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BOTANICAL CHARACTERIZATION, CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF ROMANIAN LAVENDER (*LAVANDULA ANGUSTIFOLIA* Mill.) FLOWERS

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Abstract: The aim of our paper was the botanical characterization, evaluation of chemical composition and antioxidant activity of Romanian lavender (*Lavandula angustifolia* Mill.) flowers, harvested at different time of flowering (June and September). The herbal product's identity was determined based on macroscopic and microscopic exams. For phytochemical screening, we have used both qualitative (specific chemical reactions and thin layer chromatography - TLC) and quantitative assays. The antioxidant capacity was evaluated based on the scavenger activity towards DPPH, ABTS·+ free radicals and ferric reducing power. Analyzed samples are a source of flavones, phenolcarboxylic acids, essential oil and anthocyanidins. TLC analysis revealed the presence of linalool in both lavender samples. The highest content of polyphenols, tannins and anthocyanidins was determined for flowers harvested in June, while the essential oil prevailed in September. We have found a good correlation between the antioxidant activity and total phenolic content.

Keywords: phenolic compounds, antioxidant activity, linalool, lavender flowers, essential oil.

INTRODUCTION

Lavender (*Lavandula angustifolia* Mill.) also known as medicinal lavender, true lavender or common lavender is a perennial sub-shrub, native to the Mediteraneean region, which is widely cultivated throughout the world (Zhao et al., 2015). Lavender is one of the most important aromatic medicinal plants and its essential oil is widely applied in perfumery, cosmetics industry and aromatheraphy (Prusinowska et al., 2014; Demasi et al., 2018). The most valuable substance isolated from lavender is the essential oil, which has a complex chemical composition represented by linalool (9.3-68.8%), linalyl acetate (1.2-59.4%) (Prusinowska et al., 2014), lavandulyl acetate (5.9%), lavandulol (0.6-4.3%), lavandulol acetate (0.3-21.6%), E-caryophyllene (3.8%), caryophyllene oxide (1.6%), terpinen-4-ol (5.1%), borneol (1.7 %), 1,8-cineole (3.4%), D-fenchone (29.2%) and other compounds (cumene, α-pinene, camphene, myrcene, camphor, limonene, α-copaene, germacrene D, hexyl acetate etc) (Nurzyńska-Wierdak et al., 2015; Sebai et al., 2013; Mariń et al., 2016; Martucci et al., 2015). Lavender flowers are also a rich source of anthocyanidins, flavones (luteolin and apigenin, kaempferol glycosides, rutin, quercitrin, hesperidin), phenolcarboxylic acids (rosmarinic acid, caffeic acid) (Zhao et al., 2015; Zheng et al., 2019; Rădulescu et al., 2017), polysaccharides (Georgiev et al., 2017) and mineral elements (calcium, magnesium, zinc, manganese) (Imelouane et al., 2011; Prusinowska et al., 2014).

Regarding lavender flowers and the essential oil beneficial effects, they are widely used in therapeutics, due to anxiolytic activity (Saki et al., 2014; Farshbaf-Khalili et al., 2018, Rahmati S et al., 2017), neuroprotective activity (Hăncianu et al, 2013), amelioration of depression-like behaviour (Sánchez-Vidaña et al., 2019), antimicrobial, antimycotic, antileishmanial properties (Kunicka-Styczyńska et al., 2015; Baptista et al., 2015; Mariń et al., 2016; Martucci et al., 2015; Shokri et al., 2017), antioxidant activity (Bajalan et al., 2016; Baptista et al., 2015; Nurzyńska-Wierdak et al., 2015; Masuda et al., 2015), hypoglycemic (Sebai et al., 2013) and cardioprotective effects (Ziaee et al., 2015).

Taking into consideration the scientific data, the aim of our paper was the botanical characterization, chemical composition and antioxidant activity of indigenous lavender flowers, harvested at different time of flowering.

MATERIAL AND METHODS

Material

Lavender flowers were collected at flowering in the months of June (**LJ**) and September (**LS**) from Valea Călugărească, Prahova county, Romania, in 2018. Flowers were dried in the shade and stored in laboratory conditions.

Reagents and solvents

All reagents and solvents were purchased from Karl Roth (Germany) unless otherwise stated. 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet}$+ free radical) were from Sigma-Aldrich (Germany). Trichloroacetic acid was acquired from Merck (Germany).

Botanical characterization (macroscopic and microscopic exams)

The identity of the herbal product was determined through macroscopic and microscopic exams. The macroscopic exam was performed on dried flowers. For macroscopic examination, we have also used a Zeiss Stemi 305 stereomicroscope with Axiocam ERc5s camera (Zeiss, Germany). For microscopic examination we have used 800 g/L chloral hydrate as a clearing agent and 10 g/L fluoroglucin as a colour agent, according to European Pharmacopoeia method

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(European Pharmacopoeia 9th edition, 2016). A Carl Zeiss Imager D1 microscope with Canon PC 1145 camera was used for microscopic examination.

Phytochemical screening

Qualitative assays consisted in specific chemical reactions for phenolcarboxylic acids, flavones, tannins, anthocyanins (Gîrd et al, 2010) and thin layer chromatography (TLC) for essential oil analysis.

Preparation of samples for qualitative/ quantitative assays and antioxidant activity: 2.5 g of LJ and LS herbal products were heated twice with 25 mL 50% ethanol (v/v) on a reflux condenser, for 30 min. After cooling, the solutions were filtered and the combined filtrates were brought in a 50 mL volumetric flask for further analysis. The solutions were encoded as follows: **LJS** (for lavender flowers collected in June) and **LSS** (for lavender flowers collected in September).

Preparation of lavender flowers essential oil: 50 g of LJ and LS herbal products were heated with 500 mL water for three hours using a Neo Clevenger apparatus.

Thin layer chromatography analysis (TLC)

Thin layer chromatography was used for essential oil compounds identification. It was performed on an aluminium - coated TLC plate (10x20cm, Merck, Germany), which was kept for one hour at 105°C, before use. A mixture of toluene: ethyl acetate = 93 : 7 (v/v) have been used as eluent system (Gîrd et al., 2009). The plate was spotted with LJ and LS essential oils. Linalool (0.1 mg/mL methanolic solution) was used as standard reference. The plate was developed over a path of 16 cm, air dried, sprayed with a 10 g/L vanillin sulphuric acid solution and kept for 10 min at 105°C. After spraying with the detection reagent, the plate was examined in visible light.

Spectrophotometric and volumetric assays

The total phenolic content (expressed as gallic acid equivalents) was determined with Folin-Ciocalteu reagent (Singleton et al., 1965, Costea et al., 2016). Tannins content (also expressed as gallic acid equivalents) was determined as previously described (Costea et al., 2014). The flavones content (expressed as quercetin equivalents) was estimated based on the chelating reaction with aluminium chloride (European Pharmacopoeia 9th edition, 2016, Gîrd et al, 2015). Anthocyanidins content (expressed as cyanidin chloride equivalents) was determined according to European Pharmacopoeia 9th edition (*Myrtilli fructus* recens monography) (European Pharmacopoeia 9th edition, 2016). The essential oil content was determined based on a volumetric method using Neo Clevenger apparatus (European Pharmacopoeia 9th edition, 2016). Calibration curves of: gallic acid (1.22-7.22 µg/mL, R^2 = 0.9989, n = 6), quercetin (2.06-14.42 µg/mL, R^2 = 0.9983, n = 8) and cyanidin chloride (1.63-9.79 µg/mL, R^2 = 0.9995, n = 11) were used to determine the content of active substances. All spectrophotometric determinations were performed using a Jasco V-530 spectrophotometer (Jasco, Japan) at different wavelengths: λ = 763 nm (for total phenolic content and tannins assay), λ = 420 nm (for flavones content) and λ = 550 nm (for anthocyanidins content).

Antioxidant activity

The antioxidant capacity was evaluated by means of well known methods: the scavenger activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet}$+) free radicals and ferric reducing power.

- **DPPH free radical scavenging capacity**

Briefly 0.5 mL of LJS and LSS solutions (0.2-4 mg/mL) were treated with 3 mL of a 0.1 mM ethanolic solution of DPPH. The mixture was kept in the dark, at room temperature and the absorbance was measured at λ = 516 nm, before (A_{start}) and 30 min. after adding the samples (A_{end}). Ethanol was used as a blank (Brand-Williams et al., 1995).

The ability to scavenge the DPPH free radical was calculated using the following formula: DPPH radical scavenging activity (%) = ($A_{start} - A_{end}$) / A_{start} x 100.

- **$ABTS^{\bullet}$+ free radical scavenging capacity**

Briefly, the $ABTS^{\bullet}$+ radical cation was generated by incubation of ABTS diammonium salt (7 mM) with potassium persulphate (2.45 mM) in the dark, at room temperature for 16 hours. The absorbance of the $ABTS^{\bullet}$+ radical solution was equilibrated to a value of 0.700 ± 0.02, at λ = 734 nm after dilution with ethanol. Briefly 0.5 mL of LJS and LSS solutions (0.2-1 mg/mL) were mixed with 3 mL reagent and the absorbance was measured at λ = 734 nm, before (A_{start}) and 6 min. after adding the samples (A_{end}) (Re et al., 1999). The $ABTS^{\bullet}$+ scavenging activity was calculated according to the following formula: $ABTS^{\bullet}$+ radical scavenging activity (%) = ($A_{start} - A_{end}$) / A_{start} x 100.

- **Ferric reducing power**

The reducing power assay was determined according to Oyaizu method (Oyaizu, 1986). Briefly, 2.5 mL of LJS and LSS solutions (0.3-2.24 mg/mL) were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 10 g/L potassium ferricyanide solution. Samples were kept at 50°C in a water bath (Raypa, Spain) for 20 min. After, 2.5 mL of 100 g/l trichloroacetic acid was added and the mixture was centrifuged at 2500 rpm for 5 min. (Universal 16 centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL water and 0.5 mL of 1 g/L ferric chloride solution. The absorbance was measured at λ = 700 nm, after 10 min., against a blank that contained all reagents except for samples. A higher absorbance indicates stronger reducing power.

Antioxidant activity was assessed by means of EC_{50} (mg/mL) and trolox equivalents (µM/g of herbal product). EC_{50} values were determined graphically from the linear regression curve plotted between percent (%) of inhibition (for DPPH, $ABTS^{\bullet}$+ assays) / absorbance (for ferric reducing power assay) values and extractive solutions concentration (mg/mL).

The antioxidant activity was also expressed as trolox equivalents, which were calculated based on calibration curves: 6-60.9 µg/mL, R^2 = 0.9976, n = 5 (for DPPH assay), 1.01-20.32 µg/mL, R^2 = 0.9916, n =

7 (for ABTS•+ assay) and 6-121.9 µg/mL, R^2 = 0.9984, n = 8 (for ferric reducing power).

Lower EC_{50} values and higher trolox equivalents reflect higher antioxidant capacity.

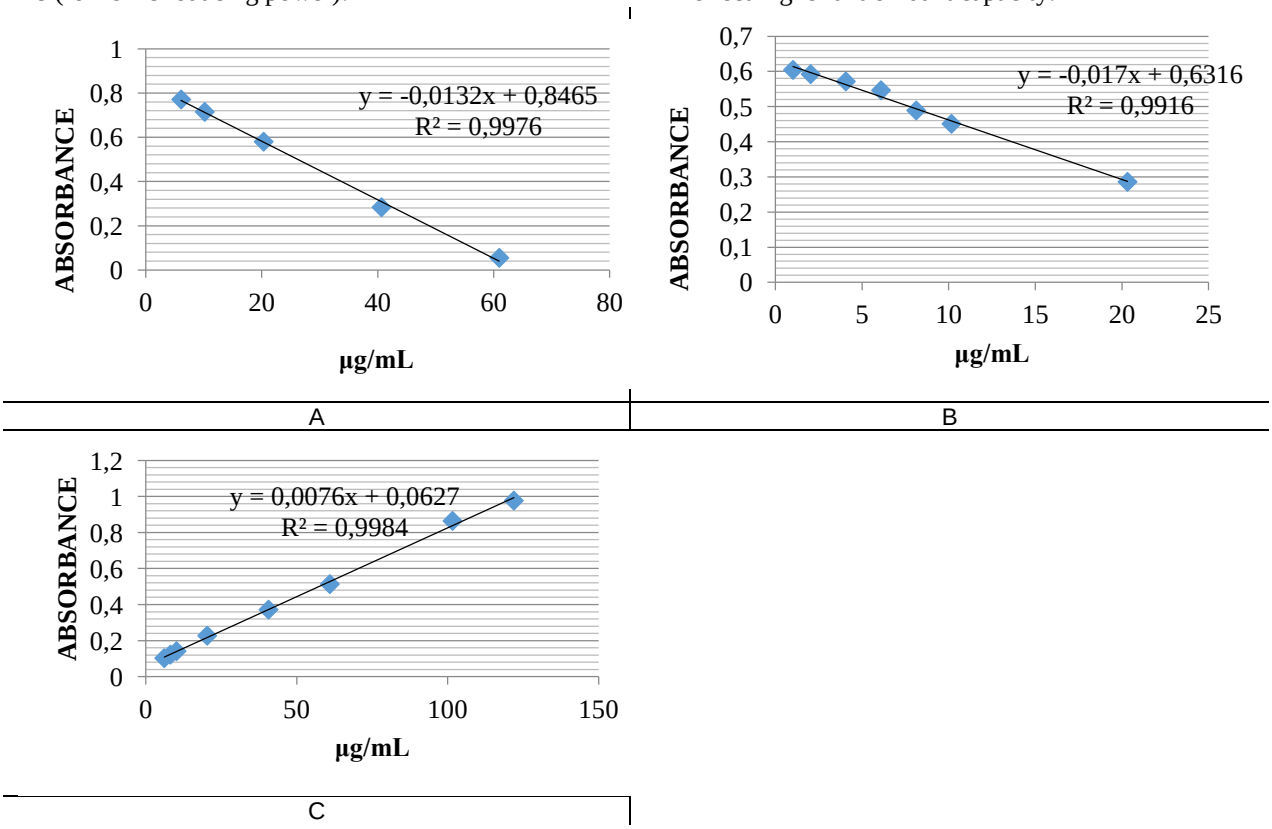


Fig. 1. Calibration curves of trolox . A – DPPH assay, B – ABTS•+ assay, C – ferric reducing power.

Statistical analysis

Results for spectrophotometric and antioxidant assays represent the mean ± standard deviation (SD) of three replicates, from three independent determinations. Results were statistically analyzed using GraphPad Prism 6 software (GraphPad, USA), by means of student's t-test. A value of $p < 0.05$ was considered the threshold for a statistically significant difference.

RESULTS AND DISCUSSION

The macroscopic exam (fig. 2A) revealed that LJ and LS flowers are violet and grow in spikes, arranged in circles (3-5 flowers per circle). Analyzing the flowers using the stereomicroscope (fig. 2B, 2C, 2D), one can note the presence of numerous glandular and non-glandular trichomes.

The microscopic exam (fig. 3, 4) revealed the presence of anatomic elements characteristic to flowers such as: pollen grains, endotecium, papillae, epidermis, cellulosic fibers, small xylem vessels, non-glandular (dichotomic) and glandular trichomes (octocellular trichomes specific to *Lamiaceae* family).

Thin layer chromatography revealed the presence of linalool in both analyzed samples. However, analyzing the plate, one can note several other spots, probably corresponding to lipophilic compounds, that were not identified due to lack of standard references (fig.5). Our results are in agreement with other authors, that found linalool, as one of the main constituents of lavender flowers essential oil (Demasi et al., 2018; Mariń et al., 2016; Martucci et al., 2015).

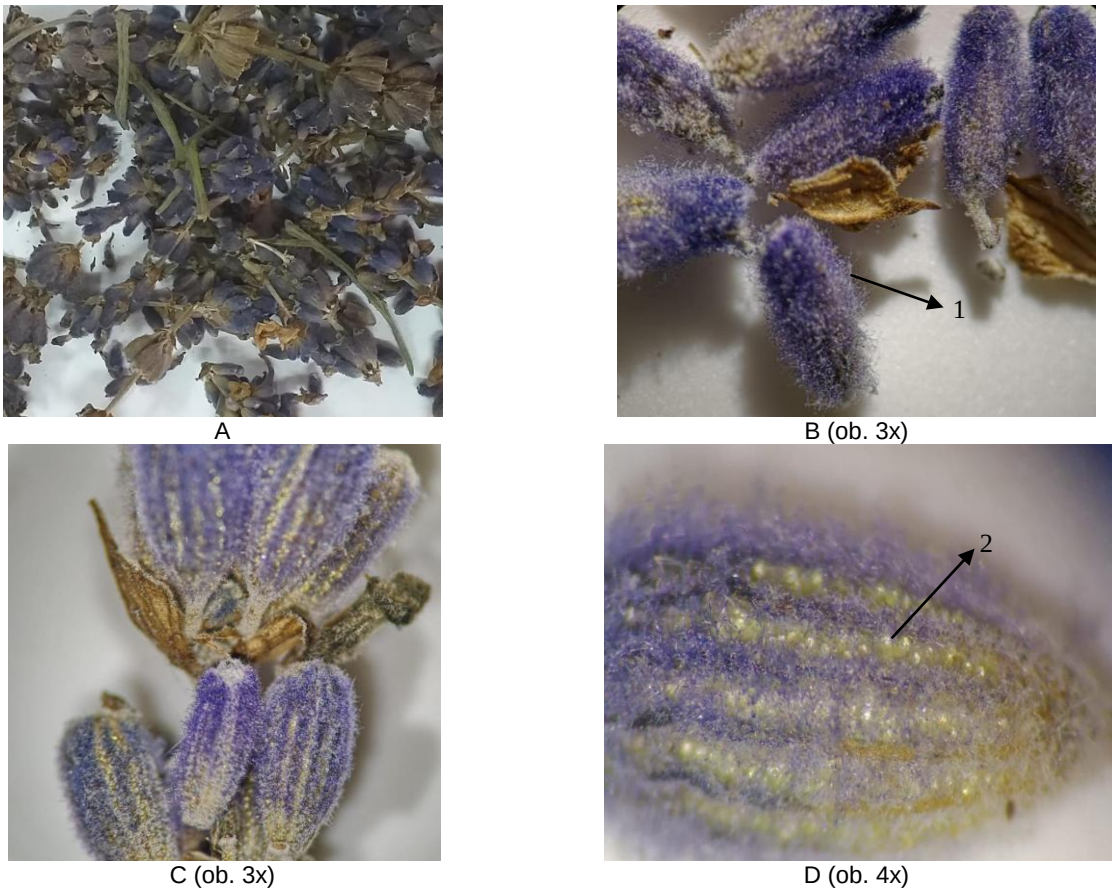
According to our qualitative and quantitative assays, both analyzed samples are a source of tannins, anthocyanidins and essential oil. However, lavender flowers collected in June have a higher content of phenolic compounds compared to flowers collected in September, although the essential oil content is significantly lower (table I).

Still, the essential oil content, for both analyzed samples, is in agreement with European Pharmacopoeia 9th edition, that foresees a concentration of minimum 1.3 mL/100 g dried herbal product (European Pharmacopoeia 9th edition, 2016). Significant differences between LJ and LS have been found regarding tannins and flavones contents ($p < 0.05$) (table I).

Our total phenolic content is higher compared to Bajalan and co-workers, who found 0.03-0.105 g polyphenols (expressed as gallic acid equivalents)/100 g herbal product (Bajalan et al., 2016). However our results regarding the total phenolic content are lower compared to Aprotosoaie and co-workers, that found 3.10 g polyphenols (expressed as galic acid)/100 g herbal product, using 50% ethanol as extraction solvent (Aprotosoaie et al., 2013).

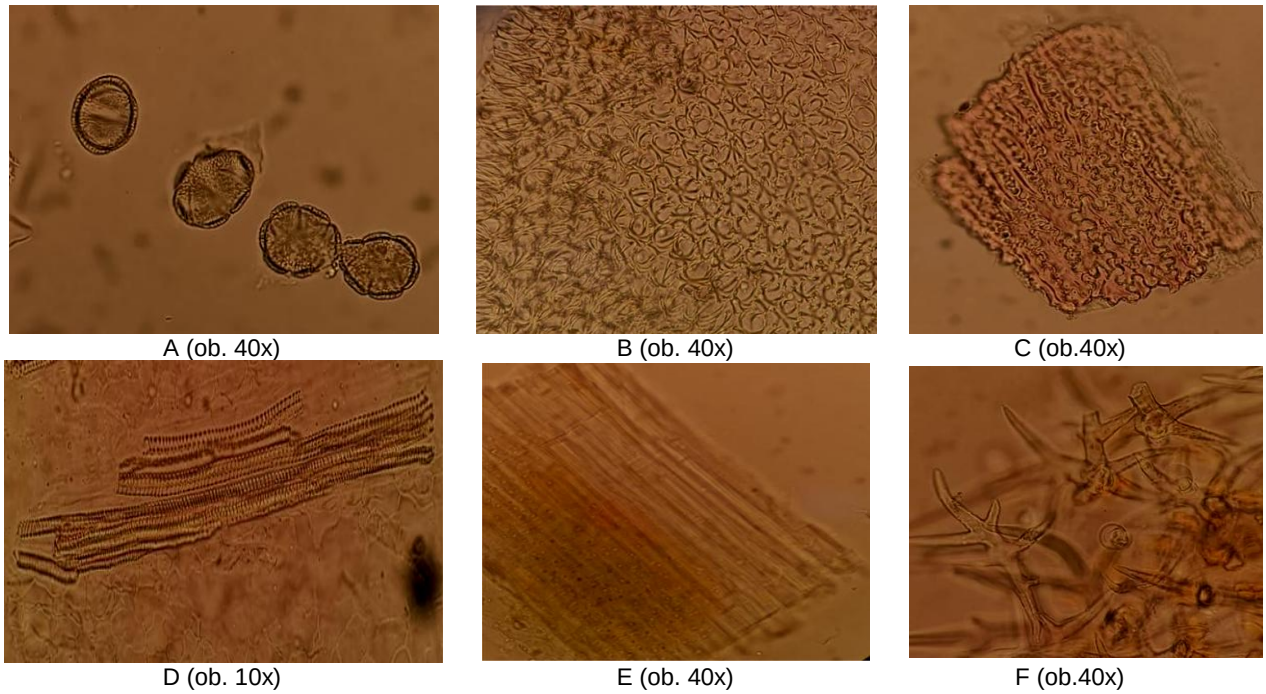
Regarding the flavones content, our results are much higher compared to Bajalan and co-workers, that found only 0.039-0.0683 g flavones (expressed as quercetin equivalents)/100 g herbal product) in lavender flowers collected from Zagros regions,

Wester Iran (Bajalan et al., 2016). Moreover our results are higher compared to Nurzynska-Wierdak and co-workers that found 0.2 g % flavones for flowers collected from Poland (Nurzyńska-Wierdak et al., 2015).



A B (ob. 3x) C (ob. 3x) D (ob. 4x)

Fig. 2. Macroscopic examination of lavender flowers. A – visual examination, B,C,D – stereomicroscope images Legend: 1 – non-glandular trichomes, 2 – glandular trichomes (with essential oil).



A (ob. 40x) B (ob. 40x) C (ob.40x) D (ob. 10x) E (ob. 40x) F (ob.40x)

Fig. 3. Microscopic exam of lavender flowers. A – pollen grains, B - endotecium, C – epidermis, D - small xylem vessels, E - cellulosic fibers, F – dichotomic non-glandular trichomes.

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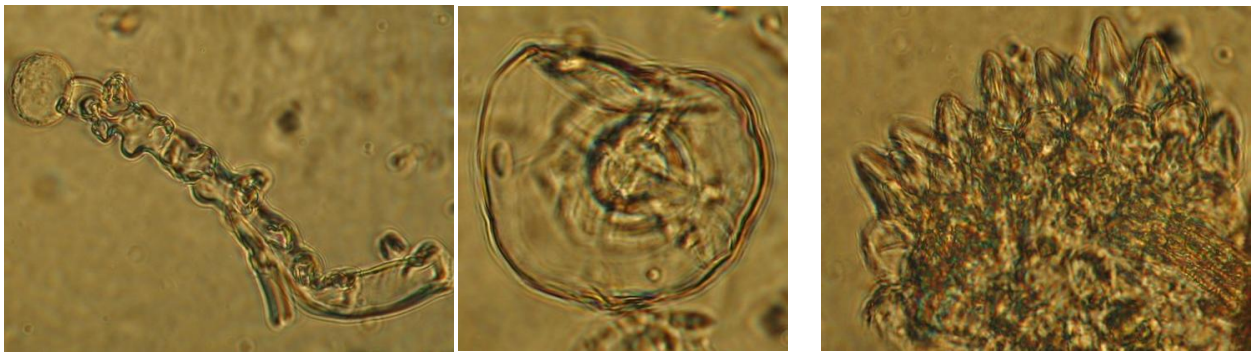


A (ob.40x) B (ob. 40x) C (ob.40x)

Fig. 4. Microscopic exam of lavender flowers: A – glandular trichomes, B – octocellular glandular trichomes (specific to *Lamiaceae* family), C – papillae.

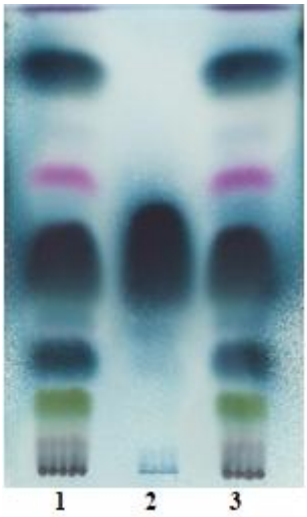


Fig. 5. TLC analysis of essential oil from lavender flowers. 1 – LJ (flowers collected in June) essential oil; 2 – linalool (standard reference); 3 – LS (flowers collected in September) essential oil.

Table 1.

Quantitative results for lavender flowers

Sample	Total phenolic content (g gallic acid/100 g herbal product)	Tannins (g gallic acid/100 g herbal product)	Flavones (g quercetin/100 g herbal product)	Anthocyanidins (g cyanidin chloride/100 g herbal product)	Essential oil (mL/100 g herbal product)
LJ	2.8980 ± 0.3049	1.3250 ± 0.31175	0.6721 ± 0.2132	0.0472 ± 0.0052	3
LS	2.0870 ± 0.2510	0.7275 ± 0.0668	nd	0.0359 ± 0.0033	4.40

Legend: LJ – lavender flowers collected in June, LS – lavender flowers collected in September, nd – under the limit of quantification.Results are mean ± SD (n = 3)

Regarding the anthocyanins (anthocyanidins) content, our results are lower compared to Nurzynska-Wierdak and co-workers, that found 0.09 g% anthocyanins in Polish lavender flowers (Nurzyńska-Wierdak et al., 2015). Cyanidin, pelargonidin and malvidin glycosides are all mentioned by scientific literature, as important constituents of lavender flowers (Zheng et al., 2019).

Regarding the antioxidant capacity, LJ and LS have shown scavenger activity towards DPPH, ABTS•+ free radicals and ferric reducing properties. Regarding DPPH assay, for the same concentration interval 0.2-4 mg/mL, at the highest concentration of 4 mg/mL, LJ and LS had a similar scavenger activity towards DPPH free radical (89.47% - for LJ and 90.12 % - for LS) (fig. 6A). Our results regarding DPPH scavenger activity are higher compared to Bajalan et al. (50-72%) (Bajalan et al., 2016), but similar to Duda and co-workers, that found a high inhibition percent (71.14-81.74%) (Duda et al., 2015).

The scavenger activity towards ABTS•+ free radical was significantly higher ($p < 0.05$) for LJ sample (for 0.2 mg/mL - 36.99% and for 1 mg/mL - 98.88%) compared to LS sample (for 0.2 mg/mL -

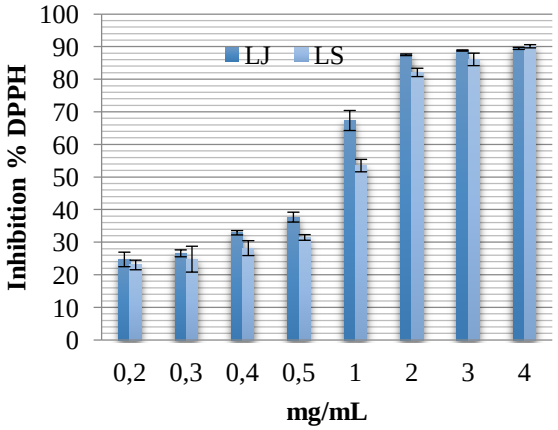
30.10% and for 1 mg/mL - 89.15%) (fig. 6B). The higher antioxidant activity observed for ABTS•+ assay, compared to DPPH method, might be the consequence of different mechanisms of action. It is well known that DPPH method is mainly based on electron transfer and it is usually applied for hydrophilic antioxidants (such as flavones, phenolcarboxylic acids, tannins, anthocyanidins etc), while ABTS•+ assay has a mixed mechanism (both electron and proton transfer) and lipophilic compounds (triterpenes, essential oil constituents) are also responsible for the overall antioxidant effect (Dudonné et al, 2009). Concerning ferric reducing power (fig. 6C), at 2.24 mg/mL the absorbance was 1.292 for LJ sample compared to 0.831, for LS.

Independent of the method used, LJ had a stronger antioxidant capacity compared to LS (table II). Important antioxidant activity for lavender flowers was also reported by other authors (Baptista et al., 2015; Nurzyńska-Wierdak et al., 2015; Masuda et al., 2015), however the comparison with our results was difficult, due to different extraction procedures and pedoclimatic conditions.

Still, our results are similar to other authors, that investigated the effect of harvest time upon total phenolic content and antioxidant activity of lavender flowers. It was found that flowers collected in the first decade of June had the best antioxidant capacity, which was correlated with a higher phenolic content (Duda et al, 2015). According to Guitton and co-workers, lavender flowers collected from France, in September, had a higher essential oil content, compared to ones harvested in May. The authors concluded that that the essential oil, obtained from flowers harvested in September was richer in linalool, terpinen-4-ol and cis-ocimen (Guitton et al, 2009). Similar results, regarding the essential oil content, were found by Koleilat and co-workers. According to the authors the essential oil obtained from lavender flowers, collected in September had a higher content of α-pinene, camphene, linalool, lavandulol and linalyl acetate, compared to the one obtained from flowers harvested in July. These differences were also responsible for a better antioxidant activity of September essential oil (Koleilat et al, 2017). A higher content of essential oil for flowers collected in September compared to ones collected in June was also observed for other Lavandula species (Lavandula stoechas L. ssp. stoechas growing wild in Sardinia, Italy) (Angioni A et a, 2006).

We assume that mainly polyphenols (flavones, tannins, phenolcarboxylic acids, anthocyanidins) and minerals are responsible for the overall antioxidant capacity of LJ and LS flowers (Zhao et al., 2015; Zheng et al., 2019; Prusinowska et al., 2014; Rădulescu et al., 2017). It is well known that polyphenols are powerful antioxidants due to their free radicals scavenger activity, metal chelating properties and increased activity of the endogenous antioxidant system, represented by superoxide dismutase, catalase, glutathione peroxidase, etc (Pandey et al., 2009).

Moreover, analyzing our results one can note a positive correlation between the polyphenols content and antioxidant activity, since a higher total phenolic content was observed for flowers collected in June compared to ones harvested in September.



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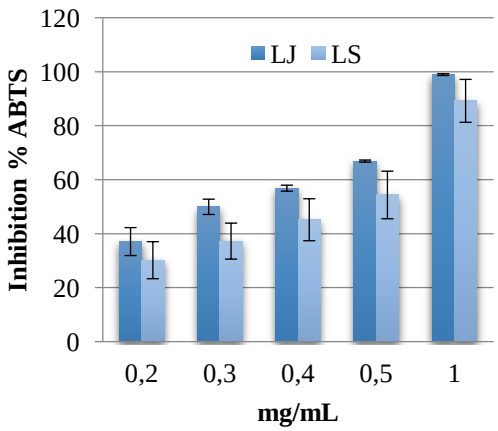

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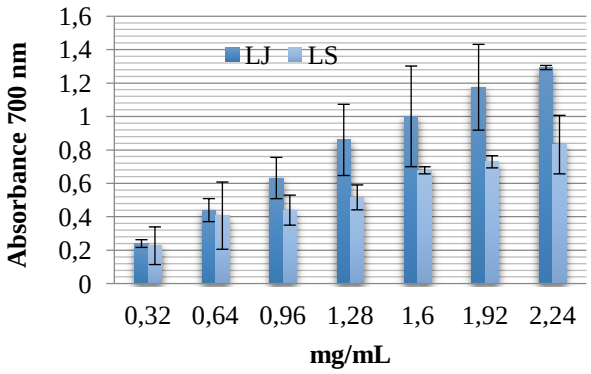

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Fig. 6. Lavender flowers antioxidant activity. A – DPPH assay; B - ABTS$^{\bullet}$+ assay; C – Ferric reducing power. LJ – flowers collected in June, LS – flowers collected in September. Results are mean ± SD (n = 3).

Table 2.

EC_{50} (mg/mL) and trolox equivalents (µM/g) for lavender flowers

Sample	ANTIOXIDANT ASSAYS					
	DPPH		ABTS$^{\bullet}$+		Ferric reducing power	
	EC_{50} (mg/mL)	µM trolox/g herbal product	EC_{50} (mg/mL)	µM trolox/g herbal product	EC_{50} (mg/mL)	µM trolox/g herbal product
LJ	1.0558 ± 0.0142	115.39 ± 3.4907	0.3191 ± 0.0369	198.47 ± 1.1518	0.7127 ± 0.0201	335.0141 ± 1.8874
LS	1.2995 ± 0.0504	94.0412 ± 2.0614	0.4652 ± 0.1026	146.63 ± 4.2961	1.1186 ± 0.0153	238.183± 5.4488

Results are mean ± SD (n = 3), LJ – flowers collected in June, LS – flowers collected in September.

CONCLUSIONS

Analyzed lavender flowers are a source of bioactive compounds, with antioxidant activity. However, the harvest time represents a key factor that strongly influences the chemical composition, since flowers collected in June have a higher content of phenolic compounds, while flowers harvested in September are a rich source of essential oil. Moreover, flowers collected in June have shown a stronger antioxidant effect, therefore they might be further used for obtaining selective dry extracts, with potential therapeutic effects, in diseases for which oxidative stress is a key factor.

AUTHORS CONTRIBUTION

Conceptualization: Costea T., Gîrd C. E.; Methodology: Costea T., Străinu A. M.; Data collection: Costea T., Străinu A. M.; Data processing: Costea T., Străinu A. M.; Writing – original draft preparation: Costea T., Străinu A. M., Gîrd C. E.; Writing – review and editing: Costea T., Gîrd C. E.

CONFLICT OF INTEREST

The authors delcare no conflict of interest.

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