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Changes in the metabolite profile and antioxidant capacity of *Levisticum officinale*, a wild medicinal plant by salicylic acid elicitation

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Abstract

Levisticum officinale (Apiaceae) is an important medicinal plant. It is in danger of extinction in Iran due to the fact that it was only grows in a small circumscribed area in south west of Iran. All parts of the plant are aromatic and are used in folk medicine. The aim of this study was to evaluate the effect of foliar application of salicylic acid (SA) on the plant metabolite profile) including: total phenolic contents, total flavonoid contents, total anthocyanin contents and free radical scavenging. Free radical scavenging activity was determined according to the elimination of DPPH radicals. The total phenol and Total flavonoid content was determined by the Folin–Ciocalteu reaction and Aluminum chloride colorimetric method, respectively. Results showed that SA foliar application had a significant effect on all measured traits. This effect was more significant at 750ppm concentration. These results indicate that the metabolite profile in *L. officinale* can be increased by suitable concentration of SA foliar application.

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Introduction

Many plants are sources of numerous biologically active chemical compounds, which act as a natural defense system in plants and have been used in many aspects such as: pharmaceuticals, fragrances, flavor compounds, dyes, and Agrochemicals (Rathore *et al*., 2013). Numerous human diseases are resulted from oxidative stress leading to the damage of lipids, proteins and DNA (Schieber & Chandel, 2014). Antioxidants are compounds that can effectively help protect our body cells from these damages (Zheng & Wang, 2001). They can effectively neutralize free radicals. Plants contain great amounts of secondary metabolites such as phenolic and flavonoid which can act as antioxidants and inhibit the free radicals. Phenolic compounds are secondary metabolites which are synthesized through pentose phosphate, shikimate and phenyl propanoid pathways in plants (Pandey, 2014). Flavonoids including flavones, flavonoles, isoflavones, flavonones and chalcones inhibit production of reactive oxygen species (ROS) in cells (Rahman & Abdalla, 2014). Today, there is a great attention on the use of naturally occurring antioxidants in foods. Due to the importance of this class of compounds, attention to antioxidant or radical-scavenging capacity has been increased (Christophe *et al*., 2013). *Levisticum officinale* is an important and endangered wild medicinal plant belongs to Apiaceae family. It is used for disorders of the stomach; reduce excess gas and uses as a diuretic (Hog *et al*., 2001). All parts of the plant contain compounds that can act as natural antioxidants (Raghavan, 2007). Elicitation is an attractive strategy mimic stresses, resulting enhancement of secondary metabolite production in plants (Gadzovska Simic *et al*., 2014). Salicylic acid is a chemical stimulus enables to enhance the strength and antioxidant activity of the plant, as well as increasing phenolic compounds as a regulator of the internal growth (Rivas-San Vicente, 2011). No information on SA effects on the chemistry of *L. officinale* is available. Such data would be beneficial to provide information on the availability of high levels of useful components. The aim of the study was the comparative assessment of the some secondary metabolites of *L. officinale in* response to Salicylic Acid.

Material and methods

Reagents and chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, aluminum trichloride, Quercetin and sodium nitrite were purchased from Sigma Aldrich. Sodium carbonate, Gallic acid, Ascorbic acid, Salicylic acid, Acetic acid and Methanol were purchased from MERCK chemical chemistry. All the chemicals were of analytical grade.

Plant cultivation and salicylic acid application

The seeds of *L.officinale* were collected from the only natural habitat located in Hezar Mountain in South East of Iran, Kerman Province. Seedlings from seeds were grown in a greenhouse at the Shahid Bahonar University. After 90 days, the plants were randomly treated with different concentrations of the SA as an elicitor. Four different concentrations of the SA were used: 250ppm, 500ppm, 750ppm and 1000ppm. The control plants were treated with distilled water. The treatments were carried out by spraying the aerial parts of the plant at dew point (approximately 100mL per plant) 3 times at days 90, 93 and 96. Finally leaves were collected for analysis at day 100.

Extract preparation and determination of total phenolic and flavonoids content

Aliquot 0.5 g from fresh leaves was weighed and phenolic and flavonoids compounds were extracted with 50mL of 80% aqueous methanol on an ultrasonic bath for 20min. Then, the extracts were centrifuged for 5 min at 14000rpm. Then the supernatant was collected and filtered thoroughly. The total phenolic content (TPC) was determined according to the Folin–Ciocalteu colorimetric method. Briefly, 1mL of extract was mixed with 9mL of water and 1mL of Folin-Ciocalteu reagent. After 5 min, 10mL of Na2CO3 solution (7.5%) was added and the samples were brought to final volume of 10mL with water. After 90 min at room temperature, the absorbance was measured at 750nm using a UV spectrophotometer. Gallic acid was used as a standard and the results were expressed asmg of Gallic acid (GA) equivalents per g of dry matter (mg GAE/g) (Singleton *et al*., 1999). The calibration curve of Gallic acid was drawn.

The total flavonoid content was evaluated using aluminum chloride colorimetric assay. 1mL of extracts were taken in a test tube, then 4ml of distilled water was added, followed by the addition of 0.3mL of sodium nitrite (5% NaNO2, w/v) and allowed to stand for 5 min. Following this, 0.3mL of aluminum chloride (10% AlCl3) was added and the samples were brought to a final volume of 10mL with distilled water. The mixture incubated for 15 min and absorbance was measured at 510nm. Distilled water was used as blank. Quercetin was used as standard and flavonoid contents were expressed inmg of quercetin equivalents (QE) per gram of extract (Zhishen *et al.*, 1999). The calibration curve of quercetin was drawn.

Determination of total anthocyanin

Aliquot 0.1 g fresh leaf was homogenized in 10mL acidified methanol and the content was kept at 25°C for 24 h in the dark conditions. The homogenate was centrifuged at 4000rpm for 10 min and the absorbance of the supernatant was determined at 535nm. The extinction coefficient of 33,000 (mol-1 cm-1) was used to calculate the amount of total anthocyanin and it was expressed as µ mol g-1 FW (Wagner, 1979).

DPPH free radical scavenging assay

The DPPH radical scavenging assay was done according to the method of Zhu *et al* with some modifications (Zhu *et al.*, 2006). Aliquot 0.5g fresh material of *L. officinal* was extracted in 50mL of 80% aqueous methanol with random shaking. After 24 hours the methanolic extract was centrifuged at 30000g for 20 min to abolish plant residues. Then the supernatant was collected and filtered. The filtrate was concentrated through the rotary vacuum evaporator at 35°C up to dryness. A solution of 0.004% (w/v) DPPH in 80% aqueous methanol was prepared. 2mL of DPPH solution was mixed with 2mL of the samples dissolved in methanol at different concentrations (0-100mg/ml). The reaction mixture was shaken and incubated in the dark at room temperature for 60 min, and the absorbance was read at 517nm against the blank.

Controls were prepared in a similar way as for the test group except for the replacement of the antioxidant solution with the corresponding extraction solvent (methanol). Ascorbic acid was used as standard and calibration curve was drowned. The experiment was carried out in triplicate. The inhibition of the DPPH radical by the sample was calculated according to the following formula:

% DPPH radical-scavenging= A0 - A 1 / A0 × 100.

Where A0 was the Absorbance of control reaction and A1 was the Absorbance in presence of test or standard sample. Results were expressed as the half maximal inhibitory concentration (IC50) and compared with Ascorbic acid standard.

Statistical analysis

The experimental design was completely randomized, consisting of five treatments (different concentration of Salicylic acid), with three replicate. All results were expressed as the mean ± standard deviation (SD). Data were analyzed by one-way ANOVA and differences among treatments were determined by comparison of means using LSD's test. The level of statistical significance was considered at p values< 0.0

Results

Based on the results of ANOVA (Table 1), the SA foliar application showed a significant effect on all measured traits, including total phenolic and flavonoid content and DPPH radical scavenging (P≤0.05).

Table 1. Analysis variance mean squares of SA treatments on TFC, TPC and TAC of *Levisticum officinale*.

Source	DF	TFC	TPC	TAN
T	4	0.01047507 **	0.03205610**	0.31685573**
Error	10	0.00002827	0.00001040	0.00844080
C.V.		3.86	0.68	3.87

TFC: Total Flavonoid Contents, TPC: Total Phenolic Contents, TAC: Total Anthocyanin Contents

In the present study, the effect of different concentrations of SA significantly increased total phenolic contents. The treated plants by different concentrations of SA showed higher TPCs content in comparison to control plants.

The results showed that TPCs increased from 28.68mg GAE/g in non-treated plants (control) and reached to its maximum value (46.44mg GAE/g) when 750ppm of SA was used. Application of SA 750ppm was found to result in a 1.61 fold enhancement of the TPCs (Fig. 1).

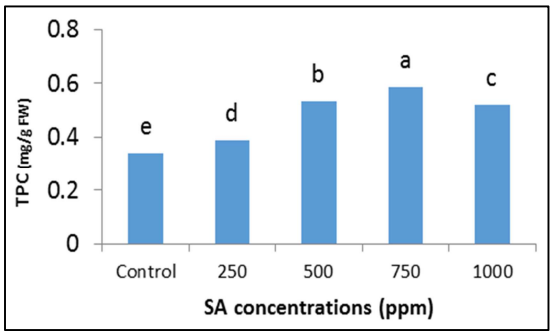

Fig. 1. Effect of different concentrations of salicylic acid on total phenolic contents (TPC) of *Levisticum officinale*.

According to the Fig. 2, the highest means of total flavonoid contents (0.142mg QE/ g) were observed in the plants treated with 250ppm of SA. Application of SA 250ppm caused significant increasing in TFC content compare to control (0.093 QE/ g). It was observed with increasing in SA concentrations from 250ppm to 1000ppm TFC contents significantly decreased. The highest mean anthocyanin content was observed in plants treated with 500ppm and 750ppm of SA with any significant difference. Treating plants with 250ppm of SA was not effective to increase TACs compare to control. The total anthocyanin content ranged from 2.92mg for 750ppm of SA to 1.92mg for 1000ppm of SA (Fig. 3).

The results of DPPH radical scavenging assay expressed as IC50 values determined by DPPH assays. IC50 determines the concentration of the sample necessary to cause 50% inhibition free radical activity, which was obtained by interpolation from linear regression analysis (Yang *et al.*, 2008). It was found that extract obtained from plants treated with 750ppm of SA had the highest DPPH radical scavenging activity, while control plant's extract showed the lowest DPPH radical scavenging activity (Table 2 and Fig. 4).

The antioxidant activities were highest for Ascorbic acid (IC50: 11.49µg/mL), followed by750ppm of SA (IC50: 20.63µg/mL), 500ppm of SA (IC50: 32.24µg/mL), 500ppm of SA (IC50: 88.94µg/mL), 250ppm of SA (IC50: 199.97µg/mL) and lastly control (IC50: 219.28µg/mL) (Table 2 and Fig. 4).

Table 2. IC50 (mg/ml) values of plant extracts treated with SA for free radical scavenging activity by DPPH radical. Lower IC50 value indicates higher antioxidant activity.

Salicylic acid (ppm)	Ic50 (µg/mL)
Control	61.37
SA 250	50.83
SA 500	33.24
SA750	20.63
SA1000	42.18
ASA	12.11

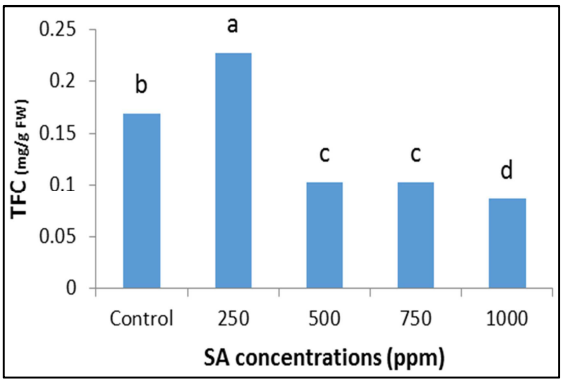

Fig. 2. Effect of different concentrations of salicylic acid on total flavonoid contents (TFC) of *Levisticum officinale*.

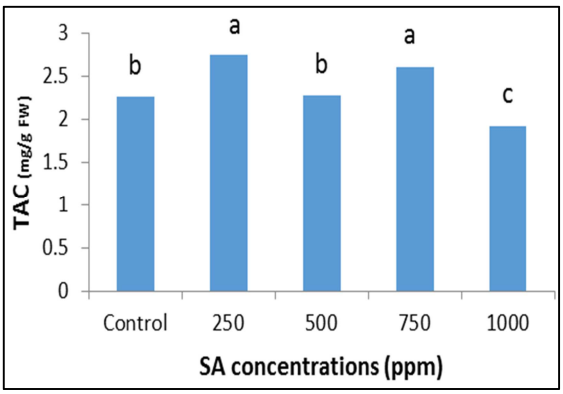

Fig. 3. Effect of different concentrations of salicylic acid on total anthocyanin contents (TAC) of *Levisticum officinale*.

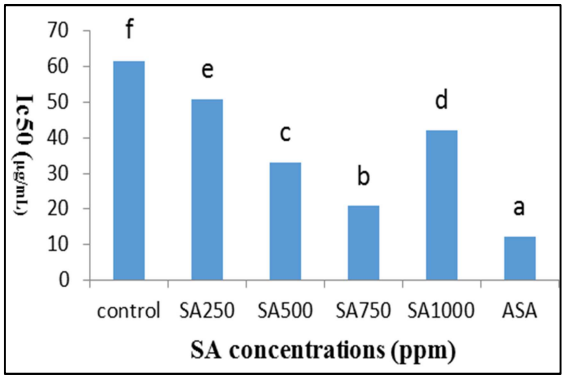

Fig. 4. IC50 (µg/mL) values of plant extracts treated with SA for free radical scavenging activity by DPPH radical. Lower IC50 value indicates higher antioxidant activity.

Discussion

Recently, phenolic compounds are known as powerful antioxidants and proved to be more efficient antioxidants than Vitamin C and E and carotenoids (Rice-Evans *et al.*, 1996). They can restrict free radicals activity due to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals. According to the results from present study, total phenolic content of *L. officinale* varied between 28.68mg to 46.44 GAE/100g. Plants treated with different SA concentrations (250, 500, 750 and 1000ppm) showed an increase in total phenolic compounds of 12.53%, 49.14%, 61.98% and 45.41% compare to control respectively. Regards to these values, it can be noted that along with increasing in SA concentrations up to 750ppm the TPCs slightly increased too. These findings are in accordance with those of obtained by Chung, who demonstrated that SA stimulates phenolic compounds in *Momordica charantia* (chung *et al.*, 2016). Conversely to TPC, high values of TFC were observed when the lowest concentration of SA, 250ppm, was applied. According to the Wen *et al* (2008) Salicylic acid induces gene regulation responsible to the biosynthesis of secondary metabolites in plants (wen *et al.*, 2008). When SA is applied exogenously, it can trigger cell signaling and regulate the expression of genes which encode enzymes related to the phenyl propanoid pathway production (among them the flavonoids), increasing the amount or the activity of these enzymes.

However, with increased SA concentration of 250ppm, this resulted in the highest values in TPC and the lowest value in TFC. These results suggest that SA application is capable to change or modify the concentration of flavonoids and phenolic acids in Lovage. These findings are in accordance with those obtained from foliar application of SA in sweet cherry fruit (Yao & Tian, 2005) and *Thymus vulgaris* (Khalil *et al.*, 2018). Conversely, reverse effect was reported by Ghasemzadeh (2012), who demonstrated that in zinger plants along with increasing the level of SA, the production of TFC increased and synthesis of TPC decreased (Ghasemzadeh & Jaafar, 2012). The treatment of the plants with SA 500 and 750ppm caused a significant enhancement in anthocyanin levels (Fig. 3). Between the different SA levels, 750ppm resulted in the maximum value of anthocyanin. In comparison to control, it was observed a 21% increase in the anthocyanin accumulation at 750ppm. Previously, similar results on the enhancement of anthocyanin production under foliar application of SA were also reported SA as a signal molecule increases Ca flux, leading to the anthocyanin biosynthesis (Sudha & Ravishankar, 2003). It was reported the production of anthocyanin in broccoli sprouts significantly increased when plants were treated with SA (Natella *et al.*, 2016).

Free radicals are involved in many disturbances and disease. Antioxidants are The DPPH is a simple method commonly used to determine radical scavenging activity of different samples (Alam *et al.*, 2013). IC50 determines the concentration of the sample necessary to cause 50% inhibition free radical activity, which was obtained by interpolation from linear regression analysis (Yang *et al.*, 2008). Regards to IC50 values, the inhibitory activity of the plants under SA elicitation increased with the increasing elicitor concentration. The extracts obtained from plants treated with SA 750ppm concentrations produced the most significant effects and showed the lowest IC50 values. This treatment reduced IC50 values by 66% compared to control. This effect can be related to increasing phenolic compounds. There was a positive relationship between phenolic compounds t

and antioxidant activity enhancement. Our results are in a good line with those of obtaining by Perez *et al.* (2014), who reported increasing in antioxidant activity of the peppermint plant under SA elicitation could be due to the increase in phenolic compounds (Pérez *et al.*, 2014).

This study demonstrated that foliar application of SA could be promoting the production of secondary metabolites and antioxidant activity of *L.officinale*. SA at 750ppm concentration found to be the most suitable elicitor to promote secondary metabolite improvement. Finally, SA at 250ppm was best concentration for the enhancement of total flavonoids.

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