Enhancement of Antioxidant Properties of Lavender (*Lavandula officinalis* L.), Sage (*Salvia officinalis* L.) and Basil (*Ocimum basilicum* L.) by Application of Natural Biostimulants

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Summary

The aim of this study was to examine the effects of seaweed extract (Algaren), nettle extract and microbial biostimulant (Slavol) on total phenolics and flavonoids and antioxidant capacity of leaves of lavender (*Lavandula officinalis* L.), sage (*Salvia officinalis* L.) and basil (*Ocimum basilicum* L.). The study was carried out in a greenhouse located in Vitez, Bosnia and Herzegovina. The experimental trial was set up in a randomized block design with four biostimulant treatments in three replications. The first application of biostimulant was carried out immediately after the transplanting of seedlings, and the second and third treatments 15 and 30 days later, respectively. Total phenolic and flavonoid contents and ferric-reducing/antioxidant power (FRAP) assay were used to evaluate the antioxidant properties of each leaf extract. The nettle extract was found to be the most effective in increasing total phenolics, flavonoids, and antioxidant capacity in leaves of studied plants. In this study, the antioxidant capacity of leaf extracts was in line with total phenolics/flavonoids, regardless of plant species and biostimulant treatments, suggesting that phenolic compounds greatly contribute to antioxidant capacity of studied plants.

Key words

antioxidant capacity, flavonoids, health, phenolics

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Introduction

Agrochemicals play a crucial role in horticulture and have been widely used for crop protection and increasing plant yield and quality. Despite their usefulness, the continuous long-term use of agrochemicals poses potential risks to the environment, food safety and consequently to human health (Durán-Lara et al., 2020). Accordingly, modern agricultural practice tends to reduce the use of fertilizers in agricultural activities and partially or completely replace them with natural plant biostimulants (PBs). PBs were defined by du Jardin (2015) as any natural substance or microorganism applied to plants with the aim to improve nutrition and water use efficiency, tolerance to abiotic stress and/or crop quality traits, regardless of its nutrient content. Based on this definition, PBs include diverse bioactive natural substances: (i) humic and fulvic acids, (ii) animal and vegetal protein hydrolysates, (iii) seaweeds extracts and (iv) beneficial microorganisms: arbuscular mycorrhizal fungi and nitrogen-fixing bacteria (Pylak et al., 2019).

It is well known that biostimulants enhance antioxidant capacity of plants as well as plant growth and development, but the mode of their action is still not well understood (Drobek et al., 2019). Namely, biostimulants are extracted from a wide range of natural organic materials containing multiple bioactive natural substances that, together, may provide a specific effect on plants. Various hypotheses have been proposed to explain the connection between plant metabolism and main active components in biostimulants responsible for the beneficial effects on plant growth and development and available evidence suggests that secondary metabolic pathways are recognized as targets of biostimulants (Bona et al., 2017; Rouphael and Colla, 2020). Accordingly, biostimulants beneficially affect secondary metabolites contents, primarily phenolics and flavonoids contents in plant tissues (Yang et al., 2018).

The presence of phenolic compounds in plants has been associated with health promoting properties including antioxidant, anti-allergic, cardioprotective, anti-inflammatory and antimicrobial activities (Shahidi and Yeo, 2018). Therefore, the use of biostimulants has become a regular practice for enhancing secondary metabolites synthesis in medicinal plants and improving their quality. In addition, biostimulant's application reduces the need for fertilizers without compromising soil productivity and plant health (Rouphael et al., 2018).

In recent years, biostimulants that have sparked an interest of many crop producers in Bosnia and Herzegovina are Slavol, Algaren and nettle extract.

Slavol (Agrounik, Serbia) is a liquid microbial biostimulant composed of nitrogen fixing and phosphorus mineralizing bacteria, while the Algaren (Green Has, Italia) is a highly concentrated liquid seaweed extract made from *Ecklonia maxima* (Osbeck) Papenfuss. The product specification given in an Algaren guide manual states the following active ingredients: auxins, cytokinins, polyamines, gibberellins, abscisic acid, brassinosteroids, several B vitamins and several micro- and macronutrients.

Nettle extract (OPG Pezelj, Croatia) is a plant-based fertilizer derived from leaves, stem and other parts of the stinging nettle (*Urtica dioica* L.). Many studies have reported that the nettle extract provides a wide variety of bioactive compounds including minerals (nitrogen, calcium, iron, magnesium, phosphorus, potassium and sodium), vitamins (A, C and K, as well as several B vitamins), phenolic compounds, carotenoids and organic acids (Dar et al., 2013; Dziamski and Stypczyńska, 2014; Kregiel et al., 2018). However, very few studies have focused on the use of nettle extract (*Urtica dioica* L.) as biostimulant in horticulture and its effect on metabolic pathways and consequently on antioxidant properties of medicinal plants cultivated under standard growth conditions.

Hence, the objective of this study was to evaluate the effect of microbial biostimulant (Slavol), seaweed extract (Algaren) and nettle extract on total phenolic and flavonoid contents and antioxidant capacity of lavender (*Lavandula officinalis* L.), sage (*Salvia officinalis* L.) and basil (*Ocimum basilicum* L.) under standard growth conditions.

Materials and Methods

Chemicals

Chemicals with analytical purity were used in the present study. The solvents (ethanol) used in this investigation was purchased from Semikem (Sarajevo, Bosnia and Herzegovina). Folin-Ciocalteu reagent, Na_2CO_3, NaOH, $NaNO_2$, $AlCl_3$, 2,4,6-tripyridyl-s-triazine, $FeSO_4 \times 7\ H_2O$, HCl, $FeCl_3$ was obtained from Sigma-Aldrich (Steinheim, Germany).

Experimental Design

The experiment was carried out during 2020 in a double-span polyethylene-covered greenhouse with natural ventilation in Vitez, Bosnia and Herzegovina (44° 09'30'' N, 17° 47'18'' E). A pot trial was set up in a randomized block design with four biostimulant treatments in three replications for each studied plant species (lavender, sage and basil). Prior to beginning the experiment, lavender, sage and basil seedlings were transplanted into individual pots (22 cm diameter), containing commercial substrate Florahum-SP. There were no significant differences in terms of size and appearance among seedlings within each studied plant species. All biostimulant treatments were applied at the concentrations recommended by the manufacturers: (1) Algaren 0.3% (2) Slavol 1% (3) nettle extract 1% and (4) untreated plants (control). The first treatment with biostimulant was carried out immediately after the transplanting of seedlings, and the second and third treatment 15 and 30 days later, respectively. Algaren and nettle extract were applied on plant leaves, while Slavol application was performed through the soil. 12 individuals per plant species were used for each treatment, summing up to 48 individuals per species and 144 individuals in total.

Approximately 10 days after the last biostimulant treatment, the leaves were collected and then dried in an oven at 40°C. Thereafter, the dried samples were ground to a fine powder using an electric blender and stored in paper bags until chemical analysis.

Preparation of Leaf Extract

1 g of dried-leaf powder was extracted with 30 mL of 60% ethanol aqueous solution. The mixture was kept for 24 hours at room temperature. Thereafter, the extract was filtered through

Whatman filter paper (11 µm pore size) into the volumetric flask and diluted to the 50 mL mark with extract solution. The extract thus obtained was used for the estimation of total phenolic content, total flavonoid content and total antioxidant capacity.

Estimation of Phenolic Contents

Total phenolics content (TPC) was determined by Folin-Ciocalteu colorimetric method (Ough and Amerine, 1988) as follows: A sample of each leaves extract solution (0.25 mL) was transferred into a 25 mL flask and then mixed thoroughly with 15 mL distilled water and 1.25 mL of Folin-Ciocalteu reagent (Folin-Ciocalteu's reagent was diluted with distilled water in the 1:2 ratio before use). After mixing for 3 min, 3.75 mL of 7.5% (w/v) Na_2CO_3 was added. After cooling to room temperature, the absorbance of the mixture was determined at 765 nm. TPC quantification was based on a standard curve of gallic acid (0 -500 mg L^{-1}), and the results were expressed as mg of gallic acid equivalents per gram of dry matter of leaf sample (mg GAE g^{-1} DW).

Estimation of Flavonoid Contents

Total flavonoids content (TFC) was determined according to the aluminium chloride colorimetric assay (Zhishen et al., 1999) as follows: In a 10 mL tube, 1 mL of each extract, 4 mL of distilled water, 0.3 mL of 5% $NaNO_2$, 0.3 mL of 10% $AlCl_3$ were added and mixed, followed by 5 min incubation and the addition of 2 mL of 1 M NaOH. After 15 min, the absorbance of the mixture was determined at 510 nm. TFC quantification was based on a standard curve of catechin (0 - 100 mg L^{-1}), and the results were expressed as mg of catechin equivalents per gram of dry matter of leaf sample (mg CE g^{-1} DW).

Total Antioxidant Activity Estimation

The reducing powers of the samples (total antioxidant capacity) were determined following the method of Benzie and Strain (1996). In a 10 mL test tube, 80 µl of each extract solution was mixed with 240 µl of distilled water and 2080 µl of FRAP reagent (prepared immediately before use by mixing 10 parts of 0.3 mol L^{-1} acetate buffer (pH = 3.6), 1 part of 10 mmol L^{-1} 2,4,6-tripyridyl-triazine (TPTZ) in 40 mM HCl, and 1 part of 20 mM $FeCl_3 \times 6H_2O$ in dH_2O). The incubation for this mixture was set at 37 °C for 10 min. After cooling to room temperature, the absorbance was measured at 595 nm. Total antioxidant capacity quantification was based on a standard curve of $FeSO_4 \times 7\,H_2O$ (0 - 2000 µmol L^{-1}), and the results were expressed as µmol Fe^{2+} per gram of dry matter of leaf sample (µmol Fe^{2+} g^{-1} DW). The absorbance in all spectrophotometric assays was measured by using a UV/VIS Amersham ultrospec 2100 pro spectrophotometer (Biochrom, USA).

Statistical Analysis

All the experimental measurements were performed in triplicates and all the data were recorded as mean ± standard deviation. The obtained data were statistically processed employing the Microsoft Excel 2010 software. Statistical analysis included the analysis of variance (ANOVA, $P \leq 0.05$) between samples. Significant differences between the means of samples were further analyzed by the least significant difference (LSD, $P \leq 0.05$).

Results

Total phenolic content in leaves of lavender and sage seedlings increased significantly after nettle extract treatment. The application of Slavol also significantly increased total phenolic contents in sage leaves compared to control (no treatment) seedlings. On the other hand, there was no statistically significant difference between basil seedlings with respect to phenolic contents in leaves, regardless of biostimulant treatments (Table 1). In terms of phenolics, the results of this study revealed that biostimulants acted differently on different plant species, indicating that plant response to biostimulant treatments is difficult to predict.

Table 1. Total phenolic contents in leaves of studied medicinal plants

Treatment	Total phenolic content (mg g^{-1} dry matter)		
	Lavender	Sage	Basil
Slavol	41.98 ± 3.11^{ab}	61.42 ± 6.27^{b}	69.94 ± 2.23
Algaren	39.72 ± 4.23^{bc}	61.04 ± 4.11^{b}	70.45 ± 1.19
Nettle extract	43.59 ± 3.76^{a}	70.79 ± 2.93^{a}	70.35 ± 3.03
Control	38.42 ± 2.11^{c}	63.05 ± 9.77^{b}	68.91 ± 1.55
$LSD_{0.05}$	2.985	5.671	-

Note: Results are expressed as means ± standard errors. Different superscript lower-case letters in a column represent statistically significant differences between mean values for each main effect at P < 0.05 obtained by a LSD test

The content of flavonoids in lavender and basil leaves significantly increased compared to the control plants upon the use of nettle extract. The use of Algaren resulted also in an increased total flavonoids content of basil leaves with respect to controls. However, the statistical analysis indicated that the differences between the effects of biostimulants on total flavonoids content of sage leaves were insignificant (Table 2).

Table 2. Total flavonoid contents in leaves of studied medicinal plants

Treatment	Total flavonoid content (mg g^{-1} dry matter)		
	Lavender	Sage	Basil
Slavol	19.78 ± 2.01^{b}	28.75 ± 3.11	28.20 ± 1.66^{c}
Algaren	19.07 ± 1.78^{b}	28.82 ± 2.45	31.82 ± 2.44^{a}
Nettle extract	21.79 ± 2.45^{a}	29.34 ± 4.11	30.00 ± 1.97^{b}
Control	19.63 ± 2.04^{b}	28.45 ± 3.03	28.11 ± 1.11^{c}
$LSD_{0.05}$	0.905	-	0.692

Note: Results are expressed as means ± standard errors. Different superscript lower-case letters in a column represent statistically significant differences between mean values for each main effect at P < 0.05 obtained by a LSD test

The highest increase of total antioxidant capacity in leaves of lavender and sage was found for the seedlings treated with nettle extract. It was also found that the application of nettle extract and seaweed extract (Algaren) significantly increased total antioxidant capacity of basil leaves compared to non-treated plants (Table 3).

Table 3. Total antioxidant capacity in leaves of studied medicinal plants

Treatment	Total antioxidant capacity (µmol Fe^{2+} g^{-1} dry matter)		
	Lavender	Sage	Basil
Slavol	138.98 ± 12.07^{b}	252.56 ± 10.44^{b}	233.79 ± 5.01^{bc}
Algaren	138.10 ± 7.65^{b}	275.21 ± 18.53^{a}	247.45 ± 11.12^{a}
Nettle extract	161.16 ± 21.12^{a}	279.44 ± 22.11^{a}	236.45 ± 9.65^{b}
Control	128.47 ± 10.9^{b}	252.77 ± 11.33^{b}	231.01 ± 4.03^{c}
$LSD_{0.05}$	12.49	14.06	4.65

Note: Results are expressed as means ± standard errors. Different superscript lowercase letters in a column represent statistically significant differences between mean values for each main effect at P < 0.05 obtained by a LSD test

Discussion

Medicinal plants are mainly used for the prevention and treatment of human diseases. Among bioactive compounds in medicinal plants, phenolics are of considerable interest due to their antioxidant properties and potential beneficial health effects (Yuan et al., 2016). The antioxidant properties of phenolics are related mainly to the number and positions of free hydroxyl groups on the aromatic ring and to their redox metal (Cu, Fe) chelating ability (Shahidi and Ambigaipalan, 2015).

Considering the fact that phenolic compounds play an essential role in immunity, many agricultural and food scientists as well as farmers have focused on agro-technical practices that can stimulate the synthesis of phenolics and other antioxidants in plants. Biostimulants treatment is certainly one of these practices (Shukla et al. 2019).

In this study, the treatment with nettle extract significantly increased total phenolics content (TPC) in sage leaves compared to other biostimulant treatments. The results of this study also showed that nettle extract treatment as well as treatment with microbial biostimulant Slavol significantly increased TPC in sage leaves over control (no treatment) plants. The use of nettle extract also increased TPC in basil leaves, but this increase was not statistically significant. Overall, these results tend to point to the fact that the greatest increase in phenolics synthesis in plants was observed after nettle extract treatment. The efficiency of nettle extract to increase the phenol synthesis in plants has been mainly attributed to the aromatic amino acids contained in nettle leaves such as phenylalanine, tyrosine and tryptophan. Namely, these amino acids serve as crucial precursors for a wide range of phenolic compounds that are important for overall plant growth and development. Several studies have confirmed the high aromatic acids content in nettle extracts as well as their ability to increase phenol synthesis in plants (Fattahi et al., 2014, Rajput et al., 2018; D'Abrosca et al., 2019).

The results of this study also showed that TPCs were significantly higher in the extracts of sage and basil leaves than in the extracts of lavender leaves, indicating that the synthesis of phenols in leaves also depends on the genetic background of plant species.

Among the several classes of phenolic compounds, the flavonoids are considered as the most important antioxidative barrier. The configuration and total number of -OH groups within flavonoids basic structure strongly influence mechanisms of their antioxidant activity such as free radical scavenging and metal ion chelation ability. In addition, flavonoids increase the activity of antioxidant enzymes, thus indirectly affecting the concentration of free radicals in the plants (Jovanova et al., 2019).

This study found that the application of nettle extract resulted in a significant increase in TFC of lavender and basil leaves compared to control plants. It was also found that the application of seaweed extract (Algaren) significantly increased TFC, but only in basil leaves. These results lead to the conclusion that among applied biostimulants nettle extract had the greatest effect on the increase of TFC in leaves of studied plants.

The application of microbial biostimulant Slavol did not have a significant effect on the TFC in the leaves of tested plants under experimental conditions. Our assumption is that a lower effect of microbial biostimulant Slavol on TFC is primarily related to its composition. Namely, Slavol contains microbial inoculants which efficiency to improve soil fertility and thus plant growth and development strongly depend on the environmental conditions, plant-microbe interactions, soil pH and soil physical properties. Since environmental factors and plant-microbe interactions vary widely over time, it is not surprising that the microbiological activity in soil varies considerably, and thus their impact on the plant metabolism. Therefore, the success of microbial inoculants as biostimulants may not be predictable.

The results of this study also showed that TFC was significantly higher in the sage and basil leaves than in the lavender leaves, regardless of biostimulant treatments. It is interestinge that the same observation also refers to the phenolics content in plants, indicating that the studied plants differ greatly in terms of the ability to accumulate phenolic compounds in their leaves under experimental conditions.

The results of this study demonstrated that the treatment of lavender, sage and basil seedlings with nettle extract caused significant increase in total antioxidant capacity (TAC) compared to non-treated seedlings, suggesting that nettle extract can enhance antioxidant activity of studied medicinal plants (Matamane et al., 2020). The results of this study also demonstrated that seaweed extract has potential to enhance the antioxidant capacity of basil and sage seedlings under experimental (without stress) conditions.

It is commonly believed that the application of biostimulants can improve antioxidant capacity of plants only when they are cultivated in adverse conditions (Kwiatkowski and Juszczak, 2011). However, the results of this study indicate that the application of biostimulants can be also effective when the plants are cultivated under standard growth conditions. These findings are consistent with the results of Przybysz et al. (2010).

In this study, the antioxidant capacity of leaf extracts increased in parallel with the total phenolics/flavonoids content, regardless of plant species or biostimulant treatments. Numerous studies have also reported positive relationship between total antioxidant activity and total phenolics/flavonoids content, indicating that phenolic compounds, in general, are one of the major groups of endogenous antioxidants in plants (Sadeghi et al., 2015; Dastoor et al., 2018).

Conclusions

The nettle extract was found to be the most effective in increasing total phenolics, flavonoids and antioxidant capacity in leaves of basil, lavender and sage. Total phenolics/flavonoids content as well as total antioxidant capacity were significantly higher in sage and basil than in lavender, regardless of biostimulant treatments. These results lead to the conclusion that studied plants differ greatly in terms of the ability to accumulate phenolic compounds in their leaves under experimental conditions. The antioxidant capacity of leaf extracts was in line with total phenolics/flavonoids, regardless of plant species and biostimulant treatments, suggesting that phenolic compounds greatly contribute to plant antioxidant defense system.

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