh PD, Tsai HL. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem. 2002; 79(3): 307-313. [CrossRef]

[16] Ellman GL, Courtney KD, Andres V Jr, Feather-stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7(2): 88–95. [CrossRef]

[17] Orhan I, Şenol FS, Koca U, Erçetin T, Toker G. Evaluation of the antioxidant and acetylcholinesterase inhibitory activities of *Arnebia densiflora* Ledeb. Turk J Biol. 2011; 35(1): 111-115. [CrossRef]

[18] Noshadi B, Erçetin T, Luise C, Yüksel MY, Sippl W, Şahin MF, Gazi M, Gülcan HO. Synthesis, Characterization, Molecular Docking, and Biological Activities of Some Natural and Synthetic Urolithin Analogs. Chem Biodivers. 2020; 17(8): e2000197. [CrossRef]

[19] Gürdal EE, Turgutalp B, Gülcan HO, Erçetin T, Şahin MF, Durmaz I, Yarım M. Synthesis of Novel Benzothiazole-Piperazine Derivatives and Their Biological Evaluation as Acetylcholinesterase Inhibitors and Cytotoxic Agents. Anticancer Agents Med Chem. 2017; 17(13): 1837-1845. [CrossRef]

[20] Norouzbahari M, Burgaz EV, Erçetin T, Fallah A, Foroumadi A, Firoozpour L, Gülcan HO. Design, synthesis and characterization of novel urolithin derivatives as cholinesterase inhibitor agents. Lett Drug Des Discov. 2018; 15(11): 1131-1140. [CrossRef]

[21] Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958; 181: 1199-1200. [CrossRef]

[22] Sharopov F, Valiev A, Sobeh M, Arnold E, Winka M. Bioactivity of three *Salvia* species in relation to their total phenolic and flavonoid contents. Pharm Chem J. 2018; 52(7): 596-600. [CrossRef]

[23] Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965; 16(3): 144-158.

[24] Woisky RG, Salatino A. Analysis of propolis: some parameters and procedures for chemical quality control. J Apic Res. 1998; 37(2): 99-105. [CrossRef]