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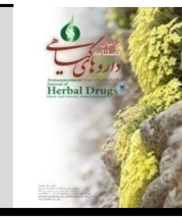

Effect of different concentrations of L-phenylalanine on chemical compositions and yield of essential oil of lemon balm (*Melissa officinalis*)

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ABSTRACT

Background & Aim: *Melissa officinalis* (lemon balm), belongs to the family Lamiaceae is a known herb that has long been used in traditional medicine to treat many disorders and give fragrance to different food and beverage products. In this study, the effects of the foliar spray of L-phenylalanine on essential oil content and chemical composition of lemon balm (*Melissa officinalis*), at field condition in Horeh, Saman city of Chaharmahal and Bakhtiari Province, southwestern Iran, were investigated.
Experimental: This study was conducted in randomized complete block design with three replications. Experimental treatments were including three levels of the foliar application of L-phenylalanine including 1000, 2000 and 4000 mg/L. Distilled water was used as a control.
Results: The essential oil contents of the treatments ranged between 0.11 to 0.19% (v/w). Statistical analysis indicated that there were significant differences among treatments for essential oil contents of *M. officinalis*. Neral, Z-Citral, E-Citral, Geranial, Citronellal and β-Caryophyllene were the six major compounds of *M. officinalis* essential oil**.** The results indicated that the foliar application of L-phenylalanine have significant impacts on main constitutes of the essential oil and generally the application of L-phenylalanine spraying have beneficial and effective role in improving the essential oil content and some of chemical composition of lemon balm.
Recommended applications/industries: The plant biostimulants as environmentally friendly products can be effective for increasing the quantity and quality of lemon balm essential oil. Application of L-phenylalanine can be a promising strategy in achieving organic production of medicinal plants such as lemon balm.

1. Introduction

Lemon balm (*Melissa officinalis* L.; Persian name: "Badrnajboyeh" or "Varangboo"), is a perennial herb up to 100 cm in height, belonging to the family Lamiaceae, which it grows in various areas from the eastern Mediterranean regions to western Asia and in some parts of Iran (Mozaffarian, 2008). In addition, *M. officinalis* is widely cultivated throughout Europe (Koytchev *et al.*, 1999). The most important ingredients in the plant are known to be phenolic compounds, rosmarinic acid, caffeic acid, cholinergic acid, metrilic acid; flavonoids such as luteolin, apigenin and monoterpene derivatives; the sesquiterpenes including beta-caryophyllene and germacrene;

triterpenes such as oleanolic and ursolic acid; volatile oil and tannins (Rasmussen, 2011). The main components of the essential oil of *M. officinalis* are citrals, citronellal, limonene, geraniol, β-caryophyllene, β-caryophyllene oxide and germacrene D (Mimica-Dukic *et al.*, 2004).

This herb has been used extensively in traditional medicine and the history of it goes back to more than 2,000 years ago. Avicenna, the Iranian eminent physician and philosopher introduced this plant as vitality tonic and as a sedative drug to treat neurological disorders (Naghibi *et al.*, 2005). The aerial parts of lemon balm are used for treatment of headaches, gastrointestinal disorders, nervousness and rheumatism (Bisset and Wichtl, 2001). In Iranian folk medicine, the aerial parts of lemon balm are used for treatment of indigestion, headaches, flatulence, colic, nausea, nervousness, anaemia, vertigo, syncope, malaise, asthma, bronchitis, amenorrhea, cardiac failure, arrhythmias, insomnia, epilepsy, depression, psychosis, hysteria, ulcers and wounds and also as carminative, antispasmodic, sedative, analgesic, tonic and antidiarrheal (Ghasemi-Dehkordi, 2002). The extracts and essential oil extracted from *M. officinalis* have been shown to have biological and pharmacological activities, such as an anti-bacterial (Mimica-Dukic *et al.*, 2004), anti-fungal (Mimica-Dukic *et al.*, 2004), anti-viral (Allahverdiyev *et al.*, 2004), anti-glycative (Miroliaei *et al.*, 2011), amoebicidal (Malatyali *et al.*, 2012), anti-tumor (De Sousa *et al.*, 2004), insecticidal (Koliopoulos *et al.*, 2010), antispasmodic (Masakova *et al.*, 1979), anti-diabetic (Chung *et al.*, 2010) and use for alzheimer's disease (Akhondzadeh *et al.*, 2003; Dastmalchi *et al.*, 2009), neurohttps protective (Bayat *et al.*, 2012) and gastrointestinal spasms (Sadraei *et al.*, 2003). Mantle *et al.* (2000) demonstrated that Melissa leaf had 'appreciable' levels of anti-oxidant activity. The anti-oxidant properties of a whole extract of *Melissa* are probably mainly attributable to its flavonoid content (Mantle *et al.*, 2000).

Bio-stimulators are a set of compounds that stimulate life of plants. Some of them act as effective compounds in the plant's response to environmental conditions and others play role as growth stimulators, therefore, they can stimulate the quantitative and qualitative performance of the plant (Thomas *et al.*, 2009). Among these compounds, amino acids like phenylalanine are organic molecules that these molecules can directly or indirectly affect the physiological activity associated with the growth and development of plants (Buchanan *et al.*, 2000). Phenylalanine as one of the most important amino acids plays a role in the production of aromatic compounds, antioxidants. Earlier reports on complex of amino acids revealed that foliar application of these chemicals enhanced the productivity in the harvest without affecting the quality of final produce (Mandal *et al.*, 2007). Similar studies were carried out in cotton to increase yield (Biles and Cothren, 2001).

Few studies have been carried out to investigate the effects of foliar application of amino acid such as L-phenylalanine on accumulation of essential oil and chemical components of essential oil from lemon balm cultivated in agricultural systems. Thus, the main goals of this research was to evaluate the foliar of L-phenylalanine on quantity and quality of yields of the essential oil of lemon balm for first time.

2. Materials and Methods

2.1. Plant material

This study was done in May 2018 in Horeh, Saman City of Chaharmahal and Bakhtiari Province, Southwestern of Iran (latitude. 32°35′N; longitude. 50° 51′ E; altitude. 1900 m asl).Some of the meteorological parameters during the implementation of the research are presented in Table 1.

Table 1. Average temperature, total precipitation and average humidity during 2018.

Average temperature °C	Total precipitation mm	Average humidity %
10.4	65.5	45

2.2. Soil analysis

The soil samples were air dried, crushed and passed through a 2 mm sieve for the physiochemical analyzes (Table 2). The soil texture determination and its particle size analysis were conducted using a standard hydrometer (Tandon, 1996). Electrolytic conductivity (EC) and pH value of the soil were measured after shaking the soil with deionized water at a 1:1 (w/v) ratio by by standard conductivity meter and pH meter , respectively (Janzen, 1993). Organic matter was determined by Walkley-Black procedure (Walkley and Black, 1934); total organic carbon by dry combustion (Nelson and Sommers, 1982); total N by standard Kjeldahl method (Bremner and Mulvaney, 1982); available P by Olsen procedure (Olsen, 1954). Exchangeable Kþ was extracted using 1 M NH_4OAc and analyzed using flamephotometry (Brown and Lilleland, 1946). Total Zn, Cu, Mn, B and Fe were measured by atomic absorption spectroscopy (PerkinElmer, Norwalk, Conn.) after digestion of the soil with nitric and perchloric acid at 150°C (Baker and Amacher, 1982).

Table 2. Physicochemical properties of the soil at testing site 0–30 cm deep.

Soil texture	EC (dsm^{-1})	N (%)	Organic Carbon (%)	pH	($mg.kg^{-1}$)						
					K	P	Fe	Mn	Zn	Cu	B
Clay-silt	10.400	6.5	2.594	7.29	1161	218.2	14.23	17.63	1.55	3.11	4.01

2.3. Experimental design and treatments

This study was conducted in randomized complete block design with three replications. Various treatments were inoculation of L-phenylalanine (purchased from Merck, Germany) spray with three levels including 1000, 2000 and 4000 mg/L. Distilled water was used as a control. In order to spraying of L-phenylalanine, its powder was dissolved in 5% acetic acid and desired concentrations were obtained with distilled water (Gonzalez-Arenzana *et al*., 2017). The foliar application of L-phenylalanine was done at three stages of growth, including late vegetative growth, early of flowering and 50% flowering. The L-phenylalanine solution was sprayed using an atomizer onto the leaves until it completely ran off.

2.4. Essential oil isolation

The aerial parts of lemon balm were harvested at full flowering stage (October 2018). The tissue samples were subsequently air-dried for five days in a shaded room at 30°C. The collected samples were ground to fine a powder and passed through a sieve (mesh 20) to remove large pieces of debris. The essential oil of each sample was extracted from 100 g of powdered tissue by hydro-distillation method. The hydrodistillation was also performed using the Clevenger-type (made by Glass Fabricating of Ashk-e-Shishe Co., Tehran, Iran) with 500 mL water for 3 h according to the European Pharmacopoeia and then the essential oil yields were measured (Pharmacopoeia, 1988). This process was performed in triplicate at three different times. The collected essential oil was dried over anhydrous sodium sulphate and stored at 4°C until analyzed.

2.5. Gas chromatography/mass spectrometry (GC/MS) analysis

Composition of the essential oils was determined by gas chromatography (GC) and mass spectrophotometry (GC/MS). The GC analysis was done on an Agilent Technologies 7890 GC (Agilent Technologies, Santa Clara, CA) equipped with a single injector and a flame ionization detector (FID) using a HP–5MS capillary column (30m×0.25 mm, 0.25 µm film thicknesses) coated with 5% phenyl, 95% methyl polysiloxane. The carrier gas was helium (99.999% pure) at a flow rate of 0.8 mL min−1. Initial column temperature was 60 °C and programmed to increase at 4 °C min−1 to 280 °C. The injector temperature was set at 300 °C. Split injection was conducted with a ratio split of 1:40. Essential oil samples of 1 µL were injected neat (directly). GC-MS analyses of aromatic oil samples were performed on an Agilent Technologies 7890 gas chromatograph coupled to Agilent 5975 C mass selective detector (MSD) and quadrupole EI mass analyzer (Agilent Technologies, Palo Alto, CA, USA). A HP–5MS 5% column (coated with methyl silicone) (30m×0.25 mm, 0.25 µm film thicknesses) was used as the stationary phase. Helium was used as the carrier gas at 0.8 mL min^{-1} flow rate. The temperature was programmed from 60 to 280 °C at 4 °C min−1 ramp rate. The injector and the GC-MS interface temperatures were maintained at 290 °C and 300 °C, respectively. Mass spectra were recorded at 70 eV. Mass range was from m/z 50–550. The ion source and the detector temperatures were maintained at 250 and 150°C, respectively. The samples (1 µL) were injected neat with 1:40 split ratio. 2.6. Identification of components Essential oil constituents were identified based on their retention indices (determined with reference to homologous series of C5eC24 nalkanes), by comparison of their mass spectra with those reported in the literature (Adams, 2007) and stored in NIST 08 (National Institute of Standards and Technology) and Willey (ChemStation data system) libraries. The percentage composition was computed from the GC peak areas without using any correction factors.

2.6. Statistical analysis

The data was statistically analyzed using one way analysis of varianceby the program SPSS. Treatment means were compared by leastsignificant differences (LSD) at P<0.05 level.

3. Results and discussion

3.1. Essential oil content

The essential oil of *M. officinalis* extracted was a clear, yellow liquid. The essential oil contents of studied treatments ranged between 0.11 to 0.19% (v/w), based on dry weight. Statistical analysis indicated that there were significant differences among treatments for essential oil contents of *M. officinalis* (Table 1). Results in this study indicated the foliar spray L-phenylalanine increased quantity yield of the essential oil from *M. officinalis* (Fig. 2), although content of essential oil decreased by increasing amino acids concentrations (Fig. 2). The highest essential oil yields were observed from the foliar spray of L-phenylalanine (1000 mg L) and their difference with control were statistically significant. In this respect several investigators studied the effect of different amino acids on the total oil percentage and oil yield (ml / plant). Gamal El. Din *et al.*, (1997) on *Cymbopogon citratus* hort, Karima *et al.*, (2005) on *Matricaria chamomilla* L. and Mona and Talaat (2005) on *Pelargonum graveolens* L. Talaat *et al.* (2002) and Omer *et al.* (2013), mentioned that foliar application of amino acids significantly increased essential oil percentage and yield. Similarity, EL-Zefzafy *et al.* (2016) showed that phenylalanine spraying on *Artemisia abrotanum* aerial parts significantly increased the essential oil content and yield by increasing the concentration of phenylalanine (EL-Zefzafy *et al.*, 2016). Overall, L-phenylalanine spraying increased essential oil content, possibly it acted as a potent inducer for increasing the biosynthesis of secondary metabolites.

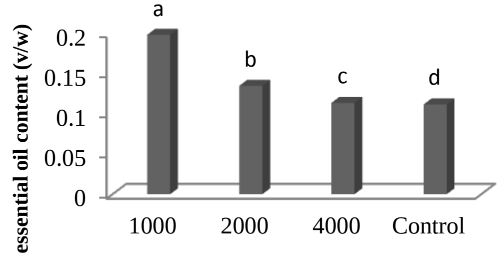

Fig. 1. Effects different concentrations of L-phenylalanine on yield of essential oil of *M. officinalis* [Different letters indicate a significant difference according to LSD test (P≤0.01)].

3.2. Chemical compositions of the essential oil

The GC-FID and GC/MS analysis of essential oils indicated some major constituents, such as, Neral, Z-Citral, E-Citral, Geranial, Citronellal and β-caryophyllene. Results of other investigations on GC/MS analysis of the essential oil from cultivated *M. officinalis* in Iran also indicated that the main constituents in the essential oil are neral, geranial, geranyl acetate, citronellal, caryophyllene oxide and β-caryophyllene and (Sadraei *et al.*, 2003; Asgari and Sefidkon, 2004; Sharafzadeh *et al.*, 2011; Taherpour *et al.*, 2012). Mimica-Dukic *et al.* (2004) reported that the main constituents in *M. officinalis* essential oil were neral, geranial, citronellal, isomenthone, menthone and β-caryophyllene. A comparison of our results with the previous reports suggests few differences in the volatile composition of the plant could be attributed to the geographic origin of the plant sample, methods of extraction and agronomic practices (Ghasemi Pirbalouti *et al.*, 2017; Torki-Harchegani *et al.*, 2018; Moghaddam *et al.*, 2018). The essential oil content and chemical compositions are known to be affected by genetic (ecotype, chemotype, cultivar etc.), agronomic managements, ecological and environmental factors.

The result of analysis of variance of this investigation indicated that different levels of the foliar application of L-phenylalanine have significant impacts on main constitutes in the essential oil from the *M. officinalis* (Fig. 2). The highest content of Neral was obtained in the treatment of 1000 mg/L L-phenylalanine (36.36), While, the lowest content of it was showed in the treatment of 4000 mg/L L-phenylalanine (23.45 %)(Fig. 2A). The maximum content of Z-Citral was caused by application of the L-phenylalanine treatment at 4000 mg/L (1.43%), but the minimum content of it was achieved when the L-phenylalanine treatment was applied at 2000 mg/L (0.96%) (Fig. 2B). The greatest and lowest content of E-Citral was detected at control and L-phenylalanine at 4000 mg/L, respectively (3.63%, 1.15%) (Fig. 2C).

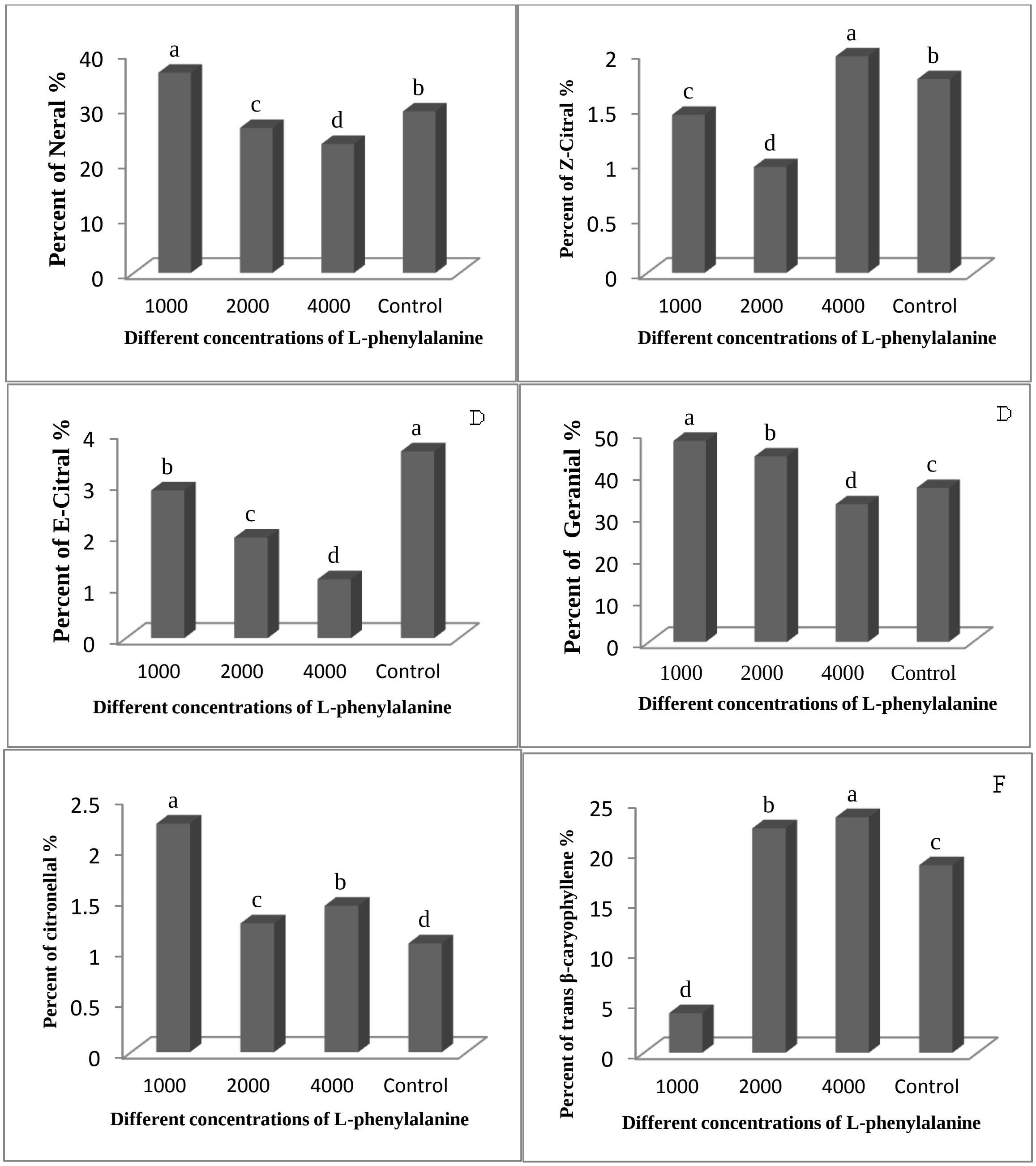

Fig. 2. Effects of different concentrations L-phenylalanine on percent of Neral (A), Z-Citral (B), E-Citral (C), Geranial (D), Citronellal (E) and β-caryophyllene (F) of *M. officinalis*. Different letters indicate a significant difference according to LSD test (P ≤0.01).

The highest content of Geranial was related to application of L-phenylalanine at 4000 mg/L (47.96%) and the lowest content of it was recorded in the treatment of L-phenylalanine at 2000 mg/L

(32.85%)(Fig. 2D). The highest content of citronellal was found in the concentration 1000 mg/L of L-phenylalanine (2.25%) and the lowest was found in control (1.07%) (Fig. 2E). The maximum content of β-caryophyllene was obtained with the application of L-phenylalanine at 4000 mg/L (23.44%) and the lowest content of it was acquired at 1000 mg/L L-phenylalanine (3.93%) (Fig. 2F). This research reported that the quantity and quality of *M. officinalis* essential oil was changed by application of the plant biostimulants based on amino acids. Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors and other industrial materials. There are several commercially available chemical compounds that could be used as elicitors to modify plant secondary metabolites and subsequently the bioactivity of medicinal plants. According to several studies, the foliar application of amino acids have important role in the biosynthesis of secondary metabolite. Various physiological and biochemical effects of elicitors, including L- phenylalanine on plant systems have been documented. Accumulation of metabolites often occurs in plants subjected various elicitors or signal molecules. Amino acids can play wide roles in plants including acting as regulatory and signaling molecules (Rai, 2002). L-phenylalanine as amino acid is important component in the signal transduction pathway metabolic. In this study, the exogenous applications of L-phenylalanine improved concentrations of neral, geranial and citronellal in the essential oils. Similar to our results, Ardebili *et al.* (2012) indicated that foliar application of amino acid, at suitable concentrations, had positive effects on the content of secondary metabolites.

Amino acids such as phenylalanine function in the synthesis of terpenoids and volatile compounds, etc. It is clear that more than one biosynthetic route is involved in the conversion of phenylalanine into plant volatiles (Gonda *et al.,* 2018; Vogt, 2010).These biostimulants may through improvement of plant nutrients uptake, induction of phytohormones biosynthesis, biotic and abiotic stress reduction and enhancing of enzymes activity related to tricarboxylic acid cycle (TCA cycle) and nitrogen metabolism caused increassing essential oil components of the plants (Calvo *et al.,* 2014).

4. Conclusion

The present study is the first report of essential oil responses of *M. officinalis* to L-phenylalanine spraying. The major compounds in the essential oils from the leaves of *M. officinalis* were Neral, Z-Citral, E-Citral, Geranial, Citronellal and β-caryophyllene. In general, results of this study indicated that the quantitative and qualitative of essential oil of *M. officinalis* were influenced by the foliar application of L-phenylalanine. Our results are in accordance with many investigators who reported that the application of amino acids increases the quantity and quality of essential oil, by incrasing the yield percentage of oil and increasing the concentration of major compounds in the oil. Positive effects of the application of L-phenylalanine can be attributed to their potentials in the supply of nutrients. Therefore, it can be concluded that with using biological stimulators such as L-phenylalanine, the ability of produce medicinal metabolites can be increased and in the direction of sustainable agriculture an effective step was taken.

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