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RESEARCH ARTICLE

Foliar application of yeast extract and salicylic acid affect chemical composition and content of lemon balm (*Melissa officinalis* L.) essential oil

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ABSTRACT: The present study investigated the effect of 14 treatments consisting yeast extract (YE) (0, 0.5, 1.0, 1.5, 2 g/l), salicylic acid (SA) (0, 40, 80, 160, 320 mg/l) and YE (1 and 1.5 g/l) in combination with SA (80 and 160 mg/l) foliar application on essential oil content and constituents of lemon balm (*Melissa officinalis* L.). The experiment was conducted in a completely randomized design with three replications under greenhouse conditions. Essential oils analyzed by GC/MS and a total of 39 compounds were identified that the major constituents were citronellol, trans-carveol, γ-3-carene, linalool, citral and carvacrol acetate, respectively (42.8 to 48.0% in total). Citronellol was the main constituent of essential oils with 11.05%. SA and YE significantly altered the amount of 23 constituents of lemon balm essential oil ($P<0.01$). The highest citronellol, linalool and citral (14.50, 7.9 and 8%, respectively) production was obtained at 1.5 g/l YE+160 mg/l SA treated plants that was 103, 88 and 203% higher than control plants, respectively. The highest essential oil content (0.336% v/w) that was 49% higher than control was achieved by 1.0 and 1.5g/l YE+160 mg/l SA treatments. The principal component analysis (PCA) and heatmap indicated that the content of compounds varied with different treatment and also revealed a clear separation between control and treatment groups. The results suggested that SA, YE and SA in combination with YE has considerable ability to stimulate the production of major constituent such as citronellol, citral, and linalool in the lemon balm.

KEYWORDS: Citral, Citronellol, Elicitor, Lamiaceae, Medicinal plant.

INTRODUCTION

In the last few decades, medicinal plants have served as a valuable source for bioactive compounds [20]. The increased demand of natural remedies has caused an enhancing industrial request in the production of standardized plant material and extracts [19]. The quality of medicinal plants used for the production of pharmacologically useful compounds is usually assessed by the content of biologically active compounds [17]. Essential oils are natural complex volatile compounds that are often obtained from various aromatic plants [7]. Chemical variation in essential oil is a very important property for marketing and contributes to its commercialization as a main component in pharmaceutical industries [36]. Lemon balm (*Melissa officinalis* L.) is valuable and economically important medicinal and aromatic herb belonging to the Lamiaceae family, which widely grows in Southern Europe, North America, and East Asia [30]. Several pharmacological studies have shown that extracts and essential oil extracted from lemon balm has various biological and medicinal functions such as sedative, antioxidant, antispasmodic, anti-bloating, antibacterial, antifungal, anticancer, antiviral and anti-inflammatory properties [15, 22, 28, 34]. Leaf trichomes of this herb overall present low essential oil contents

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(range from 0.02 to 0.40%), which turns it into one of the most valuable essential oil types, due to its high market value in comparison to that of essential oils extracted from other aromatics [29]. The main components of lemon balm essential oil are citronellal and citral [33, 35]. The odor of this plant is similar to that of lemon due to the presence of citral in its essential oil [29]. The essential oils content and composition are influenced by multiple factors including geographical condition, harvest time, storage conditions, drying and extraction method, and genotype [8, 14, 23, 32]. Using of elicitors as signal molecules can be a suitable approach to increase the production of valuable secondary metabolites in medicinal and aromatic plants [1]. SA (2-hydroxybenzoic acid, $C_7H_6O_3$) is a hormone-like substance and signaling molecule that plays a role in regulating of plant growth and stimulating secondary metabolite biosynthesis in stress conditions [31]. SA as highly potent, cost effective, ecofriendly and harmless to the life, and quick in action strategy to enhance the synthesis and accumulation of secondary metabolites [3] has been employed in a large number of studies in different plant species [9, 21, 24, 31]. Also, the stimulating influence of YE as biotic elicitor in improvement of secondary metabolites content was confirmed in several studies [16, 18, 21, 24]. In order to economically produce essential oil, it is necessary to use elicitors optimally in medicinal plants. The hypothesis of current study was that foliar application of abiotic and biotic elicitors such as SA and YE would have stimulation effect on accumulation of essential oil, and chemical components profile of lemon balm. In the present investigation, we have reported the influence of SA, YE and SA in combination with YE as a foliar spray on chemical composition and content of the essential oil of greenhouse-cultivated lemon balm plants.

MATERIALS AND METHODS

Experimental set up and growth conditions

Lemon balm (*Melissa officinalis* L.) seeds (Code number: 11430) were purchased from Isfahan Pakanbazr Company, Iran. The study was conducted in 2019 at a greenhouse of Razan County (latitude: 35° 12´ N, longitude: 48° 33´ E, altitude: 1696 m above sea level), located in the west of Iran and north of Hamadan province. Culturing was carried out in 84 plastic pots (20 cm in diameter and 25 cm height) containing a uniform mixture of soil: sand: and farm yard manure (1:1:1 ratio). In each plastic pot (experimental unit), 10-15 seeds were sown in superficially depth of 0.5 to 1 cm and a thin layer of rotten manure was poured on them and irrigation was carried out immediately. Five plants were kept in each pot in 4-leaf stage. Plants were grown in a naturally-lit greenhouse and temperature fluctuated between 22 and 28°C during the experimental period. During the experimental period, plants were irrigated two to three times a week, as required.

Experimental design and treatments

A completely randomized design (CRD) experiment with 14 treatments consisting yeast extract (YE), salicylic acid (SA) and the combined effect of YE with SA (0 g/l YE (distilled water), 0.5 g/l YE, 1.0 g/l YE, 1.5 g/l YE, 2 g/l YE, 0 mg/l SA (distilled water + 1% ethanol as solvent), 40 mg/l SA, 80 mg/l SA, 160 mg/l SA, 320 mg/l SA, 80 mg/l SA+1.0 g/l YE, 80 mg/l SA+1.5 g/l YE, 160 mg/l SA+1.0 g/l YE, and 160 mg/l SA+1.5 g/l YE) as a foliar spray involving three replications (two pots were treated as one replication) was carried. The doses of YE and SA for foliar application was selected based on our previous studies [21, 24]. Stock solution of SA was made by dissolving weighed quantity in minimum quantity of ethanol and final volume made by distilled water. Also, YE was dissolved in distilled water. In this experiment, distilled water foliar application was used as control for YE (YE0) and distilled water + 1% ethanol (as solvent) treated plants was used as control for SA (SA0). Foliar application was carried out between 8:00 AM and 9:00 AM, using a handheld sprayer to wet the entire aerial plant parts of lemon balm at 40% flowering stage. For each treatment, six plastic pots having five plants per pot was used. To evaluate the chemical composition and oil content of lemon balm, the sampling was performed 5 days after the foliar application of the elicitors. Aerial parts were separated and air-dried for one week under shade at room temperature (25±5 °C). In this research, the characteristics such as oil content (%) and essential oil components were measured.

Essential oil extraction and chemical analysis

The essential oil was extracted using hydro-distillation method from 50 g of dried and chopped lemon balm aerial parts by a Clevenger apparatus for 3 hours at 100°C. The extracted essential oils were dried with anhydrous sodium sulphate and stored in dark glass at 4°C before further analysis. The essential oil was weighed and its content was calculated based on the following formula:

Essential oil content (%) = [oil quantity (mg) / dry aerial parts weight (g)] × 100.

The composition of the essential oils was determined by gas chromatography-mass spectroscopy (GC/MS) using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (HP-5970 mass-selective detector-USA) and a 50 m × 0.20 mm HP-5 (cross-linked Phenyl–Methyl Silicon) column with a 0.25 µm film thickness. For GC/MS detection an electron ionization system with ionization energy of 70 eV was used. The flame ionization detector (FID) was maintained at 250 ºC. Helium (99.99%) was used as carrier gas at the flow rate of 1.0 ml/min. The column temperature was programmed from 100 °C to 250 °C at 4 °C/min. Individual components were identified by spectrometric analyses using computer library. Furthermore, percentage of each essential oil component was determined according to the level below the curve of each of the chromatogram peaks and its comparison with the total surface under the curve.

Statistical analysis

Data were statistically analyzed by one-way ANOVA using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA), followed by Duncan's Multiple Range Test and significance was assessed at 5% level ($P<0.05$). The experimental results represented as a mean ± standard error (SE). Principal component analysis (PCA) -R Studio statistical analysis software, version 4.1.1 (Using the "ggplot2", "FactoMineR" and "factoextra" packages) was used to verify the influence of elicitors in correlations to major essential oil components. The data were further analyzed by heatmap using the software ClustVis (https:llbiit.cs.ut.ee/clustvisl).

RESULTS

Essential oil content and composition

The essential oil constituents of *M. officinalis* identified by GC/MS analysis are shown in Table 1. Furthermore, the related chromatogram is shown in Fig. 1. According to results, 39 compounds were identified in the essential oils of *M. officinalis* under foliar application of YE, SA and their combination in greenhouse conditions that representing 98.9-99.9% of the volatile constituents. The results showed that citronellol, trans-carveol, γ-3-carene, linalool, citral and carvacrol acetate were major constituents of *M. officinalis* essential oils, respectively (Totally 42.8% to 48.0%). In present study, citronellol and trans-carveol were 11.05% and 9.83% of the essential oils, respectively. The results of analysis of variance (ANOVA) showed that the effects of 14 treatments consisting different concentrations of YE, SA and their combined effects on amount of 23 constituents of lemon balm including myrcene, γ-3-carene, limonene, 1,3,6,-octatriene, linalool, cis-sabinene, 3-methyl-2(methyl-2-2 butenyl), β-thujone, trans-carveol, isoborneol, citronellol, citral, 3,6,-octadienoic acid, thymol, carvacrol, carvacrol acetate, 2,6-octadienoic acid, calamenene, α-humulene, β-ionone, β-bisabolene, eugenol and α-muurolene was highly significant at 1% probability level ($P<0.01$), two constituents including citronellal and β-cubebeneand was significant at 5% probability level ($P<0.05$). Also, the effects of mentioned treatments on 14 constituents including 1,3- octadiene, alpha-pinene, 1, octen-3-ol, alpha phellandrene, para cymene, eucalyptol, γ-terpinene, 5-hepten-1-ol, bicyclo[2.2.1] heptan-2-one, isopulegol,

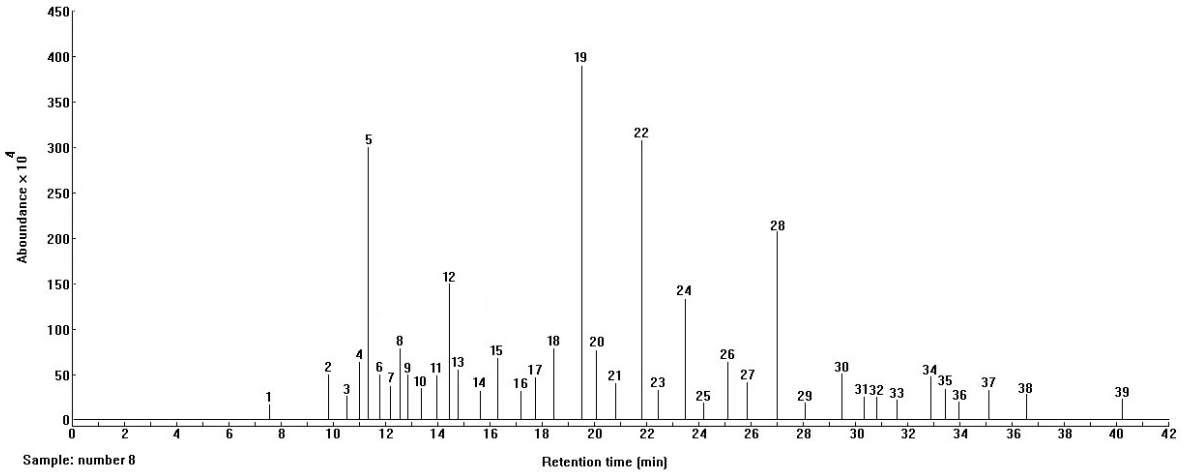

Figure 1. GC/MS chromatogram of *M. officinalis* essential oil treated with 2 g/l yeast extract foliar application (horizontal axis is the time diagram and vertical axis is the frequency).

Table 1. Mean comparison for effects of yeast extract, salicylic acid and combination of both elicitors on essential oil components in *M. officinalis*

Treatments	Compounds (%)							
	1,3- octadiene	Alpha-pinene	1,Octen-3-ol	Myrcene	γ-3-Carene	Alpha phellandrene	Para cymene	Limonene
YE_0-0	1.05±0.09^{a}	1.60±0.12^{b}	0.95±0.09^{a}	2.60±0.12^{a}	10.05±0.09^{a}	1.80±0.11^{a}	1.30±0.06^{a}	2.60±0.06^{abcd}
$YE_{0.5}$-0.5	1.00±0.06^{a}	2.00±0.17^{ab}	1.10±0.06^{a}	2.60±0.06^{a}	9.50±0.06^{ab}	1.90±0.12^{a}	1.25±0.14^{a}	2.45±0.03^{cd}
YE_1-1.0	1.15±0.03^{a}	2.20±0.17^{a}	1.15±0.26^{a}	2.20±0.06^{bc}	8.65±0.32^{c}	1.80±0.12^{a}	1.40±0.17^{a}	2.90±0.06^{abc}
$YE_{1.5}$-1.5	1.10±0.06^{a}	2.05±0.03^{ab}	1.05±0.14^{a}	1.95±0.03^{c}	8.70±0.17^{bc}	1.85±0.37^{a}	1.35±0.03^{a}	3.05±0.14^{a}
YE_2-2.0	0.85±0.09^{a}	1.90±0.00^{ab}	1.10±0.00^{a}	2.35±0.09^{ab}	7.95±0.20^{cd}	1.70±0.11^{a}	1.35±0.14^{a}	3.00±0.06^{ab}
SA_0-0	1.15±0.09^{a}	1.95±0.15^{ab}	1.25±0.14^{a}	2.20±0.12^{bc}	9.95±0.09^{a}	2.20±0.06^{a}	1.70±0.11^{a}	2.40±0.17^{d}
SA_{40}-40	1.00±0.06^{e}	1.95±0.14^{ab}	1.00±0.00^{a}	2.60±0.06^{a}	8.10±0.23^{c}	2.00±0.12^{a}	1.40±0.23^{a}	2.60±0.06^{abcd}
SA_{80}-80	1.00±0.12^{a}	2.15±0.14^{a}	1.00±0.17^{a}	2.60±0.06^{a}	7.15±0.14^{de}	1.95±0.03^{a}	1.55±0.03^{a}	3.00±0.00^{ab}
SA_{160}-160	1.20±0.06^{a}	2.00±0.12^{ab}	1.20±0.17^{a}	2.10±0.11^{bc}	6.35±0.26^{ef}	1.75±0.32^{a}	1.60±0.06^{a}	2.85±0.14^{abcd}
SA_{320}-320	1.15±0.09^{a}	1.90±0.06^{ab}	1.25±0.09^{a}	2.15±0.09^{bc}	5.65±0.14^{fg}	2.00±0.23^{a}	1.30±0.00^{a}	2.60±0.17^{abcd}
YE_1+SA_{80}	1.15±0.14^{a}	1.80±0.11^{ab}	1.20±0.23^{a}	2.60±0.06^{a}	5.40±0.35^{g}	2.10±0.11^{a}	1.50±0.17^{a}	2.65±0.09^{abcd}
$YE_{1.5}$+SA_{80}	1.10±0.00^{a}	1.95±0.14^{ab}	1.05±0.03^{a}	2.55±0.09^{a}	5.60±0.29^{fg}	1.95±0.09^{a}	1.50±0.00^{a}	2.95±0.09^{ab}
YE_1+SA_{160}	1.10±0.12^{a}	1.90±0.12^{ab}	1.45±0.03^{a}	2.20±0.06^{bc}	5.10±0.12^{g}	1.90±0.23^{a}	1.55±0.14^{a}	2.55±0.09^{bcd}
$YE_{1.5}$+SA_{160}	1.05±0.03^{a}	2.05±0.09^{ab}	1.50±0.12^{a}	2.15±0.09^{bc}	6.25±0.14^{g}	1.80±0.11^{a}	1.30±0.12^{a}	2.70±0.12^{abcd}

Treatments	Compounds (%)							
	Eucalyptol	1,3,6,-Octatriene	γ-Terpinene	Linalool	Cis-Sabinene	3-methyl-2(methyl-2-2butenyl)	β-Thujone	5-Hepten-1-ol
YE_0-0	1.80±0.11^{a}	1.35±0.03^{b}	1.85±0.09^{a}	4.20±0.06^{e}	2.60±0.11^{ab}	1.25±0.09^{abc}	2.30±0.17^{bc}	1.20±0.06^{ab}
$YE_{0.5}$-0.5	1.80±0.24^{a}	1.55±0.03^{ab}	1.70±0.11^{a}	5.30±0.11^{cde}	2.15±0.20^{bc}	1.15±0.03^{abc}	2.80±0.00^{ab}	1.35±0.20^{ab}
YE_1-1.0	1.90±0.21^{a}	1.90±0.11^{a}	1.90±0.11^{a}	6.05±0.14^{bc}	2.30±0.00^{abc}	1.15±0.20^{abc}	2.70±0.11^{abc}	1.35±0.03^{ab}
$YE_{1.5}$-1.5	2.15±0.24^{a}	1.40±0.06^{b}	1.75±0.03^{a}	6.00±0.23^{bc}	1.90±0.00^{c}	0.90±0.11^{cd}	2.65±0.20^{abc}	1.45±0.09^{ab}
YE_2-2.0	2.20±0.15^{a}	1.60±0.06^{ab}	1.95±0.03^{a}	5.55±0.32^{cd}	2.25±0.09^{bc}	1.15±0.09^{abc}	2.60±0.00^{abc}	1.35±0.09^{ab}
SA_0-0	1.70±0.23^{a}	1.80±0.06^{ab}	1.55±0.09^{a}	4.65±0.14^{de}	2.75±0.14^{a}	1.05±0.09^{bcd}	2.20±0.23^{c}	1.40±0.06^{ab}
SA_{40}-40	2.20±0.12^{a}	1.55±0.03^{ab}	1.80±0.17^{a}	5.85±0.32^{bcd}	2.45±0.14^{ab}	1.30±0.00^{ab}	2.75±0.03^{abc}	1.20±0.11^{ab}
SA_{80}-80	2.15±0.25^{a}	1.65±0.14^{ab}	1.55±0.20^{a}	5.50±0.23^{cd}	2.10±0.06^{bc}	1.15±0.03^{abc}	3.00±0.06^{a}	1.35±0.14^{ab}
SA_{160}-160	2.00±0.18^{a}	1.90±0.12^{a}	1.60±0.06^{a}	6.15±0.26^{bc}	2.10±0.11^{bc}	0.75±0.03^{d}	2.50±0.06^{abc}	1.35±0.03^{ab}
SA_{320}-320	1.95±0.20^{a}	1.40±0.06^{b}	1.95±0.09^{a}	6.90±0.23^{ab}	2.15±0.14^{bc}	1.10±0.00^{abc}	2.60±0.23^{abc}	1.40±0.11^{ab}
YE_1+SA_{80}	2.10±0.12^{a}	1.60±0.12^{ab}	1.95±0.14^{a}	7.10±0.40^{ab}	2.55±0.14^{ab}	1.40±0.00^{a}	2.90±0.00^{a}	1.04±0.03^{b}
$YE_{1.5}$+SA_{80}	2.00±0.12^{a}	1.60±0.23^{ab}	1.60±0.17^{a}	7.15±0.49^{ab}	2.20±0.06^{bc}	1.15±0.03^{abc}	2.85±0.14^{ab}	1.40±0.06^{ab}
YE_1+SA_{160}	1.80±0.00^{a}	1.70±0.11^{ab}	2.00±0.23^{a}	7.90±0.52^{a}	2.60±0.00^{ab}	0.90±0.06^{cd}	2.55±0.14^{abc}	1.20±0.06^{ab}
$YE_{1.5}$+SA_{160}	2.10±0.12^{a}	1.65±0.03^{ab}	2.00±0.06^{a}	7.90±0.40^{a}	2.15±0.09^{bc}	0.95±0.03^{cd}	2.55±0.03^{abc}	1.60±0.12^{a}

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Table 1. Continue…

Treatments	Compounds (%)							
	Bicyclo[2.2.1] heptan-2-one	Isopulegol	Trans-carveol	Citronellal	Isoborneol	Citronellol	1,3,8,-P-menthatriene	Citral
YE_0-0	2.15±0.03^{ab}	2.05±0.09^{a}	13.40±0.06^{a}	2.65±0.09^{ab}	2.10±0.12^{a}	7.15±0.03^{g}	1.65±0.09^{a}	3.95±0.03^{e}
$YE_{0.5}$-0.5	1.80±0.11^{ab}	2.40±0.29^{a}	12.30±0.23^{ab}	2.55±0.09^{ab}	1.90±0.06^{ab}	8.35±0.26^{fg}	1.60±0.06^{a}	4.35±0.09^{de}
YE_1-1.0	1.90±0.00^{ab}	2.05±0.03^{a}	11.40±0.23^{bc}	2.70±0.23^{ab}	1.75±0.14^{abc}	9.20±0.29^{ef}	1.60±0.17^{a}	4.65±0.20^{de}
$YE_{1.5}$-1.5	2.05±0.14^{ab}	2.60±0.06^{a}	11.35±0.20^{bc}	2.80±0.11^{ab}	1.75±0.03^{abc}	9.30±0.29^{ef}	1.40±0.06^{a}	4.75±0.20^{de}
YE_2-2.0	1.85±0.09^{ab}	2.90±0.11^{a}	10.55±0.37^{cd}	2.75±0.09^{ab}	1.85±0.14^{abc}	10.00±0.29^{de}	1.40±0.06^{a}	5.10±0.11^{cde}
SA_0-0	2.25±0.26^{a}	2.15±0.32^{a}	13.00±0.20^{a}	2.45±0.26^{ab}	1.70±0.06^{abc}	7.55±0.14^{g}	1.45±0.14^{a}	4.00±0.06^{e}
SA_{40}-40	1.95±0.09^{ab}	2.25±0.20^{a}	10.65±0.23^{cd}	2.90±0.06^{a}	1.50±0.11^{bc}	10.35±0.26^{de}	1.45±0.14^{a}	4.95±0.09^{cde}
SA_{80}-80	1.95±0.20^{ab}	2.25±0.43^{a}	9.70±0.26^{de}	2.95±0.09^{a}	1.55±0.03^{bc}	11.20±0.29^{cd}	1.45±0.03^{a}	5.40±0.11^{cd}
SA_{160}-160	1.85±0.03^{ab}	2.40±0.06^{a}	8.75±0.26^{ef}	2.65±0.14^{ab}	1.80±0.06^{abc}	12.25±0.37^{bc}	1.45±0.09^{a}	6.10±0.29^{bc}
SA_{320}-320	2.00±0.00^{ab}	2.50±0.17^{a}	7.85±0.43^{fg}	2.50±0.06^{ab}	2.05±0.14^{a}	13.10±0.35^{ab}	1.45±0.03^{a}	7.10±0.23^{ab}
YE_1+SA_{80}	2.05±0.09^{ab}	1.95±0.03^{a}	7.55±0.49^{fg}	2.40±0.17^{ab}	1.45±0.09^{c}	13.55±0.55^{ab}	1.45±0.14^{a}	7.25±0.49^{ab}
$YE_{1.5}$+SA_{80}	1.80±0.11^{ab}	2.10±0.29^{a}	7.55±0.43^{fg}	2.75±0.09^{ab}	1.50±0.00^{bc}	13.65±0.55^{ab}	1.35±0.03^{a}	7.30±0.58^{ab}
YE_1+SA_{160}	1.90±0.06^{ab}	2.25±0.32^{a}	6.75±0.40^{g}	2.45±0.03^{ab}	1.85±0.09^{abc}	13.65±0.66^{a}	1.60±0.06^{a}	8.00±0.46^{a}
$YE_{1.5}$+SA_{160}	1.70±0.06^{b}	2.35±0.20^{a}	6.80±0.35^{g}	2.30±0.06^{b}	1.90±0.06^{ab}	14.55±0.52^{a}	1.60±0.06^{a}	8.00±0.35^{a}

Treatments	Compounds (%)							
	3,6,-Octadienoic acid	Thymol	Carvacrol	Carvacrol acetate	2,6-Octadienoic acid	β-Caryophyllene	Caryophyllen epoxide	Calamenene
YE_0-0	1.10±0.11^{abc}	2.35±0.09^{abc}	1.85±0.09^{bc}	6.40±0.06^{a}	1.05±0.09^{ab}	2.60±0.06^{a}	1.70±0.11^{a}	1.10±0.12^{ab}
$YE_{0.5}$-0.5	1.15±0.09^{ab}	2.20±0.12^{abc}	1.80±0.00^{bc}	5.75±0.20^{b}	0.90±0.06^{abcd}	2.70±0.00^{a}	1.50±0.00^{a}	1.15±0.03^{ab}
YE_1-1.0	1.00±0.06^{abcd}	2.00±0.12^{bc}	2.00±0.00^{abc}	5.15±0.09^{c}	0.90±0.11^{abcd}	2.60±0.23^{a}	1.30±0.00^{a}	1.00±0.11^{abc}
$YE_{1.5}$-1.5	1.10±0.06^{abc}	2.50±0.23^{ab}	1.95±0.14^{abc}	5.10±0.23^{cd}	0.80±0.06^{bcd}	2.60±0.00^{a}	1.35±0.03^{a}	0.80±0.00^{c}
YE_2-2.0	0.80±0.00^{d}	2.55±0.03^{a}	2.15±0.14^{ab}	4.65±0.09^{de}	0.75±0.03^{cd}	2.50±0.23^{a}	1.45±0.20^{a}	0.95±0.03^{abc}
SA_0-0	1.05±0.09^{abcd}	1.90±0.06^{c}	1.75±0.09^{c}	6.15±0.14^{ab}	0.95±0.09^{abcd}	2.80±0.11^{a}	1.30±0.06^{a}	1.10±0.06^{ab}
SA_{40}-40	1.15±0.03^{ab}	2.05±0.03^{abc}	1.90±0.06^{abc}	4.80±0.06^{cde}	1.00±0.00^{abc}	2.35±0.37^{a}	1.35±0.09^{a}	1.15±0.03^{ab}
SA_{80}-80	0.95±0.03^{bcd}	2.50±0.11^{ab}	1.95±0.03^{abc}	4.50±0.06^{ef}	0.95±0.09^{abcd}	2.60±0.35^{a}	1.40±0.17^{a}	1.05±0.03^{abc}
SA_{160}-160	0.90±0.06^{bcd}	2.40±0.23^{abc}	2.25±0.03^{a}	4.45±0.03^{efg}	0.90±0.00^{abcd}	2.60±0.12^{a}	1.35±0.03^{a}	1.00±0.11^{abc}
SA_{320}-320	0.90±0.06^{bcd}	2.45±0.03^{ab}	1.90±0.11^{abc}	4.15±0.03^{fg}	0.70±0.00^{d}	2.60±0.00^{a}	1.60±0.12^{a}	0.90±0.00^{bc}
YE_1+SA_{80}	1.25±0.03^{a}	2.15±0.03^{abc}	1.85±0.09^{bc}	4.00±0.17^{g}	1.10±0.00^{a}	2.20±0.40^{a}	1.25±0.14^{a}	1.20±0.06^{a}
$YE_{1.5}$+SA_{80}	1.00±0.06^{abcd}	2.50±0.00^{ab}	1.85±0.09^{bc}	4.05±0.09^{fg}	0.85±0.03^{abcd}	2.55±0.26^{a}	1.40±0.17^{a}	0.95±0.03^{abc}
YE_1+SA_{160}	0.85±0.03^{cd}	2.10±0.06^{abc}	1.95±0.03^{abc}	3.95±0.09^{g}	0.90±0.00^{abcd}	2.20±0.11^{a}	1.50±0.12^{a}	1.15±0.03^{ab}
$YE_{1.5}$+SA_{160}	1.05±0.03^{abcd}	2.15±0.14^{abc}	1.75±0.03^{c}	3.95±0.03^{g}	0.90±0.06^{abcd}	2.45±0.14^{a}	1.45±0.03^{a}	0.90±0.06^{bc}

Continued …

Table 1. Continue…

Treatments	Compounds (%)							
	α-Humulene	Germacrene-D	β-Ionone	β-bisabolene	Eugenol	α-Muurolene	β-Cubebene	Oil content (%, v/w)
YE_0-0	1.00±0.00^{abc}	2.15±0.26^{a}	0.85±0.03^{ab}	1.00±0.06^{abc}	0.95±0.09^{ab}	1.10±0.06^{a}	0.95±0.09^{ab}	0.225±0.01^{h}
$YE_{0.5}$-0.5	1.10±0.06^{abc}	1.75±0.26^{a}	0.90±0.06^{ab}	1.00±0.06^{abc}	0.95±0.14^{ab}	0.90±0.06^{abc}	1.05±0.03^{ab}	0.243±0.02^{fgh}
YE_1-1.0	1.15±0.09^{ab}	1.90±0.06^{a}	1.10±0.12^{a}	1.20±0.06^{abc}	0.75±0.03^{b}	0.75±0.09^{bc}	0.95±0.14^{ab}	0.253±0.03^{efg}
$YE_{1.5}$-1.5	1.15±0.03^{ab}	1.85±0.08^{a}	0.65±0.03^{b}	1.20±0.11^{abc}	0.90±0.00^{ab}	0.75±0.03^{bc}	1.10±0.06^{a}	0.257±0.03^{ef}
YE_2-2.0	0.85±0.03^{c}	2.05±0.08^{a}	1.20±0.11^{a}	0.85±0.03^{c}	1.15±0.09^{a}	0.95±0.03^{abc}	1.05±0.09^{ab}	0.269±0.06^{de}
SA_0-0	1.05±0.09^{abc}	2.10±0.17^{a}	0.90±0.11^{ab}	1.35±0.09^{ab}	0.95±0.03^{ab}	0.90±0.00^{abc}	0.95±0.03^{ab}	0.231±0.01^{gh}
SA_{40}-40	0.95±0.03^{bc}	1.95±0.14^{a}	0.95±0.09^{ab}	0.95±0.09^{bc}	1.20±0.00^{a}	1.00±0.00^{ab}	0.90±0.00^{ab}	0.273±0.05^{de}
SA_{80}-80	0.85±0.03^{c}	1.90±0.00^{a}	1.15±0.14^{a}	1.10±0.12^{abc}	1.10±0.17^{ab}	0.70±0.06^{c}	1.20±0.06^{a}	0.284±0.05^{cd}
SA_{160}-160	1.10±0.06^{abc}	2.35±0.03^{a}	0.85±0.09^{ab}	1.40±0.06^{a}	1.05±0.09^{ab}	0.95±0.09^{abc}	0.95±0.09^{ab}	0.300±0.03^{bc}
SA_{320}-320	1.10±0.06^{abc}	1.90±0.06^{a}	0.85±0.03^{ab}	1.05±0.03^{abc}	0.85±0.09^{ab}	0.90±0.00^{abc}	1.05±0.03^{ab}	0.313±0.06^{ab}
YE_1+SA_{80}	0.90±0.00^{bc}	2.20±0.23^{a}	1.00±0.00^{ab}	0.80±0.00^{c}	1.10±0.00^{ab}	1.00±0.00^{ab}	0.95±0.09^{ab}	0.321±0.08^{ab}
$YE_{1.5}$+SA_{80}	0.85±0.14^{c}	1.85±0.09^{a}	1.05±0.14^{a}	1.10±0.06^{abc}	0.90±0.00^{ab}	0.95±0.03^{abc}	0.95±0.03^{ab}	0.321±0.10^{ab}
YE_1+SA_{160}	0.95±0.03^{bc}	2.10±0.12^{a}	1.10±0.00^{a}	1.15±0.26^{abc}	1.00±0.00^{ab}	0.75±0.03^{bc}	0.75±0.03^{b}	0.333±0.09^{a}
$YE_{1.5}$+SA_{160}	1.25±0.03^{a}	1.75±0.14^{a}	0.90±0.06^{ab}	0.95±0.03^{bc}	0.75±0.03^{b}	0.95±0.02^{abc}	0.95±0.03^{ab}	0.336±0.09^{a}

Mean values followed by different letter (s) in each column are significantly different by Duncan's multiple range test at P<0.05.
The values are mean of three replicates ± standard error (SE). YE: Yeast extract (g/l). SA: Salicylic acid (mg/l).

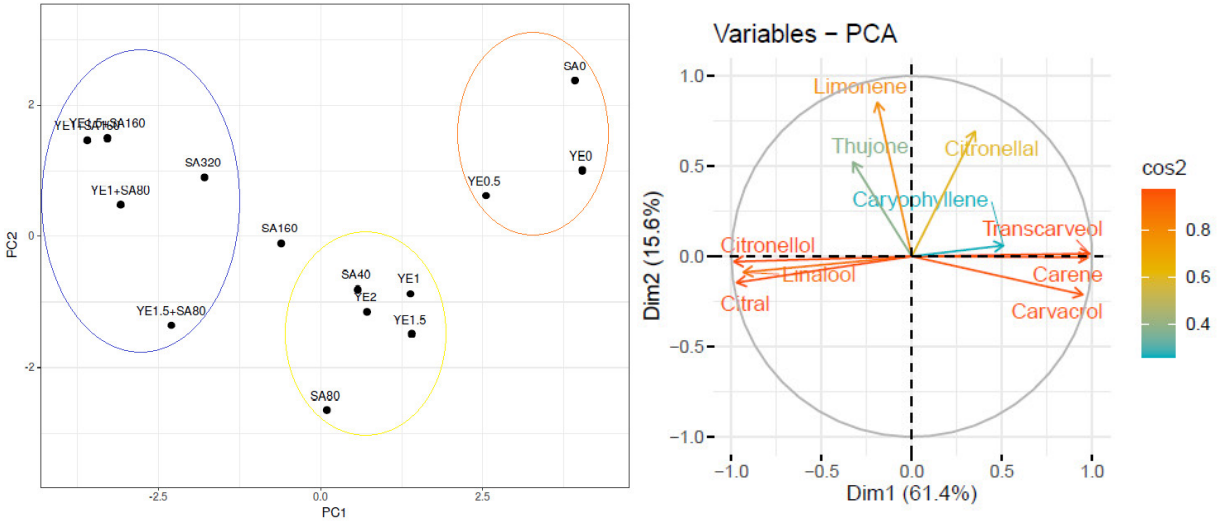

Figure 2. Principal component analysis (PCA) on the matrix correlation built using data for major essential oil components: γ-3-carene, limonene, linalool, β-thujone, trans-carveol, citronellal, citronellol, citral, carvacrol acetate, and β-caryophyllene contents of *M. officinalis* subjected to yeast extract and salicylic acid foliar application. In this figure, x and y axes represent the principal component 1 (PC1) = 61.4% and PC2 = 15.6% of the total variance, respectively.

1,3,8,-p-menthatriene, β-caryophyllene, caryophyllen epoxide, germacrene-D was not significant at 5% probability level (P>0.05) (Data not shown). Statistical analysis indicated that there were highly significant differences among treatments for amount of 8 major constituents of lemon balm including trans-carveol, γ-3-carene, linalool, citral, carvacrol acetate, limonene, β-thujone and citronellol at 1% probability level (P<0.01) and citronellal at 5% probability level (P<0.05). Mean comparison for effects of mentioned elicitors on essential oil components (%) in *M. officinalis* is shown in Table 1. Of the compounds identified in the essential oils presented in Table 1, only the process of changes in the 10 major constituents are explained here. The mean comparison results showed that the amount of citronellol varied from 7.15% to 14.50%. The highest citronellol production 14.50% was obtained at 1.5 g/l YE+160 mg/l SA treated plants that was 103% higher compared to control (distilled water). The lowest level of citronellol production was observed in control (YE0 = 7.15%) and distilled water+1% ethanol (SA0 = 7.55%). The results showed that with increasing the doses of YE from 0.5 to 2 g/l, the amount of citronellol increased. Also, with increasing the concentration of SA from 40 to 320 mg/l, the amount of citronellol increased from 10.35 to 13.10%. The results of mean comparison revealed that the amount of trans-carveol varied from 6.75 to 13.40%. The highest trans-carveol production (13.40%) was obtained at control plants, that was 98.5% higher compared with elicited plants by 1.0 g/l YE+160 mg/l SA. This was followed by 1.5 g/l YE+160 mg/l SA. Also, the results showed that with increasing the doses of YE from 0.5 to 2 g/l, and SA from 40 to 320 mg/l, the amount of trans-carveol has decreased. The results showed that the highest citral content (8% of essential oil) was obtained at 1.0 g/l YE+160 mg/l SA and 1.5 g/l YE+160 mg/l SA treated plants. This amount, considering the amount of citral in the control plants (3.95%), is a significant amount that was 2.03-fold higher compared to control. Four levels of YE did not have a significant effect on citral content in *M. officinalis* in greenhouse conditions, but treatments of 160 and 320 g/l SA significantly increased the amount of citral. It is observed that external application of YE in combination with SA has a positive effect on the production of citral in lemon balm essential oil compared to YE and SA individually. The results of mean comparison of mentioned treatments (Table 1) showed that the amount of γ-3-carene varied from 5.1% to 10.05%. The highest level of γ-3-carene production was observed in control (10.05%) and distilled water+1% ethanol (9.95%). The lowest level of γ-3-carene production was obtained at YE in combination with SA treated plants. The results showed that with elicitation by 1.0 g/l YE+160 mg/l SA the accumulation of γ-3-carene was 97% lower than the control plants. Also, the results showed that with increasing the concentration of YE from 0.5 to 2 g/l, the amount of γ-3-carene decreased. It is observed that with increasing the concentration of SA from 40 to 320 g/l, the amount of γ-3-carene has decreased from 8.1 to 5.65%. Linalool content was significantly affected by YE, SA, and YE in combination with SA spray treatments (Table 1). The highest (7.9%) content of linalool was obtained in the plants sprayed by high SA concentration with YE. The highest linalool content that was 88% higher compared to control was achieved by 1.0 and 1.5 g/l YE in combination with 160 mg/l SA. The results of mean comparison showed that the highest (6.4%) content of carvacrol acetate was obtained in the non-sprayed plants and the lowest content was achieved in the plants sprayed by SA in combination with YE. In present study, the obtained results showed that there were high significant differences (P<0.01) in the essential oil contents among the treatments in lemon balm. The highest essential oil amount (0.336 % v/w) that was 49% higher compared to control was achieved by 1.0 g/l YE+160 mg/l SA and 1.5 g/l YE+160 mg/l SA treatments, while no treatment control yielded the lowest level of essential oil content (0.225 % v/w) in our experimental condition (Table 1).

Principal component analysis and heatmap

In this study, data evaluations have shown the chemical composition of lemon balm essential oil were different in plants sprayed with SA and YE elicitors. Data of the most abundant components (i.e. the ten major essential oil constituents) was subjected to multivariate analyses so that it could be visualized. The principal component analysis (PCA) was performed to illustrate the relationships among major essential oil components (γ-3-carene, limonene, linalool, β-thujone, trans-carveol, citronellal, citronellol, citral, carvacrol acetate, β-caryophyllene) of lemon balm under YE and SA foliar application (Fig. 2). The analysis applied to ten major essential oil constituents together has explained 77% of total variation in the PCA, as shown in Fig. 2. There was

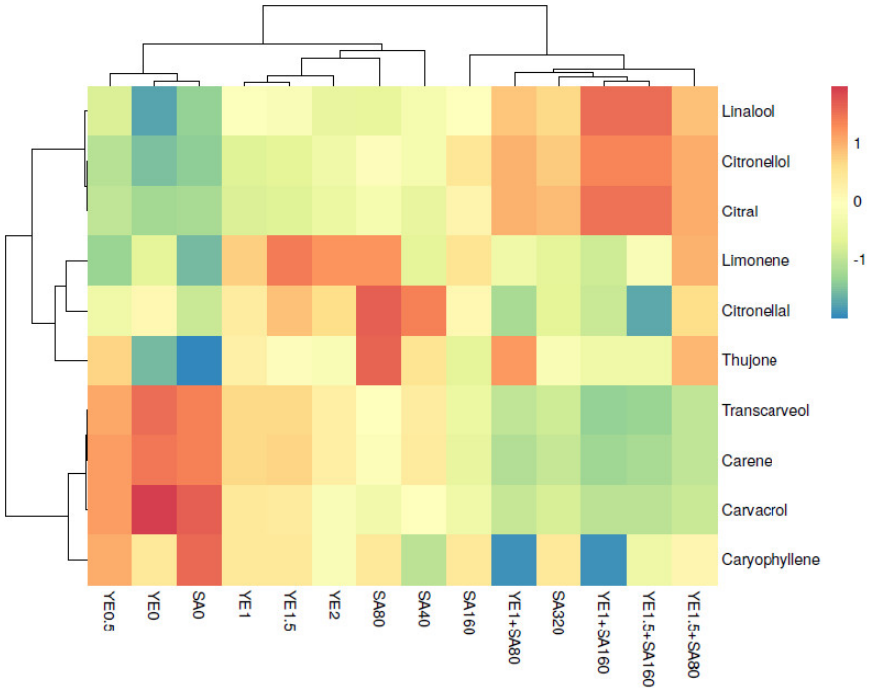

Figure 3. Heatmap diagram based on the relative concentration of 10 major essential oils constituents of *M. officinalis* treated with yeast extract and salicylic acid foliar application. Each column and each row represent different concentrations of elicitors and individual constituents, respectively. Essential oil constituents that are classified in the same group responded similarly to the experimental treatments. Color scale of heatmap represents relative intensity values. The colors (blue to red) of the tiles in the heatmap represent the values varying from low to high.

61.4% variation associated with PC1, whereas PC2 explained 15.6% of the total variance, respectively. It was noted from Fig. 2 that among the ten main constituents of essential oil, six components including γ-3-carene, linalool, trans-carveol, citronellol, citral, and carvacrol acetate had higher loading in the PC1 component; whereas limonene had positive higher loading on PC2. In other words, loading analysis allowed observing that foliar application with SA, YE and SA in combination with YE had a greater effect on trans-carveol, citronellol, citral, γ-3-carene, carvacrol acetate, and linalool contents in the current study. Based on the common variation across the elicitors, three metabolite clusters were observed. The compounds γ-3-carene, carvacrol acetate, and β-caryophyllene grouped together; citral, linalool, and citronellol also grouped together as a separate group. Additionally, limonene, citronellal, and β-thujone also grouped together. The major essential oil compounds that are classified in the same group were highly correlated each other; while the compounds on both sides of the loadings plot are negatively correlated with each other. For instance, the citronellol content showed a significant positive correlation with citral and linalool content and showed a significant negative correlation with γ-3-carene, carvacrol acetate, and β-caryophyllene content. In other words, as the amount of citronellol, citral, and linalool of the essential oil increased, the amount of γ-3-carene, carvacrol acetate, and β-caryophyllene decreased. Three separate groups based on the examined parameters for PCA of all the reference treatments were observed: first, control treatments (SA0 and YE0) and 0.5 g/l YE; second, YE (1.0, 1.5 and 2 g/l) and SA (40 and 80 mg/l) treatments; and third, YE in combination with SA (80 mg/l SA+1.0 g/l YE, 80 mg/l SA+1.5 g/l YE, 160 mg/l SA+1.0 g/l YE, and 160 mg/l SA+1.5 g/l YE) and 320 mg/l SA treatments. Multivariate statistical analyses revealed a clear separation between control and treatments groups. The results showed that the control treatments were clearly separated in the PCA diagram from the foliar spraying with SA and YE (11 other treatments), particularly SA in combination with YE treatments. Loading analysis allowed observing that elicitation with YE in combination with SA, had positive influence on citronellol, citral, and linalool contents in the current study. Plants sprayed without SA and YE also presented higher contents of γ-3-carene, carvacrol acetate, and β-caryophyllene. The present study has shown that the content of major constituents can be

significantly changed in lemon balm due to the use of SA and YE separately or together.

The heatmap was made after conducting clustering analysis within experimental treatments and major essential oil constituents, respectively (Fig. 3). Among the fourteen treatments (YE, SA, and YE in combination with SA), the lemon balm aerial parts foliar sprayed with SA in combination with YE and high concentrations of SA (320 mg/l) grouped in one cluster; YE (1.0, 1.5 and 2 g/l) and low concentration of SA (40 and 80 mg/l) treatments also grouped together; while other treatments (SA0, YE0, and 0.5 g/l YE) formed another cluster. The heatmap diagram (Fig. 3) showed these three clusters are completely separated from each other. Therefore, elicitor type (SA and YE separately or in combination with together) and their concentrations were a key factor for the differences observed within treatments, while SA0, YE0, and 0.5 g/l YE treatments had similar characteristics. The obvious difference in the ten major constituents of lemon balm essential oil elicited with elicitors were observed in SA in combination with YE and high concentration of SA treatments, and differences in these compounds were not prominent in low concentrations of YE and SA. SA in combination with YE treatments showed elevated citronellol, citral, and linalool contents. Essential oil constituents that are classified in the same group responded similarly to the experimental treatments.

DISCUSSION

Elicitation is the process of inducing or increasing synthesis of biologically active compounds by the plants to ensure their survival, persistence and competitiveness [10]. In present study, the influence of SA and YE and their combinations on the accumulation of bioactive ingredient of lemon balm was investigated and the identified essential oil compounds are listed in Table 1. A total of 39 components were identified in the essential oil of this plant. The most abundant essential oil components found in lemon balm subjected to YE and SA applied either alone or together were citronellol, trans-carveol, linalool, citral and carvacrol acetate. The essential oil contents ranged between 0.22 to 0.33% (v/w). Ghasemi Pirbalouti at al. (2019), reported that a total of 35 components were identified in the essential oils from leaves of cultivated lemon balm that the major compounds were geranial, neral, carvacrol, β-caryophyllene, citronellal, thymol, geranyl acetate, and caryophyllene oxide and the essential oil contents of studied treatments ranged between 0.53 to 0.61% (v/w) [9]. Hatami et al. (2021) reported that a total of fifteen compounds were identified in *M. officinalis* using GC/MS analysis that major essential oil components were neral, geranial, and geranyl acetate. Also, the essential oil contents ranged between 0.16 to 0.32% (v/w) [13].

Results of other researches indicated that the main components of lemon balm essential oil were citronellal and citral [33, 35]. A comparison of our results with the previous reports suggests few differences in essential oils content and major chemical constituents of the cultivated lemon balm plants that may be due to several factors, such as geographical conditions of the plant sample, harvest time, storage conditions, growth season, drying and extraction method, genotype [8, 14, 23, 32] and elicitors [24]. In this research, SA from 0-320 mg/l, YE from 0-2 g/l and their combinations exhibited different eliciting effects on essential oil content and constituents of lemon balm. SA and YE significantly altered the amount of 25 constituents of lemon balm essential oil. The foliar application of suitable concentrations of SA and YE significantly enhanced oil content and major constituents (citronellol, linalool and citral) in lemon balm aerial parts. When essential oil content was evaluated, it was determined that SA at 320 mg/l and SA in combination with YE application were more effective than YE and low and moderate SA concentrations. In present study, high concentration of YE showed a moderate stimulating effect on content and major compounds of essential oils. Similarly, Motiee and Abdoli, (2021) reported that with increasing the concentrations of YE from 0.75 to 1.5 g/l, the amount of menthone, neomenthol and γ-terpinene in *M. piperita* essential oil increased [24]. In our experiment, among the four applied concentrations, 0.5 g/l of YE had no significant effect on oil constituents, as compared with the control. The stimulating influence of YE on secondary metabolites production was confirmed in other investigations [16, 18, 21]. The majority of biotic stimulants are recognized by specific receptors bound to the cell membrane, and transferred to the cell by a signal transduction system, producing changes that ultimately lead to the formation of phytoalexins [5]. The results of analysis of variance showed that SA significantly altered the amount of 23 constituents and oil content of lemon balm at 1% probability level ($P<0.01$). Similar results of increasing in essential oil content and composition in response to SA application have been reported in several

other plant species such as *Ruta graveolens* [4], *Achillea millefolium* [11], *Mentha piperita* [6, 24, 27], *Tanacetum vulgar* [12], and *Rosmarinus officinalis* [2]. Biotic and abiotic elicitors when applied to plants, induce the expression of enzyme coding genes associated to plant defense response and to the accumulation of secondary metabolites [5, 11]. Also, in accordance with our results, it is reported that foliar application with SA considerably enhanced the monoterpene oxygenated and sesquiterpenes secondary metabolites in lemon balm plants. However, essential oil content was not affected significantly by SA foliar application [9] which this is not in agreement with the results obtained in this study. Results of present study highlights the differences in quantity and quality of essential oil among concentrations and type of elicitors. The combination of elicitors SA and YE was more effective in enhancing citronellol, linalool and citral biosynthesis than control and SA or YE alone. As shown, the highest citronellol, linalool and citral (14.50, 7.9 and 8%, respectively) content was obtained at 1.5 g/l YE+160 mg/l SA treated plants that was 103, 88 and 203% higher than control plants, respectively. While there are many studies of elicitor potential use in improving secondary metabolism biosynthesis individually, few reports were found with the combination of both biotic and abiotic forms of elicitors. In accordance with our results, it reported that application of combined elicitors in cell suspension culture [25] and hairy root culture [26] of feverfew were more useful for parthenolide production. Often the major components of lemon balm essential oil in the conditions of this study had a positive and significant relationship with each other. The essential oil of lemon balm was highly correlated with type and concentration of elicitors (Fig. 2, 3). The major essential oil compounds that are classified in the same group were highly correlated each other. In this study, citronellol as a most abundant constituent showed a significant positive correlation with citral and linalool content and showed a significant negative correlation with γ-3-carene, carvacrol acetate, and β-caryophyllene content. In other words, as the amount of citronellol, citral, and linalool of the essential oil increased, the amount of γ-3-carene, carvacrol acetate, and β-caryophyllene decreased. The results demonstrated the inverse relationship between SA and YE foliar application and trans-carveol, γ-3-carene and carvacrol acetate content. In agreement with the results of present study, it was found that most major constituents of essential oil of *M. piperita* and *M. officinalis* both had significant positive correlation with each other [24]. The results also revealed a clear separation between control and treatments groups.

CONCLUSION

The current study provided evidence that lemon balm is a medicinal plant that is well responsive to foliar application of SA and YE applied either alone or together. Thirty-nine components were identified in the lemon balm essential oils that citronellol, trans-carveol, γ-3-carene, linalool, citral and carvacrol acetate were major constituents, respectively. The combination of both elicitors SA and YE was more effective in enhancing citronellol, linalool and citral biosynthesis than control and SA or YE alone. In order to obtaining maximum essential oil content of *M. officinalis* application of 1.0 g/l YE +160 mg/l SA recommended. The citronellol showed a significant positive correlation with citral and linalool and significant negative correlation with γ-3-carene, carvacrol acetate, and β-caryophyllene content. PCA and heatmap indicated that the content of compounds varied with different treatments and also revealed a clear separation between control and treatments groups. SA and YE application changed the percentage of some major and minor constituents of lemon balm and we can select better elicitor and concentration due to our purpose.

AUTHOR CONTRIBUTION STATEMENT

M. Hedayati performed the experiments and data gathering. M. Abdoli designed/supervised the research, analyzed the data and wrote the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare no competing financial interest.

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تاثیر محلول پاشی عصاره مخمر و اسید سالیسیلیک بر ترکیب شیمیایی و میزان اسانس بادرنجبویه (*Melissa officinalis* L.)

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چکیده

این پژوهش، اثر ۱۴ تیمار شامل عصاره مخمر (۰، ۰/۵، ۱، ۱/۵ و ۲ گرم در لیتر)، اسید سالیسیلیک (۰، ۴۰، ۸۰، ۱۶۰، ۳۲۰ میلی گرم در لیتر) و اثر توام عصاره مخمر (۱ و ۱/۵ گرم در لیتر) با اسید سالیسیلیک (۸۰ و ۱۶۰ میلی گرم در لیتر) را بر میزان و ترکیبات اسانس بادرنجبویه مورد بررسی قرار داد. آزمایش در قالب طرح کاملاً تصادفی با سه تکرار در شرایط گلخانه انجام شد. در مجموع، ۳۹ ترکیب توسط دستگاه GC/MS در اسانس بادرنجبویه شناسایی شد که اجزای اصلی آن به ترتیب سیترونلول، ترنس کارول، گاما-۳-کارن، لینالول، سیترال و استات کارواکرول بودند (مجموعاً ۴۲/۸ تا ۴۸٪). سیترونلول با ۱۱/۰۵ درصد، ترکیب اصلی اسانس بود. اسید سالیسیلیک و عصاره مخمر مقدار ۲۳ ترکیب و میزان اسانس بادرنجبویه را به طور معنی‌داری در سطح احتمال ۱ درصد تغییر دادند. بیشترین میزان تولید سیترونلول، لینالول و سیترال (به ترتیب ۱۴/۵۰، ۷/۹ و ۸ درصد) در گیاهان تیمار شده با ۱/۵ گرم در لیتر عصاره مخمر+۱۶۰ میلی گرم در لیتر اسید سالیسیلیک به دست آمد که به ترتیب ۱۰۳، ۸۸ و ۲۰۳ درصد بیشتر از گیاهان شاهد بودند. بیشترین مقدار اسانس (۰/۳۳۶٪) که ۴۹ درصد بیشتر از شاهد بود، با تیمارهای ۱ و ۱/۵ گرم در لیتر عصاره مخمر+۱۶۰ میلی گرم در لیتر اسید سالیسیلیک بدست آمد. تجزیه مؤلفه اصلی و نمودار حرارتی نشان داد که مقدار ترکیبات در تیمارهای مختلف، تفاوت دارند و شاهد و سایر تیمارها در گروههای مجزایی قرار گرفتند. نتایج نشان داد که محرک های اسید سالیسیلیک، عصاره مخمر و اثر توام آنها توانایی زیادی در تحریک تولید ترکیباتی مانند سیترونلول، سیترال و لینالول در بادرنجبویه دارند.

کلمات کلیدی: سیترال، سیترونلول، محرک، نعناعیان، گیاه دارویی