



Biological activities of Cretan *Salvia pomifera* extracts

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ABSTRACT: The polyphenolic content and biological activities of dichloromethane, chloroform, ethyl acetate, and ethanol extracts of Cretan *Salvia pomifera* L. (Lamiaceae) were analysed. The ethyl acetate extract showed the highest content of total phenolics and total flavonoids. The ethanol extract exhibited the highest activity in the DPPH and FRAP assays, while the dichloromethane extract had the highest activity in the ABTS test. The ethyl acetate extract showed the highest activity in the β -carotene-linoleic acid system. The antioxidant activity of extracts was positively correlated with the total content of phenolics. Extracts demonstrated weak antibacterial activity. The ethyl acetate extract had the highest acetylcholinesterase inhibition at 50 $\mu\text{g}/\text{mL}$, while the ethanol and dichloromethane extracts showed the highest activity of tyrosinase inhibition at 25 $\mu\text{g}/\text{mL}$. In view of the significance of antioxidants in prevention and treatment of neurological diseases, the noticeable antioxidant and anti-neurodegenerative effects of the ethanol and ethyl acetate extracts recorded in this study make further research on *S. pomifera* seem promising.

KEYWORDS: antibacterial activity, anti-neurodegenerative activity, antioxidant activity, extracts, *Salvia pomifera*

Received: 27 June 2017

Revision accepted: 29 January 2018

UDC: 635.74:615.451.1 (495.9)

DOI: 10.5281/zenodo.1468304

INTRODUCTION

Salvia L. (sage), the largest genus of the Lamiaceae family, includes nearly 1000 species distributed worldwide (WALKER *et al.* 2004). Numerous *Salvia* species have been used since ancient times for medicinal and culinary purposes, and in modern times in the food, cosmetics, and pharmaceutical industries, owing to biological properties of the essential oils and extracts obtained from various species (HAMIDPOUR *et al.* 2014).

Salvia pomifera L. (apple-bearing sage) is an East Mediterranean species distributed in Greece and Asia Minor (HEDGE 1982). Its name originates from the round fruit-like galls (pomes) which are formed on the branches. Candied with sugar to make sweets, the galls are considered a great delicacy by the Greeks, one possessing healing properties. The leaves have a strong odour

and flavour, resembling a mixture of common sage and lavender, which are used for food flavouring or making tea (DWECK 2000). The essential oil from *S. pomifera* has been analysed, and high total thujone content was found (BELLOMARIA *et al.* 1992; BASER *et al.* 1993; KAORUSOU *et al.* 1998). Chemical composition of the essential oil and the larvicidal effect of *S. pomifera* against the West Nile virus mosquito *Culex pipiens* were evaluated by KOLIOPULOS *et al.* (2010). Antifungal activity of the oil was analysed by PITAROKILI *et al.* (1999) and GLAMOČLIJA *et al.* (2006). The antioxidant activity of aqueous extracts was investigated by LIONIS *et al.* (1998), who found a decrease of lipid peroxidation in cultured lung cells exposed to iron. GOUGOULIAS (2012) tested antioxidant activities and phenolics of leaf methanol extracts of four *Salvia* species cultivated in Greece and found a high antioxidant potential for *S. pomifera* in three applied tests.

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The aerial parts of Cretan *S. pomifera* were extracted by n-butanol and ethyl acetate, after which the phenolic components and antiradical activity were analysed by ATWI *et al.* (2016). COULADIS *et al.* (2003) examined ethanol extracts of aerial parts of 21 Lamiaceae species and obtained a high antioxidant potential for *S. pomifera* subsp. *calycina*. STAGOS *et al.* (2012) investigated 24 extracts of Greek Lamiaceae, among them aqueous and methanol extracts of *S. pomifera*, in an attempt to find a correlation of polyphenolic content with antioxidant and antibacterial effects, which was not established for the investigated species. DUIJKER *et al.* (2015) investigated the potential of using the extracts of three Cretan aromatic plants, including *S. pomifera*, as antiviral therapeutics. ERDOGAN *et al.* (2011, 2014) studied leaf methanol extracts of three Turkish *Salvia* species (including *S. pomifera*), which exhibited strong antioxidant activity, as well as high phenolic and flavonoid contents.

Since data on the biological activities of *S. pomifera* extracts are scarce, the aim of this study was to analyse the antioxidant, antibacterial, and anti-neurodegenerative activities of four extracts of *S. pomifera* aerial parts.

MATERIAL AND METHODS

Plant material. Aerial parts of *S. pomifera* were collected in July of 2013 near the town of Chania on the Greek island of Crete, and determined by Prof. Dr. P. D. Marin. Plant material was air-dried and kept in the dark at room temperature for further processing. The voucher sample is deposited in the herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, University of Belgrade, Faculty of Biology (BEOU 17356).

Preparation of plant extracts. Dry, milled plant material (10 g) was successively extracted by 100 mL of dichloromethane, chloroform, ethyl acetate, and ethanol (ORHAN *et al.* 2013; ALIMPIĆ *et al.* 2017a, b). Crude extracts were stored at +4°C for further experiments.

Determination of total phenolic and flavonoid contents. Total phenolic content (TPC) was quantified using the Folin-Ciocalteu phenol reagent (SINGLETON & ROSSI 1965), while total flavonoid content (TFC) was measured using aluminium nitrate nonahydrate (PARK *et al.* 1997) according to a slightly modified experimental procedure (ALIMPIĆ *et al.* 2017a, b), the results being expressed as mg GAE/g and mg QE/g of dry extract, respectively.

Evaluation of antioxidant activity. For testing of antioxidant activity, stock solutions of dry extracts were prepared in methanol at a concentration of 500 µg/mL. Four spectrophotometric assays were applied and antioxidant activity was monitored from change of the reaction mixture's colour in the presence of the sample. 2,2-Dyphenyl-1-picrylhydrazyl (DPPH) (BLOIS 1958),

2,2'-azino-bis-(3)-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS) (MILLER *et al.* 1993), ferric-reducing ability of plasma (FRAP) (BENZIE & STRAIN 1996), and β-carotene-bleaching (β-CB) (DAPKEVICIUS *et al.* 1998) assays were performed using modified experimental protocols as described previously (ALIMPIĆ *et al.* 2017a, b). Results were presented as IC₅₀ values (µg/mL), as ascorbic acid equivalents per gram of dry extract (mg AAE/g), as µmol FeSO₄ × 7H₂O /g dry extract, and as inhibition of β-carotene bleaching, respectively.

Evaluation of antibacterial activity. The antibacterial activity of extracts and the standard antibiotics streptomycin and rifampicin was tested against four Gram-negative (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC15442, *Salmonella enteritidis* ATCC12076, and *Shigella flexneri* ATCC9199) and four Gram-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Listeria innocua* ATCC 33090) bacterial strains. The lowest concentrations without visible growth, i.e., the minimal inhibitory concentrations (MICs), were determined according to KOLAREVIĆ *et al.* (2016).

Evaluation of anti-neurodegenerative activity. Acetylcholinesterase (AChE) and tyrosinase (TYR) inhibitory activity assays were performed by the spectrophotometric method (ELLMAN *et al.* 1961; MASUDA *et al.* 2005) using 96-well plates with slight modifications as described before (ALIMPIĆ *et al.* 2017a, b). The applied concentrations of extracts and standards (galanthamine and kojic acid) were 25, 50, and 100 µg/mL. Results were expressed as percents of inhibition.

Statistical analysis. All measurements were carried out in triplicate and expressed as means ± standard deviation. Pearson's correlation coefficients were calculated between the content of phenolic components and values obtained applying different antioxidant assays, and were interpreted according to TAYLOR (1990). All calculations were performed using MS Office Excel 2007.

RESULTS AND DISCUSSION

Yield of extracts, total phenolic and flavonoid content. The yield of extracts ranged from 0.89% for the ethyl acetate extract to 7.06% for the dichloromethane extract (Table 1). The less polar solvent dichloromethane yielded more compared to other solvents. ORHAN *et al.* (2013) also obtained the highest yield for several *Salvia* species with dichloromethane extracts, up to 8.53%, for *Salvia viscosa* aerial parts.

The ethyl acetate extract possessed the highest content of total phenolics (82.8 mg GAE/g) and flavonoids (28.8 mg QE/g). In the study of STAGOS *et al.* (2012), methanol and aqueous extracts of *S. pomifera* ssp. *calycina* had the

Table 1. Yield, TPC, TFC, and antioxidant activity of *S. pomifera* extracts evaluated by DPPH, ABTS, FRAP, and β -CB assays.

Extracts	Yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)	DPPH assay (IC ₅₀ , μ g/mL)	ABTS assay (mg AAE/g) ^b	FRAP assay [μ mol Fe(II)/g]	β -CB assay (% inhibition)
Dichloromethane	7.06	40.99 \pm 7.91 ^a	14.26 \pm 0.82 ^a	141.04 \pm 4.65	1.46 \pm 0.05 ^b	484.33 \pm 22.19 ^b	28.19 \pm 2.26 ^b

Tested at: ^a1 mg/mL; ^b0.5 mg/mL; ^c0.1 mg/mL.

highest level of total phenolics among 24 tested extracts (575 and 551 mg GAE/g DW, respectively). GOUGOULIAS (2012) found TPC of 12.51 mg GAE/ g DW in leaf methanol extracts of Greek *S. pomifera*, which is significantly lower than our results. ERDOGAN *et al.* (2014) examined populations of three *Salvia* species and obtained the highest TPC and TFC for *S. pomifera*, but significantly lower TPC and higher TFC values compared to our results. ORHAN *et al.* (2013) obtained higher flavonoid content for ethyl acetate extracts in their study of 14 *Salvia* species. Finally, AKKOL *et al.* (2008) more efficiently extracted total phenols and flavonoids using water and methanol. In reporting their results, they emphasised influence of the extraction solvent, plant species, location, and harvesting time.

Evaluation of antioxidant activity. The ethanol extract showed the highest scavenging activity against DPPH (43.7 μ g/ml), while that of the chloroform extract was the weakest (152 μ g/ml) (Table 1). In the FRAP assay, similar results were obtained, i.e., the ethanol extract had the strongest antioxidant effect [603 μ mol Fe (II)/g], better than BHA [583 μ mol Fe (II)/g], while the chloroform extract showed the weakest effect [299 μ mol Fe (II)/g]. In the ABTS assay, the ethanol extract was the weakest (0.81 mg AAE/g), while the dichloromethane extract was the strongest (1.46 mg AAE/g) antioxidant agent. In the β -carotene-linoleic acid system, the ethyl acetate extract achieved the best result (93.6%), the chloroform extract the poorest (27.4%).

ORHAN *et al.* (2013) obtained similar results for *Salvia crypantha*, *S. viscosa*, and *S. argentea*, recording the strongest antioxidant activity for ethanol extracts in the DPPH assay. Among 24 extracts of Greek Lamiaceae species, STAGOS *et al.* (2012) obtained very high antioxidant activity for aqueous extracts of both subspecies of *S. pomifera* (8 and 11 μ g/mL) in the DPPH test, and also high activity in the ABTS assay (17 and 24 mg AAE/g). ERDOGAN *et al.* (2014) obtained high antioxidant activity of Turkish *S. pomifera* (450 μ mol TE/100 g DW) in the DPPH test, which was correlated with phenolic content. Leaf methanol extracts of *S. pomifera* from Greece achieved values of 25.5 μ mol DPPH/g DW, 24.3 μ mol TP/g DW, and 9.05 μ mol FRAP/g DW in the DPPH, ABTS, and FRAP tests, respectively (GOUGOULIAS 2012). The n-butanol and ethyl acetate extracts of Cretan *S.*

fruticosa had the highest values in applied antioxidant tests, followed by *S. pomifera* and *S. sclarea* (ATWI *et al.* 2016). In chemical analysis of *S. pomifera* extracts, they found rosmarinic acid, two flavones, and three flavone glycosides, while the ethyl acetate extract was characterised by the presence of the flavone hispidulin (6-methoxy apigenin). Rosmarinic acid and hispidulin were proved to be powerful antioxidant agents (PETERSEN & SIMMONDS 2003; PATEL & PATEL 2017).

Besides polyphenols, active agents of the antioxidant activity of *S. pomifera* extracts are diterpenes, especially pomiferin (ULUBELEN & TOPCU 1992), which has been confirmed as a valuable antioxidant (DIOPAN *et al.* 2008).

Correlation between antioxidant assays and total phenolic and flavonoid contents. Because polyphenols are considered to be the main bioactive compounds possessing antioxidant activity, correlation coefficients were calculated. Antioxidant activity was moderately to strongly correlated with total phenolic content ($0.50 < r < 0.98$). Correlation of antioxidant activity with total flavonoid content was weak, except in the case of the β -CB assay ($r = 0.69$). Results of the DPPH test were strongly correlated with total phenolic and flavonoid contents. TOSUN *et al.* (2009) also found strong correlation between DPPH test results and total phenolics, which constitutes evidence for the contribution of phenolics to antioxidant activity. Results of the ABTS test were moderately correlated with DPPH results, while STAGOS *et al.* (2012) found weak correlation among results of DPPH, ABTS results, content of phenols, and flavonoid content in the tested *Salvia* species.

Antibacterial activity. *Salvia pomifera* extracts showed various levels of antibacterial activity against the tested bacterial strains (Table 2). The most sensitive bacterial strain to the examined extracts was *B. subtilis*, while *L. innocua* was sensitive to all extracts. *Enterococcus faecalis* did not show sensitivity to the ethanol extract. The ethyl acetate and chloroform extracts showed better antibacterial activity in comparison with the other tested extracts. The examined *S. pomifera* extracts had no antibacterial effect on *S. aureus*, *E. coli*, and *S. enteritidis* (data not shown). In the previous study of STAGOS *et al.* (2012), aqueous extracts of *S. pomifera* showed a MIC value of 4.0 mg/mL against *S. aureus*. The ethanol

Table 2. Antibacterial activity of *S. pomifera* extracts presented as minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

	Extracts (mg/mL)								Antibiotics ($\mu\text{g/mL}$)			
	Dichloromethane		Chloroform		Ethyl acetate		Ethanol		Streptomycin		Rifampicin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria												
<i>B. subtilis</i>	-	0.078	-	≤ 0.078	-	≤ 0.078	-	-	-	3.125	-	-
<i>E. faecalis</i>	0.625	1.25	0.625	2.5	-	1.25	-	-	-	200	≤ 3.125	50
<i>L. innocua</i>	-	0.156	0.156	0.3125	0.156	0.3125	-	1.25	-	25	≤ 3.90625	7.8125
Gram-negative bacteria												
<i>P. aeruginosa</i>	5	-	5	-	5	-	5	-	25	50	-	-
<i>S. flexneri</i>	-	5	2.5	5	2.5	5	-	5	-	1.5625	-	-

extract of *Salvia officinalis* in the study of NASCIMENTO *et al.* (2000) had no effect on the growth of *S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, and *Shigella* spp., while in the study of MOSAFA *et al.* (2014) it had an antibacterial effect on *S. aureus*, *E. coli*, and *P. aeruginosa*. *Escherichia coli* and *P. aeruginosa* were completely non-susceptible to *Salvia* extracts (TEPE *et al.* 2005). Gram-negative bacteria are generally more resistant than Gram-positive bacteria, due to arrangement of the outer membrane, which provides a barrier to penetration by macromolecules (NIKAIDO 2003).

Anti-neurodegenerative activities of extracts. Except in the case of the ethyl acetate extract, an increase of extract concentration resulted in decrease of AChE inhibition, while galanthamine showed a linear correlation with concentration increase (Table 3). The strongest activity for ethyl acetate was obtained at 50 $\mu\text{g/mL}$ (36.9%), whereas for the dichloromethane and ethanol extracts it was obtained at 25 $\mu\text{g/mL}$, but in both cases activity was lower than for galanthamine at the same concentrations. Contrary to the anticipated result that an increase in concentration of the extract will cause an increase of activity, the tested extracts showed the strongest inhibition at the lowest concentration, an inconsistency that was also noticed in previous experiments (ŞENOL *et al.* 2010; ORHAN *et al.* 2013; ALIMPIĆ 2016).

Plant extracts are considered to be a potential source of inhibitors of the enzyme AChE, which is involved in development of Alzheimer's disease, the most widespread neurodegenerative disorder (MURREY *et al.* 2013). ORHAN *et al.* (2013) found the strongest AChE inhibition for dichloromethane and ethanol extracts of *Salvia crypantha* at 100 $\mu\text{g/mL}$ (56.2%) among 14 Turkish species. In another study, ORHAN *et al.* (2007) applied the chloroform extract of *S. cyanescens* in a concentration of 1 mg/mL, and it exhibited high activity against AChE (80.2%). In

previous work of ours dealing with *Salvia amplexicaulis* (ALIMPIĆ *et al.* 2017a), AChE inhibition was lower compared to that obtained in the current research.

An increase of extract concentration from 25 to 100 $\mu\text{g/mL}$ resulted in a decrease of tyrosinase inhibition (Table 3). The strongest activity of the ethanol extract (20.32%) and that of the dichloromethane extract (20.05%) were both obtained at 25 $\mu\text{g/mL}$, but these activities were weaker than the activity of kojic acid at the same concentration (35.73%). Tyrosinase catalyses the oxidation of tyrosine to melanin, which plays a critical role in dopamine toxicity in Parkinson's disease (PD) (ORHAN *et al.* 2017). Screening of medicinal plants for their tyrosinase inhibition activity could be important as a way of finding an alternative and valuable source of drugs for the treatment of Parkinson's disease (LI *et al.* 2013). The antityrosinase activity of *Salvia dorisiana*, *S. elegans*, and *S. officinalis* was estimated as very weak (1.7-4.5%) by LIN *et al.* (2011). During our research on different *Salvia* species, important findings were recorded indicating inhibition of tyrosinase activity stronger than by standard kojic acid (AL SHEEF 2015; ALIMPIĆ 2016; ALIMPIĆ *et al.* 2017b), with better results obtained for ethanol than for aqueous extracts.

Because oxidative stress plays the most important role in the pathophysiology of neurodegenerative diseases, antioxidant therapy is suggested in the prevention and treatment of neurological disorders (KIM *et al.* 2015; SINGH 2015). In our study, the considerable antioxidant effect and inhibition of neurodegenerative enzymes obtained using ethanol and ethyl acetate extracts are worthy of attention.

CONCLUSION

During the last decades, researchers have focused their attention on finding novel plant constituents or products

Table 3. Inhibition of enzymes AChE and TYR by *S. pomifera* extracts.

Extracts/standards (µg/mL)	Inhibition of AChE (%)			Inhibition of TYR (%)		
	25 µg/mL	50 µg/mL	100 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
Dichloromethane	33.39±0.87	30.35±1.17	26.69±0.29	20.05±1.08	16.91±3.99	9.36±0.64
Chloroform	27.49±1.57	22.06±2.88	17.05±3.53	17.02±0.32	15.00±4.24	7.55±1.44
Ethyl acetate	21.60±2.87	36.90±0.87	34.20±0.84	14.89±1.33	10.85±2.42	9.79±1.81
Ethanol	33.26±0.98	31.32±1.18	22.48±0.60	20.32±1.44	14.68±2.58	7.66±0.92
Galanthamine	42.38±0.74	50.56±0.51	57.11± 1.68	-	-	-
Kojic acid	-	-	-	35.73±5.46	33.93±3.78	51.81±2.55

that are safer, more potent, and more efficacious remedies compared to synthetic drugs. In keeping with the current trend, the present research constitutes a continuation of our investigation of representatives of the genus *Salvia*, renowned for medicinal properties.

The ethanol extract of *S. pomifera* showed higher antioxidant activity in the DPPH and FRAP tests and stronger tyrosinase inhibition, while the ethyl acetate extract exhibited better antioxidant activity than other extracts in the β -carotene test and the highest activity of AChE inhibition. These extracts could thus be considered promising in further study of the chemical characteristics and other bioactivities of *S. pomifera*.

Acknowledgement — This work was supported by the Ministry of Education, Science, and Technological Development of Serbia (Grants No. 173029 and 172058).

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REZIME

Biološke aktivnosti ekstrakata *Salvia pomifera* sa Krita

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U ovom radu su analizirani sadržaj polifenola i biološke aktivnosti dihlorometanskog, hloroformskog, etil acetatnog i etanolnog ekstrakta *Salvia pomifera* L. (Lamiaceae) sa Krita. Etil acetatni ekstrakt je pokazao najviši sadržaj ukupnih fenola i flavonoida. Etanolni ekstrakt se pokazao najefikasnijim u DPPH i FRAP testu, dihlorometanski ekstrakt u ABTS testu, dok je etil acetatni ekstrakt ispoljio najjače dejstvo u β -karoten-linolna kiselina testu. Antioksidativna aktivnost ekstrakata je bila pozitivno korelisana sa sadržajem ukupnih fenola. Testirani ekstrakti su ispoljili slabo antibakterijsko dejstvo. Etil acetatni ekstrakt je pokazao najviši procenat inhibicije acetilholinesteraze pri koncentraciji od 50 $\mu\text{g}/\text{mL}$, dok su etanolni i dihlorometanski ekstrakti bili najefikasniji u inhibiciji tirozinaze na koncentraciji od 25 $\mu\text{g}/\text{mL}$. S obzirom na značaj antioksidanasa u prevenciji i tretmanu neuroloških oboljenja, značajni antioksidativni i antineurodegenerativni efekti etanolnog i etil acetatnog ekstrakta dobijeni u ovom radu mogu biti perspektivni za buduća istraživanja *S. pomifera*.

KLJUČNE REČI: antibakterijska aktivnost, antineurodegenerativna aktivnost, antioksidativna aktivnost, ekstrakti, *Salvia pomifera*