

***In vitro* evaluation of antioxidant, antineurodegenerative and antidiabetic activities of *Ocimum basilicum* L., *Laurus nobilis* L. leaves and *Citrus reticulata* Blanco peel extracts**

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Ocimum basilicum (sweet basil) and *Laurus nobilis* (bay leaves or laurel) have been used in traditional medicine for centuries, and also extensively employed as spices for adding aroma and flavor to various food products. *Citrus reticulata* (mandarin) is mainly used in food industry for juice production, while its peel as main byproduct contains high concentration of valuable substances. The samples were collected in Lastva Grbaljska (Montenegrin coast) and purchased from the market. Since the oxidative stress results in development of numerous diseases, among them neurodegeneration and diabetes, the antioxidant activity, antineurodegenerative and antidiabetic activities were analyzed, aiming to compare potential of plants cultivated under natural conditions and commercially purchased from the market, as well as to compare the effect of different solvents applied in the extraction process. Water, methanol and acetone extracts of leaves and peel were tested by DPPH and total reducing power (TRP) methods for determination of antioxidant activity, and by acetylcholinesterase (AChE) and α -glucosidase inhibition assays for analyzing the other activities. Total phenolic (TPC) and flavonoid (TFC) contents were also determined. The acetonetic extract of *L. nobilis* from Lastva showed the highest TPC, DPPH, TRP, and α -glucosidase inhibition, while water extract of commercial *L. nobilis* exhibited the highest AChE inhibition. The leaves of *L. nobilis* are demonstrated to be promising antioxidant, antineurodegenerative and antidiabetic agent.

Key words: *Ocimum basilicum*, *Laurus nobilis*, *Citrus reticulata*, extracts, bioactivities

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ABBREVIATIONS

AD - Alzheimer's disease

AChE - Acetylcholinesterase

AGLU - α -glucosidase

DM - Diabetes mellitus

DPPH - 2,2-Diphenyl-1-picrylhydrazyl

TFC - Total flavonoid content

TPC - Total phenolic content

TRC - Total reducing power

1. INTRODUCTION

Free radicals are highly reactive species having unpaired electrons in their outermost shell leading to oxidative stress, which causes tissue damage and results in large number of diseases. Oxidative stress plays a major role in progression of neuro-

logical diseases, such as Alzheimer's disease (AD) and also in diabetes mellitus (DM). When disbalance of the production of reactive oxygen species (ROS) and cellular antioxidant defenses appears, proteins and nuclear acids damages occur, having destructive effects in AD and DM (Reddy et al., 2009). Numerous molecular, clinical, epidemiological, etc. data supports a pathophysiological link between AD and DM (Ahmad et al., 2017). Cognitive decline related to DM is characterized by mild to moderate impairment, and an increased risk of developing AD and other forms of dementia (Toth, 2014). Considering the fact that AD and DM reach epidemic proportions, different approaches for their prevention and treatment are present among scientists worldwide. Natural products are extensively studied in the last decades for suppressing ROS production, and might be promising in AD and DM therapy. Antioxidants of natural or synthetic origin neutralize the effects of reactive oxygen species and thus help in preventing

diseases. There is a growing interest both in various industrial sectors and among people worldwide towards the use of natural compounds obtained from plants in cosmetic, food and pharmaceutical industries which could represent alternatives to synthetic chemicals, because natural compounds have lesser environmental and human health impacts. Natural antioxidants can be taken up through diet as they are present in fruits, vegetables and spices which contain a large amount of flavonoids and antioxidant supplements that contribute to protection against different types of health problems (Hamid et al., 2010). The beneficial effects of medicinal plants in AD and DM have been confirmed in numerous studies (Bazzari and Bazzari, 2018; Nasri et al., 2015). Several species belonging to the family Lamiaceae are of high socio-economic value, having therapeutical, culinary, horticultural, ornamental or industrial applications due to the high content of bioactive phenolic compounds (Trivellini et al., 2016). The Lauraceae family comprises species which contain considerable concentrations of essential oils, including genera of high commercial value, such as cinnamomum, laurus and persea. Different kinds of *Citrus* fruits are used in food industry for fresh juice production, with the peel as the main waste fraction, which had been widely studied because of high content of numerous biologically active compounds including natural antioxidants such as phenolic acids and flavonoids (Xu et al., 2007). *Ocimum basilicum* L. (Lamiaceae), commonly known as sweet basil, is a perennial herb, native to Asia, Africa, South America, and the Mediterranean but widely cultivated in many countries (Grayer et al., 1996). This important medicinal plant and culinary herb has been used traditionally for treatment of anxiety, diabetes, cardiovascular diseases, headaches, nerve pain, cough, cold, fevers, headaches and migraines, insect bites, menstrual cramps, sinusitis, as an anticonvulsant, anti-inflammatory and antithrombotic agent, and in a variety of digestive and neurodegenerative disorders (Purushothaman et al., 2018). *Laurus nobilis* L. (Laureaceae), commonly known as bay leaves or laurel, is a native plant from the Mediterranean region and is cultivated mainly in Europe and USA as an ornamental plant (Garg et al., 1992). It is widely used as a spicy fragrance and flavor in traditional meat dishes, stews and rice (Dias et al., 2014). Its leaves and extracts are used to suppress high blood sugar, fungal and bacterial infections, to treat eructation, flatulence and gastrointestinal problems and also exhibits anti-inflammatory, anticonvulsive, antiepileptic, cytotoxic, antioxidant and neuroprotective properties (Barla et al., 2007; Dias et al., 2014; Maajida Aafreen M et al., 2019; Pacifico et al., 2014). China is the most important centre of origin for the species of the genus *Citrus* (Zhang et al., 2014). *Citrus* species (Rutaceae) produce widely used fruits of high nutritional and industrial value, rich in flavonoid derivatives and polymethoxylated flavonoids, with different distribution in fruit parts (Nogata et al., 2006). Dried peel of *Citrus reticulata* Blanco is recorded in the Chinese Pharmacopeia as chenpi used for activation of vital energy and circulation, elimination of phlegm, disperse physical stagnation, etc., being good source of phenolics, especially of flavanone glycosides (Tumbas et al., 2010). The mentioned substances may play a role in the prevention of diabetes, cardiovascular and other diseases (Zhang et al., 2014). The main goal of this research was to compare bioactivities (antioxidant, antineurodegenerative and antidiabetic) of extracts of *O. basilicum* and *L. nobilis* leaves and *C. reticulata* peel obtained from plants cultivated under natural conditions in the Montenegro coastal region and commercially purchased from the market, and additionally to compare the influence of the different solvents applied in the extraction process.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Methanol, ethanol and hydrochloric acid were obtained from Zorka Pharma, Šabac, Serbia. Gallic acid, ascorbic acid, acarbose, galanthamine, quercetin, 2-*tert*-butyl-4-hydroxyanisole (BHA), 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethylsulfoxide (DMSO), iron(III) chloride, sodium carbonate anhydrous (Na₂CO₃), sodium bicarbonate (NaHCO₃) sodium phosphate dibasic, sodium dihydrogen phosphate dodecahydrate, sodium phosphate monobasic, sodium phosphate monobasic dihydrate, aluminum nitrate nonahydrate (Al(NO₃)₃ × 9H₂O), potassium acetate (CH₃COOK), potassium hydrogen phosphate, potassium dihydrogen phosphate, pNPG (4-nitrophenyl β-D-glucopyranoside), DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), Trisma base, α-glucosidase (from *Saccharomyces cerevisiae*) type I, acetylcholinesterase (from *Electrophorus electricus*), acetylcholine iodide and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich, St. Louis, MO. Trichloroacetic acid and potassium ferricyanide were purchased from Superlab (Serbia), while sodium hydroxide was purchased from NRK inženjering (Serbia). Starch was purchased from Roth (Germany).

2.2. Plant material

Plants were cultivated in Lastva Grbaljska, near Budva (Montenegro). Leaves of *Ocimum basilicum* and *Laurus nobilis* were collected in August 2018, while *Citrus reticulata* fruits were collected in November 2017. Plant material was dried and kept in shade at room temperature for further processing. The voucher specimens of *L. nobilis* and *O. basilicum* were deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade, Faculty of Biology (BEOU; voucher No. 17510, and 17511). The commercially purchased basil and bay leaves are products of Premia (Serbia), while mandarins were purchased from the local market.

2.3. Preparation of extracts

Grinded plant material (5 g of *O. basilicum* and *L. nobilis* leaves and 10 g of *C. reticulata* peel) was extracted during 24 h at room temperature (5% w/v and 10% w/v, respectively) using acetone, methanol and hot water. The mixture was exposed to ultrasound 1 h before and after 24 h to improve the extraction process. Subsequently, extracts were filtered through filter paper (Whatman No.1) and evaporated under reduced pressure (Buchi rotavapor R-114). The obtained crude extracts were stored in a refrigerator at +4 °C for further experiments. The yield of the extracts was calculated using the following equation:

$$\text{Yield (\%)} = \frac{m}{M} \times 100$$

where m represents the mass of dry extract, while M is the mass of the dry plant extract used for the extraction.

2.4. Determination of total phenolic and total flavonoid contents

Total phenolic content (TPC) of the plant extracts was performed applying the spectrophotometric procedure of Singleton and Rossi (1965). The reaction mixture was prepared by mixing 100 μL of extract at the concentration of 0.5 mg/mL with 500 μL of 10% Folin & Ciocalteu's reagent dissolved in water. After 6 min, 400 μL of 7.5% sodium carbonate was added. Blank contained distilled water instead of extracts. The absorbance was measured at 740 nm after 2 h incubation at room temperature, using Jenway 7315 UV/Visible spectrophotometer. The same procedure was repeated for the standard

solution of gallic acid (GA). The phenolic content of the samples was calculated from the standard curve and expressed as mg GA equivalents per gram of dry extract, presented as mean \pm standard deviation.

Total flavonoid content (TFC) of the samples was measured spectrophotometrically using Jenway 7315 UV/Visible spectrophotometer, according to the procedure of Park et al. (1997). The reaction mixture was prepared by mixing 0.5 mL of extract at the concentration of 0.5 mg/mL with 2.05 mL 80% ethanol, 0.05 mL 10% (Al(NO₃)₃ × 9H₂O), and 0.05 mL 1 M CH₃COOK. Blank contained 96% ethanol instead of extract. After 40 min of incubation at room temperature, the absorbance was measured at 415 nm. The same procedure has been repeated for the 96% ethanol solution of quercetin (Q) in order to construct the calibration curve. The content of flavonoids in the samples was expressed as mg Q equivalents per gram of dry extract, as mean \pm standard deviation.

2.5. Evaluation of antioxidant activity

2.5.1. DPPH assay

DPPH free radical scavenging method (Blois, 1958) was used for the determination of antioxidant activity, with slight modification. In test tubes was added 100 μ L of extract at the concentration of 0.5 mg/mL and 900 μ L methanolic solution of DPPH (40 μ g/mL). Methanol was used as blank, methanol with DPPH solution was used as negative control, while BHA, BHT and ascorbic acid were used as positive controls (standards). The absorbance was measured at 517 nm after 30 min in the dark at room temperature, using Jenway 7315 UV/Visible spectrophotometer. The decrease in absorption of DPPH was calculated as follows:

$$\text{Inhibition of DPPH radical (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

where A_c represents the absorbance of the negative control, while the absorbance of the test samples is labeled with A_s . The results are presented as percentage of DPPH inhibition \pm standard deviation.

2.5.2. Total reducing power assay

The total reducing power (TRP) was determined according to the method of Tusevski et al. (2014), with slight modifications. In the reaction mixture was added 100 μ L of sample at the concentration of 0.5 mg/mL, 200 μ L phosphate buffer (0.2 M, pH 6.6) and 200 μ L 1% potassium ferricyanide. After the incubation (20 minutes at 50 °C), 200 μ L 10% trichloroacetic acid, 200 μ L distilled water and 40 μ L 0.1% iron (III) chloride were added. Blank was prepared in the same manner, with the exception of adding 100 μ L of adequate solvent instead of sample. The absorbance was measured at 700 nm after the 10 minute incubation at room temperature, using Jenway 7315 UV/Visible spectrophotometer. The total reduction power of the samples was presented as μ mol of ascorbic acid equivalents (AAE) per gram of dry extract (μ mol AAE/g dry extract).

2.6. Antineurodegenerative activity

Acetylcholinesterase (AChE) inhibitory activity assay was performed according to the procedure of Ellman et al. (1961), using 96-well plates. The reaction mixture (S) was prepared by adding 140 μ L of sodium-phosphate buffer (0.1 M, pH 7), 20 μ L DTNB solution, 20 μ L sample (at the concentration of 0.5 mg/mL) and 20 μ L AChE solution (5 U/mL) in Tris-HCl buffer (20 mM, pH 7.5). The mixture without the sample was used as a control (C, sodium-phosphate buffer instead of sample), the mixture without the enzyme was used as blank for the extracts (B), while galanthamin was used as positive control (at the concentration of 0.1 mg/mL). After 15 minutes at 25

°C, the reaction was initiated by adding 10 μ L of acetylcholine iodide. Absorbance was measured at 412 nm, using Multiscan Sky Thermo Scientific, Finland. The percentage of inhibition was calculated by using the following equation:

$$\text{Inhibition of acetylcholinesterase (\%)} = \frac{C - (S - B)}{C} \times 100$$

2.7. Antidiabetic activity

Determination of α -glucosidase inhibitory activity was performed according to the method of Wan et al. (2013), with slight modifications. Briefly, the same volumes (20 μ L) of phosphate buffer (0.1 M, pH 6.9), α -glucosidase (0.5 U/mL) and samples (S) at the concentration of 0.5 mg/mL were pre-incubated for 5 min at 37 °C. After that, 20 μ L of pNPG (15 mg/10 mL) as substrate was added to the mixture and the incubation continued for another 20 min at 37 °C. At the end, the reaction was stopped by adding 80 μ L of 0.2 M sodium carbonate and the absorbance was measured at 405 nm using Multiscan Sky Thermo Scientific, Finland. The control (C) contained buffer solution instead of sample, while blank (B) contained buffer solution instead of enzyme. Acarbose at the concentration of 0.1 mg/mL was used as a positive control. The percentage of inhibition of α -glucosidase was calculated according to the following equation:

$$\text{Inhibition of } \alpha\text{-glucosidase (\%)} = \frac{C - (S - B)}{C} \times 100$$

2.8. Statistical analysis

All experimental measurements were carried out in triplicate and the results are expressed as the average of three measurements \pm standard deviation. To assess the potential effects of the solvent type, the results were subjected to one-way ANOVA, followed by Tukey's *post-hoc* test (the results were considered statistically significant at $P < 0.05$ level). Differences between localities for all observed parameters were estimated through Student's *t*-test ($P < 0.05$). Pearson's correlation coefficients (r) were calculated among the investigated activities of extracts and phytochemical contents and presented according to Taylor (1990), where $r < 0.35$, $0.36 < r < 0.67$ and $0.68 < r < 1$ were considered weak, moderate and strong correlation, respectively. All statistical analyses were done using the software package STATISTICA v.10.0.

3. RESULTS AND DISCUSSION

3.1. Yields, total phenolic and flavonoid contents of extracts

The yields of extracts varied depending on the plant species, origin of plant material and applied solvent (Table 1). The highest extraction yields for *O. basilicum* leaves were achieved when water was used as an extraction solvent (10.08 and 10.54%). Bomma et al. (2018) analyzed six *Ocimum* species and obtained yield for methanolic extracts of *O. basilicum* ranging from 0.98 to 1.44%, while methanolic extracts in our research yielded 4.62 and 6.74%. In the case of bay leaves, the yield of acetonic extract of commercial sample (31.06%) was noticeable higher comparing to other samples. On the contrary, methanolic extracts of Moroccan bay leaves showed the highest yield (22%) comparing to other applied solvents (Taroq et al., 2018). Mandarin peel showed the highest yield in methanolic extracts of both commercial and naturally cultivated samples (23.07 and 23.77%) (Table 1). Senol et al. (2016) in the study of the ethanolic extracts of peels of *C. reticulata* cultivars obtained yields from 11.39 to 42.5%. Safdar et al. (2017) tested mandarin peels with different extraction methods and extraction solvents, and achieved the highest yield

Table 1. Yield and the total phenolic content (TPC) and flavonoid content (TFC) of the plant extracts

Species	Extract type	Yield (%)		TPC ^a		TFC		
		[%]		[mg GAE/g]		[mg QE/g]		
		Commercial	Lastva	Commercial	Lastva ^b	Commercial	Lastva	
<i>O. basilicum</i>	Acetonic	2.18	3.26	28.52 ± 1.42 d	25.45 ± 2.25 f	31.97 ± 2.70 a	44.83 ± 3.22 a	†
	Methanolic	6.74	4.62	41.60 ± 1.68 c	34.94 ± 3.00 e	† 25.66 ± 0.89 b	32.06 ± 1.35 b	†
	Aqueous	10.08	10.54	50.08 ± 3.17 b	70.21 ± 2.64 b	† 9.54 ± 0.47 d	13.52 ± 0.79 d	
<i>L. nobilis</i>	Acetonic	31.06	5.5	75.98 ± 5.40 a	83.84 ± 0.49 a	† 6.43 ± 0.05 e	27.31 ± 0.90 c	†
	Methanolic	16.3	19.88	73.37 ± 2.80 a	46.46 ± 3.22 cd	† 13.58 ± 0.05 c	24.73 ± 0.32 c	†
	Aqueous	10.54	13.96	56.57 ± 2.73 b	35.04 ± 2.17 e	† 7.02 ± 0.32 de	6.90 ± 0.61 e	
<i>C. reticulata</i>	Acetonic	5.66	4.43	52.79 ± 1.87 b	52.40 ± 2.89 c	13.61 ± 0.11 c	13.05 ± 0.23 d	
	Methanolic	23.07	23.77	34.62 ± 1.19 cd	43.00 ± 0.93 d	† 6.34 ± 0.11 e	8.05 ± 0.65 e	
	Aqueous	12.85	6.69	28.36 ± 1.19 d	25.52 ± 0.29 f	† 6.15 ± 0.43 e	6.09 ± 0.41 e	

^a Different letters denote statistical difference among means according to *post-hoc* Tuckey HSD test at level $P < 0.05$.

^b † sign denote statistically significant difference between Commercial and Lastva mean values according to the Student's *t*-test ($P < 0.05$).

for extraction with 80% ethanol (18.46%), while extraction with 100% methanol resulted in the lowest extraction yield (13.84%). In our study extraction with methanol achieved higher yield compared to the other solvents. Similarly, Zahoor et al. (2016) obtained the highest yield (44.24 g/100g) of *C. reticulata* "Merisol" peel extracted by pure methanol compared to other solvents and solvent mixtures. Plants are an important source of potentially bioactive compounds for the development of new chemotherapeutic agents. Numerous studies have shown that the bioactivities of the plant extracts are mainly due to the presence of phenolic compounds. The results of TPC are presented in Table 1. The acetonic extract of *L. nobilis* collected in Lastva showed the highest TPC (83.84 mg GAE/g), but also the commercial extract was rich in phenolics (75.98 mg GAE/g). Acetonic extracts of *O. basilicum* and water extracts of *C. reticulata* peel had the lowest TPC. Statistical analysis of the obtained results indicates that the origin of the plant material in most cases, as well as the applied solvent, have influence on TPC (Table 1). Javanmardi et al. (2003) tested a number of acetonic extracts of *O. basilicum* from different localities in Iran and found that the TPC ranged from 23.00 to 65.50 mg GAE/g, while in our study commercial *O. basilicum* acetone extract and *O. basilicum* from Lastva had 28.52 and 25.45 mg GAE/g, respectively. Ahmed et al. (2019) obtained different amounts of TPC in the study of ethanolic extracts of leaves and stems of basil collected from different locations in Egypt and concluded that this difference may be due to various climatic, seasonal and geographical conditions of the locations. Such dissimilarities can be also noticed in our results among samples of different origin. Kivrak et al. (2017) analyzed four *L. nobilis* leaves extracts and found higher TPC in ethanolic extract compared to water extract. Their findings are similar to the results obtained in this research, where methanolic extracts showed higher TPC compared to water ones. On the other hand, water extract of bay leaves collected in Morocco contained the highest TPC compared to other solvents (Taroq et al., 2018). Muñiz-Márquez et al. (2014) analyzed the effect of extraction time and different ethanol concentrations on the yields of polyphenols in bay leaves. The values for TPC were observed to grow with the increase of percentage of ethanol compared to water extract. In our study, the ace-

tonic extracts of *C. reticulata* had the highest TPC, while in the study of Zahoor et al. (2016) and Safdar et al. (2017) maximum polyphenols were extracted with methanol. Zhang et al. (2014) analyzed phenolic compounds in the 80% methanolic extracts of peels of 14 wild mandarin genotypes native to China, and obtained TPC ranging from 29.38 to 51.14 mg GAE/g. Zhang et al. (2018) have analyzed methanolic extracts of nineteen *C. reticulata* genotypes and found that TPC varied from 22.80 to 32.76 mg GAE/g, while our results ranged from 34.62 to 43.00 mg GAE/g.

The results of TFC are presented in Table 1. Both acetonic extracts of *O. basilicum* were rich in flavonoids, especially the sample from Lastva (44.83 mg QE/g). Generally, *C. reticulata* peel extracts showed the lowest values of TFC (6.09-13.61 mg QE/g). The origin of plant material had statistically significant impact in the case of *O. basilicum* and *L. nobilis*, while the effect of the used solvent was statistically significant for majority of samples (Table 1). Kaurinović et al. (2011) found considerably high TFC in water extracts of *O. basilicum* leaves, 26.42 µg RE/g. In their research, Kivrak et al. (2017) have found that ethanolic extract of *L. nobilis* possess higher TFC than water extract, which is similar to our results, with noticeable higher TFC in methanolic extracts. Likewise, the ethanolic extract of bay leaves from Morocco had the highest TFC (Taroq et al., 2018). Zhang et al. (2014) notified that TFC varied from 6.28 to 20.66 mg RE/g for methanolic extracts of peels of Chinese mandarin genotypes. Zhang et al. (2018) have found that TFC for Chinese *C. reticulata* methanolic extracts ranged from 23.29 to 56.52 mg RE/g, which was evidently higher compared to our results (6.34-8.05 mg QE/g). Generally, literature data demonstrate that the TPC and TFC vary depending on the locality, season, applied solvent and the extraction procedure (Javanmardi et al., 2003; Kivrak et al., 2017; Muñiz-Márquez et al., 2014; Safdar et al., 2017). In our study (Table 1) the statistical analysis showed that the sample origin had a significant influence on both TPC and TFC. The used solvent had statistically significant impact to TPC and TFC in majority of tested extracts.

Table 2. The activity of the plant extracts (at the concentration of 0.5 mg/mL) and standards (at the concentration of 0.1 mg/mL) against DPPH radical and the total reducing power activity (TRP).

Species	Extract type	DPPH ^a		TRP	
		[%]		[μmol AAE/g]	
		Commercial	Lastva	Commercial	Lastva
<i>O. basilicum</i>	Acetonic	15.74 ± 1.25 g	23.82 ± 1.73 h	349.06 ± 3.19 e	344.82 ± 10.72 d
	Methanolic	36.14 ± 1.54 f	33.92 ± 0.87 fg	582.70 ± 4.10 d	454.52 ± 7.73 c †
	Aqueous	52.67 ± 1.48 d	65.26 ± 2.72 c	629.97 ± 2.29 c	668.45 ± 12.73 b †
<i>L. nobilis</i>	Acetonic	79.71 ± 3.15 b	85.87 ± 2.54 b	765.42 ± 2.29 a	792.70 ± 22.27 a
	Methanolic	78.41 ± 4.08 b	47.57 ± 1.08 d †	689.97 ± 5.25 b	481.18 ± 15.45 c †
	Aqueous	59.89 ± 2.16 c	30.08 ± 1.64 g †	681.79 ± 18.92 b	353.91 ± 11.82 d †
<i>C. reticulata</i>	Acetonic	9.79 ± 0.06 h	12.70 ± 0.26 i	194.21 ± 8.15 g	184.82 ± 12.73 e
	Methanolic	8.21 ± 1.58 h	8.08 ± 0.59 j	137.55 ± 8.33 h	166.03 ± 8.40 e †
	Aqueous	16.02 ± 0.31 g	9.31 ± 0.06 ij	256.03 ± 2.29 f	199.06 ± 13.65 e †
Standards	Ascorbic acid	91.65 ± 0.21 a	91.65 ± 0.21 a	-	-
	BHA	43.33 ± 0.87 e	43.33 ± 0.87 e	-	-
	BHT	34.31 ± 0.43 f	34.31 ± 0.43 f	-	-

^a Different letters denote statistical difference among means according to *post-hoc* Tuckey HSD test at level $P < 0.05$.

[†] † sign denote statistically significant difference between Commercial and Lastva mean values according to the Student's *t*-test ($P < 0.05$).

3.2. Antioxidant activity

The antioxidant activity was evaluated applying DPPH assay which has been widely used for the determination of free radical scavenging capacity of samples and total reducing power assay (TRP) which evaluates potential of sample to reduce potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}). The results for antioxidant activity are presented in the Table 2. Acetonic extracts of leaves of *L. nobilis* showed the highest antioxidant activity in both assays. Likewise, water extracts of *O. basilicum* leaves exhibited high antioxidant potential. The influence of the solvent was statistically significant in both applied assays. The origin of the plant material was statistically significant in TRP assay for all species, while in DPPH test only in the case of *L. nobilis*.

Kaurinović et al. (2011) have obtained the best results in DPPH assay among the investigated extracts for water one of *O. basilicum*, which is in accordance with our results. Zakaria et al. (2008) showed that methanolic extracts of *O. basilicum* leaves inhibited 45.35% of DPPH radical at the concentration of 1 mg/mL, while in our study, commercial *O. basilicum* methanolic extract achieved 36.14%, and cultivated *O. basilicum* methanolic extract 33.92% at the concentration of 0.5 mg/mL. In the research of Ahmed et al. (2019) ethanol extracts of basil from different locations exhibited stronger radical scavenging activity than that of synthetic antioxidant BHT, while in our study standards were significantly stronger than extracts in applied concentrations. Antioxidant activity study of Kivrak et al. (2017) showed that water extract of *L. nobilis* had rather lower activity in all the applied assays compared to ethanolic extracts. Their findings are in accordance with our results for both used methods. Methanolic extract of *L. nobilis* possessed effective reducing power in the study of Pacifico et al. (2014), while in our research it was less potent comparing to acetonic extract. Water extract of Moroccan bay leaves exhibited the highest DPPH radical scavenging activity comparing to other tested extracts, which was probably

due to its high content of phenols (Taroq et al., 2018). Our results showed higher antioxidant activity of acetonic extracts than methanolic extracts. Dias et al. (2014) compared leaves of wild and cultivated *L. nobilis* plants and found that infusions of both samples revealed higher antioxidant activity than methanolic extracts, while in our study the methanolic extracts exhibited higher DPPH activity compared to water extracts. Water extract of Portuguese *L. nobilis* showed DPPH activity of 61% (Ferreira et al., 2006), similar as it was obtained in our research for commercial sample (59.89%) at the same concentration. Antioxidant activity of mandarin peels was studied by Xu et al. (2008) and they concluded that hot water extraction was effective in extracting of antioxidant compounds in *Citrus* peels, which was also the case in our study, with the highest antioxidant capacity obtained for the water extract of commercial sample (16.02%). Chen et al. (2011) have obtained considerably better results for ethanolic *C. reticulata* peel extract compared to our results. Zahoor et al. (2016) and Safdar et al. (2017) demonstrated high efficiency of methanolic extracts of *C. reticulata* from Pakistan, while methanolic extracts in our research had the lowest antioxidant activity. Zhang et al. (2014) reported that the DPPH values of the methanolic extracts of wild mandarins peel varied from 29.04 to 50.46 μmol Trolox equivalents/g. The FRAP values of the 14 wild mandarins, obtained by Fe^{3+} reduction assay similar to TRP used in this research, ranged from 26.50 to 46.98 μmol TE/g DW in the study of Zhang et al. (2014).

3.3. Antineurodegenerative activity

The analysis of potential antineurodegenerative activity of various extracts of leaves and mandarin peel was conducted and the results of inhibition of acetylcholinesterase, which is therapeutic target for AD, are presented in Table 3. The highest AChE inhibition was achieved by water extract of commercial *L. nobilis*, followed by acetonic and methanolic extracts. Among tested samples of *O. basilicum*, methanolic extracts

Table 3. The inhibition of α -glucosidase and acetylcholinesterase (AChE) tested on plant extracts (at the concentration of 0.5 mg/mL) and standards (at the concentration of 0.1 mg/mL).

Species	Extract type	α -glucosidase ^{a,b}			AChE		
		Enzyme inhibition [%]			Enzyme inhibition [%]		
		Commercial	Lastva ^c		Commercial	Lastva	
<i>O. basilicum</i>	Acetonic	44.83 ± 0.88 b	6.25 ± 0.68 d	†	47.68 ± 4.66 c	28.74 ± 1.26 e	†
	Methanolic	12.42 ± 2.31 d	13.65 ± 4.36 c		51.30 ± 5.27 c	43.71 ± 3.67 d	
	Aqueous	n.d.	86.48 ± 1.02 b	†	29.14 ± 3.08 d	n.d.	†
<i>L. nobilis</i>	Acetonic	23.72 ± 3.88 c	92.52 ± 4.35 ab	†	82.61 ± 3.10 b	55.77 ± 4.11 c	†
	Methanolic	48.83 ± 1.48 b	89.97 ± 0.53 ab	†	80.16 ± 4.05 b	73.99 ± 1.18 b	
	Aqueous	n.d.	11.10 ± 1.68 cd	†	90.72 ± 4.01 ab	46.60 ± 0.36 d	†
<i>C. reticulata</i>	Acetonic	n.d.	n.d.		18.69 ± 4.77 d	21.23 ± 2.12 f	
	Methanolic	n.d.	n.d.		25.07 ± 3.90 d	8.34 ± 2.94 g	†
	Aqueous	n.d.	n.d.		46.12 ± 2.48 c	22.44 ± 0.34 ef	†
Standards	Acarbose	95.51 ± 0.86 a	95.51 ± 0.86 a		-	-	
	Galantamine	-	-		96.12 ± 0.96 a	96.12 ± 0.96 a	

^a Different letters denote statistical difference among means according to *post-hoc* Tuckey HSD test at level $P < 0.05$

^b n.d. - stands for not detected activity.

^c † sign denote statistically significant difference between Commercial and Lastva mean values according to the Student's *t*-test ($P < 0.05$).

showed higher activity compared to other extracts, while the mandarin peel was the most effective in the water extracts (Table 3). The AChE inhibition was significantly affected by both plant origin and the extraction solvent. Pharmacological studies on *O. basilicum* have demonstrated potent antioxidant activities with some reports of neuroprotective actions (Manali et al., 2018). Basil essential oils were much more investigated for AChE inhibition than its extracts. The results of Sarahroodi et al. (2012) indicated that 80% ethanolic extract of *O. basilicum* significantly increased memory retention and retrieval of mice. Among ethanolic and water extracts of ten Portuguese plants analyzed by Ferreira et al. (2006), *L. nobilis* showed AChE inhibitory activity of 19.9% for water extract, but much higher for ethanolic extract (48.4%) using concentrations of 0.5 mg/mL. In our research at the same concentration, the commercial *L. nobilis* displayed higher activity of water extract (90.72%) comparing to methanolic and acetonic extracts. Senol et al. (2016) in their study of ethanolic extracts of peels of 17 Turkish *Citrus* cultivars notified that extracts at the concentration of 0.5 mg/mL did not affect AChE activity, while in our study, at the same concentration, the mandarin peels showed AChE inhibition ranging from 8.34 to 46.12%. El-Khadragy et al. (2014) tested the methanolic extract of mandarin peel on memory impairment in rats produced by scopolamine, and concluded that administration of mandarin peel extract may be of value for dementia exhibiting elevated brain oxidative status.

3.4. Antidiabetic activity

Diabetes mellitus has been proved to be linked to cognitive decline and neurodegeneration in general. Alpha glucosidase inhibitors delay carbohydrate absorption in the gastrointestinal tract, control postprandial hyperglycaemia and reduce the risk of cardiovascular and neurological complications in the development of the disease (Kalra, 2014). The results of α -glucosidase inhibition are presented in the Table 3. The best results were obtained for water extract of *O. basilicum* and

acetonic and methanolic extracts for *L. nobilis* originated from Lastva. The inhibition was not detected for the *C. reticulata* peels. Origin of the plant material and applied solvent significantly affected the α -glucosidase inhibition in the case of *O. basilicum* and *L. nobilis* (Table 3). Malapermal et al. (2017) showed that *O. basilicum* ethanolic extracts (70% and 60% ethanol) had higher antidiabetic activity than aqueous extract, which is similar to our results in the case of commercial sample. However, aqueous extract of *O. basilicum* from Lastva had the third best activity of all of the tested samples, which was far higher than the activity of the methanolic extract. The majority of data on the α -glucosidase inhibitory activity of *L. nobilis* is regarding to the essential oil, while the information on extracts is scarce. However, Kazeem et al. (2016) tested acetone extracts of bay leaves which displayed high antiglycation and antioxidant potential. Indrianingsih et al. (2015) have found that *L. nobilis* methanolic extract at the concentration of 0.2 mg/mL inhibited 47.26% α -glucosidase, which is in agreement with the results obtained in this study for the cultivated *L. nobilis*. Fayek et al. (2017) investigated the antidiabetic potency of different *Citrus* peel extracts and showed that mandarin peels decreased the glucose level in rats. Oboh and Ademosun (2011) found that α -glucosidase inhibitory effect of the Shaddock (*Citrus maxima*) peel acetonic extract achieved 89.05% at the tested concentration of 0.32 mg/mL. The enzymatic activity of α -glucosidase was tested for *Citrus limetta* peel extract and was found that at the lowest tested concentration (1.125 mg/mL) 5.2% enzyme was inhibited (Padilla-Camberos et al., 2014). It is considered that dietary α -glucosidase natural inhibitors are safe to control hyperglycemia, and medicinal plants could be useful remedies in treatment of diabetes and other health disorders (El-Beshbishy and Bahashwan, 2012).

3.5. Correlation between antioxidant activities, enzyme inhibition and total phenolic and flavonoid content

Pearson's correlation coefficients were calculated between total phenolic and flavonoid contents of tested extracts and their

antioxidant activities and α -glucosidase (AGLU) and AChE inhibition (Table 4). Antioxidant activity was more strongly correlated to total phenolic than total flavonoid content which is in accordance to Wojdylo et al. (2007). Antioxidant potential of the plant material usually correlates well with the phenolic content, which was also the case in the study of different extracts from fresh, frozen and lyophilized basil leaves (Złotek et al., 2016), where the correlation between phenolic content and antioxidant activity was positive and varied regarding to the used material. TPC displayed moderate correlation to AGLU and AChE inhibition activities. Polyphenols seem to be mostly involved in antioxidant activity and enzyme inhibition thereby possessing therapeutic potential for AD and DM. Antioxidant assays were strongly correlated to AChE inhibition, and moderately to α -glucosidase inhibition, while the enzyme inhibition tests were moderately correlated.

Table 4. Linear correlation between the assays used for analysis of the TPC and TFC, antioxidant activity and enzyme inhibition.

	TPC ^a	TFC	DPPH	TRP	AGLU	AChE
TPC	1	-0.17 ^A	0.83 ^C	0.73 ^C	0.58 ^B	0.51 ^B
TFC		1	0.03 ^A	0.12 ^A	-0.12 ^A	0.01 ^A
DPPH			1	0.96 ^C	0.54 ^B	0.78 ^C
TRP				1	0.47 ^B	0.78 ^C
AGLU					1	0.46 ^B
AChE						1

^a Different letters denote estimation of the correlation strength according to Taylor (1990), where A ($r < 0.35$), B ($0.36 < r < 0.67$) and C ($0.68 < r < 1$) were considered weak, moderate and strong correlation, respectively.

CONCLUSION

Among tested samples, *O. basilicum* and *L. nobilis* cultivated in Montenegro had higher level of antioxidant activity in both assays and also of α -glucosidase inhibition, while water extract of commercial *L. nobilis* had the highest AChE inhibition. The commercial sample of *C. reticulata* peel exhibited slightly better antioxidant and AChE inhibition activities. The origin of plant material was statistically significant in the majority of applied assays. The applied solvent was statistically significant in most cases, probably due to differences in the chemical composition of the extracts. The acetonic extract of *L. nobilis* had the highest TPC and also provided the best results in antioxidant tests. Since the acetonic extract of *L. nobilis* from Lastva showed the highest TPC, antioxidant activity and α -glucosidase inhibition, and water extract of commercial *L. nobilis* exhibited the highest AChE inhibition, the bay leaves could be promising antioxidant, antineurodegenerative and antidiabetic agent.

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