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Foliar application of copper and manganese on essential oils and morpho-physiological traits of Lemon Balm (*Melissa officinalis* L.)

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Abstract:

Lemon balm (*Melissa officinalis* L.), a member of Lamiaceae family, is an important medicinal plant that has many useful properties. Micronutrients are necessary in low dose for the growth and the development of plants. Present research was conducted to study the effects of micronutrients including manganese (Mn) and copper (Cu) on morpho-physiological traits and essential oil of lemon balm. Field trials were carried out in 2015 at Shahrekord (50°56′ E 32°18′ N) South Western Iran. Experiments were arranged in a randomized complete block design with a factorial layout and three replications. Micronutrients concentrations (Cu and Mn in 0, 150 and 300 ppm) were employed form time of planting to beginning of flowering of plants. Of the two micro nutrients, copper was more effective in stimulating the accumulation of Caryophyllene β, Citronellal, Geranial, Geraniol, Geranyl Acetate, Linalool and Neral. At 150 ppm, micro nutrients enhanced the production of citronellal, Chavicol. Although combination of $Cu^{2+}$and Mn^{2+} at 300 ppm in some of essential oils like neral, e-caryophyllene, caryophyllene oxide and 14-hydroxy-Z-caryophyllene were more produced than 150 ppm combinations most of essential oils significantly increased in 150 ppm concentration of micronutrients. Trans-piperitone epoxide was upper extracted in 300 ppm concentration of Mn^{2+} but in much combination this essential oil was not extracted in little concentration. Geranial, Geraniol, (E-) Caryophyllene, Caryophyllene oxide and Neral were the main components in all treatments.

Keywords: Medicinal plant, Micronutrients, Phytochemical.

Introduction:

Lemon balm (*Melissa officinalis* L.) is a tetraploid (2n=2x=32) and perennial plant, member of Lamiaceae family (Sekeroglu *et al.,* 2006, Bagdat 2006, Moradkhani *et al*., 2010, Abuhamdah and Chazot 2008). Also, its extraction have many medicinal uses (Farahani *et al*., 2009). Lemon balm is one of the most important medicinal and aromatic plants, with antioxidant, antimicrobial, spasmolytic, astringent and specific sensorial properties. Essential oil of the plant, is mainly made up of caryophyllene β, citronellal, geraniol, geranyl acetate, linalool, neral and ursolic acid, and is responsible for some of these effects (Bagdat and Cosge, 2006).

Use of micronutrients improves the quality and quantity crops and (Amjad *et al.,* 2014). These elements included of copper (Cu), manganese (Mn). Small amounts of Cu and Mn are essential for growth and quality of the crop because these micro nutrients also control most of the physiological activities of the crop by interrupting the level of chlorophyll content in leaves which ultimately influence the photosynthetic activity of the plant (Marschner, 1995). In micronutrients, Copper is an essential microelement in higher plants as it occurs as part of the prosthetic groups of several enzymes. It was shown to be associated with proteins or nuclear contaminants. Manganese is involved in many biochemical functions, primarily acting as an activator of enzymes such as dehydrogenases and decarboxylases involved in respiration, amino acid and lignin synthesis, and hormone concentrations (Schönherr et al., 2005). In alkaline soils, nutrient concentration may be not enough and therefore micro nutrient in this soil immobilized quickly and roots of plants can't absorb some of nutrients and no transition to leaves occurs, in these cases, application of micro nutrient may solve this problem (Dadhich and Somani, 2007). Few studies examining micronutrients fertilizers in essential oil components on lemon balm have been conducted. Present study was carried out to evaluate the some morpho-physiological characters and essential oils response of lemon balm (*Melissa officinalis* L.) to foliar application of Mn and Cu.

Material and Methods:

Seeds of Lemon balm were obtained from Iranian Seeds and Plant Improvement Institute. Seeds were planted in field condition. Table 1 shows physicochemical properties of the soil. Treatments were micronutrient Cu and Mn levels by three replications. Amounts of

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Table 1- Some physical and chemical properties of soil for experiment (0 -30) cm.

Texture	E.C	N_{total}	O.C	pH	K	P	Zn	Mn	Fe	Cu
	(ds.m^{-1})	%			mg.kg^{-1}					
Loam	8.1	0.11	0.2	8.7	770	45	0.57	1.2	4.1	1.3

essential oil components production as well as the chemical component of the essence were determined. Field trials were established in 2015 at Shahrekord (50°56′ E 32°18′ N) South Western Iran. Experiment was arranged in a randomized complete block design with a factorial layout and three replications. Topsoil of the experimental plot area was kept moist throughout the growing season when necessary. After soil test, the required nutrients were added to soil. In the 3-5 leaves phase, plants were thin out to final row distance (20 cm). During the study, usual practices were conducted to achieve the best products. At the end of the blooming stage, shoots of plants were harvested. Several parameters including leaf dry weight, leaf fresh weight, root dry weight, root fresh weight, stem length, root length, number of stem were measured.

Lemon balm shoots were washed out with distillated water, and then were dried 3 days at 40°C in oven. Micronutrients concentrations (Cu and Mn in 0, 150 and 300 ppm) were employed from the time of planting to beginning of flowering of plants.

The essential oils were analyzed by gas chromatography-mass spectrometry (GC/MS). Thermo Finnegan Trace 2000 GC/MS, made in the USA, was employed with a HP-5MS capillary column (30 m long and 0.25 mm wide, and a 0.25 µm of film thickness) at a 250°C of injector chamber. The initial column temperature was at 120°C for 5 min then raised to 280°C at the rate of 10°C/min. Helium was used as a carrier gas at a rate of 35 ml/min. MS parameters were as follows: ionization energy, 70eV; ion source temperature, 200°C; voltage, 3000 v; and mass range, 30 to 600. The compositions of the essential oil were identified by comparison of their retention indexes, retention times and mass spectra with those of authentic samples in Wiley library (Adams, 2001).

All data were subjected to ANOVA using the statistical computer package SAS ver.9 and treatment means separated using L.S.D multiple range test at $P<0.05$ level.

Results:

Results of analysis of variance (table 2) showed that: 1- Foliar application of manganese and copper had significant effects on all the studied traits except leaf dry weight. 2- In all of essential oils, amount of Caryophyllene oxide, Neral, Chavicol , Geraniol, Geranial, E-Caryophyllene and Caryophyllene oxide were most affected by micronutrients (table 2). The mean comparisons with LSD test at 0.05 were performed for micronutrient effects for all the studied traits and its results are shown in table 3. Foliar application of micronutrients effectively increased the studied traits companed to the control. There were similar results about effective application of micronutrients on leaf fresh weight, root dry weight, root fresh weight, stem length, root length and number of stem.

The most of essential oils were Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral that were affected by the treatments (tables 2, 3). Also, the Cu^{2+} application affected the caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral percentage. Similarly, the Mn^{2+} application increased the Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral of percentage by 34%. The foliar Cu^{2+} and Mn^{2+} affected the Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral of concentration an average of 55% and 150 ppm application of micronutrients compared with the control (table 3).

$Cu^{2+}$and Mn^{2+} applications affected geranial, geraniol and geranyl acetate of stems and increased with the micronutrients applications by an average of 22% during the research seasons compared with the control (table 3). There was statistical significant differences among the three rates of $Cu^{2+}$and Mn^{2+} applications (table 2). The amount of geranial, geraniol and geranyl acetate per plant increased after fertilizing compared with the control by an average of (52%), (>100%) and (71%) (table 3). Linalool and neral concentrations were affected by $Cu^{2+}$and Mn^{2+} applications and increased an average of (21%) and (12%) compared with the control (table 3). In general, the most of Caryophyllene oxide (12.62%), E-Caryophyllene (7.54%), Geranial (32.49%), Chavicol (12.65%) and Neral (27.83%) were made by treatments that had 150ppm of Cu. In most of other subsidiary components contain Linalool, exo-Isocitral, Citronellal, Z-Isoicitral, Rosefuran epoxide, E-Isoicitral, Piperitone, Piperitenone oxide, E-Caryophyllene, alpha-trans-Bergamotene, alpha-Humulene, Germacrene D, E-beta- Ionone, gamma-Cadinene, Caryophyllene oxide, Humulene epoxide II, epi-alpha-Cadinol, 14-hydroxy-Z-Caryophyllene, Hexadecanoic acid and Abieta-8(14),13(15-diene); the treatments that had 150ppm of Cu made most of components and in these treatments the treatment of Cu150 ppm with Mn150 ppm was the best (table 3).

Discussion:

In the present study, the effects of micronutrients applications were determined on the essential oils of Lemon balm. According to our knowledge, this is the first report that shows that $Cu^{2+}$and Mn^{2+} can affect the geranial and neral of Lemon balm and the same response can probably be found in other essential oils that have adapted to local soils or have high requirements for $Cu^{2+}$and Mn^{2+}. In the soil application,

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Table 2-Analysis of variation of Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol, Neral, Number of stem, Root length, Stem length, Root dry weight, Root Fresh weight, Leaf Fresh weight, Leaf dry weight in *Lemon balm* L.

Source of Variation	Degree freedom	Mean of Squares						
		Number of stem	Root length	Stem length	Root Fresh weight	Root dry weight	Leaf Fresh weight	Leaf dry weight
R	2	92345.4	82.4	10.5	745	0.0005	o.ooo1	0.005
Cu	2	3453231.2^{**}	25.9^{ns}	441.1^{**}	685.8^{ns}	0.001^{ns}	0.008^{**}	0.006^{ns}
Mn	2	679250.7^{**}	78^{ns}	172^{**}	1767^{ns}	0.001^{*}	0.0007^{ns}	0.008^{ns}
Cu×Mn	4	356946.3^{*}	209.2^{**}	80.1^{**}	3359.4^{*}	0.009^{*}	0.001^{**}	0.008^{ns}
Error	18	87788.9	45.4	5.4	598.2	0.0001	0.00003	0.007
Total	28	-	-	-	-	-	-	-
CV		10.8	11.9	14.4	15	12.2	14.8	12.1

ns,* and **: Non significant, significant at the 5% and 1% levels of probability, respectively.

Table 2- contuned-

Source of Variation	Degree freedom	Mean of Squares					
		Caryophyllene oxide	E-Caryophyllene	Geranial	Geraniol	Chavicol	Neral
R	2	0.1	0.03	0.005	0.000005	0.000005	0.000005
Cu	2	4.1^{**}	0.02^{ns}	0.004^{ns}	0.00001^{**}	0.00041^{**}	0.00031^{**}
Mn	2	0.05^{ns}	0.05^{*}	0.009^{ns}	0.00007^{**}	0.00057^{**}	0.00055^{**}
Cu×Mn	4	0.23^{**}	0.06^{**}	0.016^{**}	0.00007^{**}	0.00047^{**}	0.00088^{**}
Error	18	0.05	0.01	0.004	0.00003	0.00023	0.0002
Total	28	-	-	-	-	-	-
CV		9.9	6.5	8.5	4.3	4.3	4.3

ns,* and **: Non significant, significant at the 5% and 1% levels of probability, respectively.

Table 3- Means of essential oil percentage measured in lemon balm plants that are affected by micronutrients.

Treatments	Neral	Chavicol	Piperitone	Methyl citronellate	Geraniol	trans-Piperitone epoxide	Geranial	Methyl nerolate
Control	24.09^{b}	-	0.57^{b}	-	1.31^{b}	-	28.9^{b}	-
Cu0×Mn150	19.05^{bc}	7.33^{b}	0.64^{a}	0.46^{a}	1.44^{b}	-	20.81^{c}	5.32^{a}
Cu0×Mn300	14.14^{c}	12.28^{a}	0.56^{b}	0.4^{ab}	12.75^{a}	1.37^{a}	18.58^{c}	-
Cu150×Mn0	27.83^{a}	-	0.6^{ab}	0.27^{bc}	1.2^{b}	-	32.36^{a}	-
Cu150×Mn150	14.57^{c}	12.65^{a}	0.38^{c}	0.32^{b}	0.7^{c}	-	32.49^{a}	-
Cu150×Mn300	17.46^{c}	6.16^{b}	0.47^{c}	0.27^{bc}	1.05^{b}	-	27^{b}	-
Cu300×Mn0	26.25^{a}	-	0.7^{a}	-	1.15^{b}	-	31.39^{a}	-
Cu300×Mn150	24.61^{b}	-	0.39^{c}	-	0.81^{c}	-	19.19^{c}	3.87^{b}
Cu300×Mn300	25.59^{ab}	-	0.47^{c}	0.23^{c}	0.77^{c}	-	30.82^{ab}	-

Means within each column followed by the same latter are not significantly different (α=0.05).

Table 3-contuned-

Treatments	6-methyl-5-Hepten-2-one	Linalool	exo-Isocitral	Citronellal	Z-Isoicitral	Rosefuran epoxide	E-Isoicitral	Methyl chavicol
Control	1.43^{b}	0.45^{bc}	0.22^{b}	-	$1.15b^{c}$	0.29^{c}	1.73^{b}	0.73^{d}
Cu0×Mn150	0.42^{e}	0.44^{bc}	0.22^{b}	0.37^{c}	1.32^{b}	-	1.95^{a}	-
Cu0×Mn300	1.36^{bc}	0.49^{ab}	0.22^{b}	0.4^{c}	1.27^{b}	0.5^{a}	1.8^{ab}	0.58^{d}
Cu150×Mn0	1.06^{d}	0.44^{b}	-	0.42^{bc}	1.16^{bc}	0.39^{b}	1.65^{bc}	-
Cu150×Mn150	1.26^{c}	0.47^{b}	0.28^{a}	0.5^{ab}	1.59^{a}	0.53^{a}	2.03^{a}	2.82^{b}
Cu150×Mn300	1.31^{c}	0.56^{a}	-	0.41^{c}	1.08^{c}	0.39^{b}	1.56^{bc}	2.01^{b}
Cu300×Mn0	1.19^{cd}	0.39^{c}	0.21^{b}	0.35^{c}	1.1^{c}	0.4^{b}	1.54^{c}	4.1^{a}
Cu300×Mn150	1.72^{a}	0.25^{d}	0.22^{b}	0.56^{a}	1.28^{b}	0.45^{ab}	1.89^{ab}	4.31^{a}
Cu300×Mn300	1.26^{c}	0.48^{b}	0.23^{b}	0.45^{b}	1.18^{bc}	0.46^{ab}	1.69^{b}	1.15^{c}

Means within each column followed by the same latter are not significantly different (α=0.05).

the nutrients can be bound and therefore cannot be available for the plants. Also, since most nutrients are taken up by water, their uptake is restricted during water stress. The critical level of micronutrients were not determined for Lemon balm plants. The significant effect that $Cu^{2+}$and Mn^{2+} have on essence indicates that micronutrients play an important role in the yield of Lemon balm. Lemon balm needs $Cu^{2+}$and Mn^{2+} and it is possible that this requirement increases during flowering, as the flowering process and the reproductive tissue have higher requirements for $Cu^{2+}$and Mn^{2+} foliar applications can have a direct effect on yield. In this study, the response to micronutrients applications can be higher. This is possible since the plants grew better with

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Table 3-contuned-

Treatments	Neryl formate	Methyl geranate	Piperitenone oxide	Geranyl acetate	E-Caryophyllene	alpha-trans-Bergamotene	alpha-Humulene	Germacrene D
Control	-	0.47^{b}	-	4.9^{c}	7.54^{a}	-	0.52^{bc}	-
Cu0×Mn150	6^{a}	0.72^{a}	-	7.29^{a}	7.17^{a}	-	0.47^{c}	-
Cu0×Mn300	-	0.49^{b}	-	5.44^{b}	7.37^{a}	-	-	-
Cu150×Mn0	-	-	-	4.85^{c}	5.91^{b}	-	0.39^{c}	-
Cu150×Mn150	-	0.46^{b}	4.88^{a}	-	6.37^{ab}	0.5^{b}	1.59^{a}	0.93^{a}
Cu150×Mn300	-	-	-	5.09^{bc}	7.38^{a}	1.12^{a}	0.71^{b}	0.35^{b}
Cu300×Mn0	-	0.46^{b}	-	5.31^{b}	7.1^{a}	0.4^{b}	1.29^{a}	0.57^{b}
Cu300×Mn150	5.3^{a}	0.51^{b}	1.11^{b}	4.1^{c}	6.09^{b}	0.28^{b}	0.88^{ab}	0.4^{b}
Cu300×Mn300	-	-	0.55^{c}	6.07^{ab}	7.35^{a}	-	0.55^{b}	-

Means within each column followed by the same latter are not significantly different (α=0.05).

Table 3-contuned-

Treatments	E-beta-Ionone	gamma-Cadinene	Caryophyllene oxide	Humulene epoxide II	epi-alpha-Cadinol	14-hydroxy-Z-Caryophyllene	Hexadecanoic acid	Abieta-8(14),13(15-diene)	Total %
Control	-	-	12.46^{a}	0.72^{a}	-	0.74^{a}	0.29^{a}	2.59^{a}	91.1
Cu0×Mn150	0.24^{b}	-	9.79^{bc}	0.49^{b}	-	0.6^{b}	-	1.75^{b}	94.3
Cu0×Mn300	0.24^{b}	-	11.29^{ab}	-	-	0.65^{ab}	-	2.19^{a}	94.36
Cu150×Mn0	0.22^{b}	-	10.98^{b}	0.56^{b}	-	0.61^{b}	-	2.11^{a}	93.01
Cu150×Mn150	0.3^{ab}	1.46^{a}	6.37^{d}	-	0.4^{b}	0.39^{c}	0.24^{a}	-	94.49
Cu150×Mn300	0.42^{a}	0.7^{b}	12.62^{a}	0.64^{ab}	1.35^{a}	0.64^{ab}	0.26^{a}	1.59^{b}	92.6
Cu300×Mn0	0.3^{ab}	1.12^{ab}	8.13^{c}	0.51^{b}	0.23^{b}	0.46^{c}	-	1.22^{b}	95.89
Cu300×Mn150	0.28^{b}	-	9.89^{bc}	0.54^{b}	0.29^{b}	0.71^{a}	-	1.34^{b}	91.24
Cu300×Mn300	0.23^{b}	-	11.48^{ab}	0.61^{ab}	-	0.64^{ab}	-	2.04^{ab}	94.3

Means within each column followed by the same latter are not significantly different (α=0.05).

the $Cu^{2+}$and Mn^{2+} application, developed a bigger root system, and took up more nutrients. $Cu^{2+}$and Mn^{2+} are immobile in plants and can't be transported to developing organs. Therefore, foliar application can provide $Cu^{2+}$and Mn^{2+} to the developing organs that need it the most and, in this case, the plants do not have to take these micronutrients from the soil solution. However, the actual amount of Cu^{2+} and Mn^{2+} that reaches the reproductive tissue can be small and depends on other factors such as $Cu^{2+}$and Mn^{2+} soil and plant levels, and water stress that can restrict micronutrients movement and this is possibly one reason for finding a significant increase in the yield because of the micronutrients application. Micronutrients application affected chavicol as the plants in the control treatment and increased with micronutrients applications which were not detected in control plants. When the $Cu^{2+}$and Mn^{2+} level is too low to sustain plant growth, the plants become shorter and the total biomass is lower compared with the plants with sufficient micronutrient (Mengel *et al.*, 2001 and Habib, 2012). This effect can be explained as micronutrients affecting the carbohydrate transport, and this can affect the yield components in many plants (Marschner, 1995). Since micronutrients affect the meristems, the increase in the availability of $Cu^{2+}$and Mn^{2+} can increase the number of stems per plant as they grow better (Anwar *et al.*, 2005). In similar researches, the effect of farm yard manure reported significant on neral, (28.43%), geranial (39.86%) and geranyl acetate (8.67%) compared to other treatments (Harshavardhan *et al.*, 2007). $Cu^{2+}$and Mn^{2+} applications affected on chavicol percentage of the crop. Also Abazarian *et al.*, (2011); Yadegari *et al.*, (2012, 2015), Sinta *et al.* (2015) and Abu-Darwish (2009) showed usefulness of micronutrients on yield of plants.

Sinta *et al.* (2015) in the study on Coriander, Gomaa (2001) on amaryllis, Al-Humaid (2004) on Fennel, Youssef *et al.,* (2004) on *Ocimum sanctum,* Al-Ahl (2005) on Dill, Hanan Ali (2006) on Thyme, Samia and Mahmoud (2009) on *Tritonia crocata*, Nasiri *et al.,* (2010) on Chamomile, Khalifa *et al.*, (2009) on Iris, Heidari *et al.*, (2011) on sesame, Galavi *et al.*, (2012) on safflower, Yarnia *et al.*, (2012) on *Purple coneflower* and Younis *et al.*, (2013) on *Rosa hybrida* they found that spraying micronutrients increased essential oils and micronutrients in plants. Patora *et al.*, (2003) on lemon balm, Hanan Ali (2006) on *Thymus vulgaris,* Al-Ahl (2005) on Dill and Al-Humaid (2004) on Fennel showed beneficial application from micronutrients. The essential oil yield increased with Cu^{2+} and Mn^{2+} applications because there was a significant increase in dry matter yield. In researches of Al-Ahl (2005) on Dill, increasing on dry matter yield, essential oil content, and essential oil yield by use of micronutrients was achieved. However, more research is needed to explore these tools for Lemon balm breeding and for better Lemon balm management. There are still many unanswered questions about how $Cu^{2+}$and Mn^{2+} act in increasing essential oils for Lemon balm. One possibility is that the foliar applied micronutrients can affect dry matter accumulation and increase dry matter yield. Micronutrients can increase the number of stems per plant and slowdown leaf senescence (El-Shahawy et al., 2008; Datta et al., 2011). The obtained results are in conformity with those of Mazaheri et al. (2013) on

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Cumin, Radmanesh *et al.* (2015) on Savory, Gomaa (2001) on *Antholyza aethiopica*, Sari and Ceylan (2002) on lemon balm, Yadav *et al.,* (2002) and Rawia *et al.*, (2010) on tuberose, Al-Humaid (2004) on Fennel, Hanan Ali (2006) on Thyme, Nahed and Laila (2007) on *Salvia farinacea*, Farahat et al., (2007) on *Cupressus sempervirens* and Samia and Mahmoud (2009) on *Tritonia crocata*. The main components of the oil were Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral for all populations. This indicates that foliar application of micronutrients has a significant effect on the composition of the essential oil. Many researchers have reported that the main components of lemon balm are Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral (Manukyan, 2011; Patora *et al.*, 2003; Sari and Ceylan, 2002). However, there were significant differences among the rates of those reported components. Neral and geranial rates in the oil were reported to be 15% and 14.5% respectively by Hefendehl (1970), 19.6-36.1% and 25.3-47.5% by Tittel *et al.*, (1982), 19.5% and 31.6% by Werker *et al.,* (1985), 7.195% and 12.99% by Ozay (1990), 10.9% and 17.3% by Lawrence (1983) and 30-40% and 50-60% by Ceylan *et al*. (1994). Although many studies discussed above reported that neral and geranial were the main components of the oil, Kirimer *et al*. (1995) found that the main component of the lemon balm oil they have found in some of studies, were citronellal, linalool and geranial as major chemical compositions of the essential oil of the lemon balm (Adinee *et al.*, 2008; Bagdat and Cosge, 2006; Mrlianova *et al*., 2001; Sorensen, 2000). All the significant differences for the components of the oil among the papers discussed above may be due to the use of different genetic material and/or different environmental conditions.

Conclusion:

This study showed that the applications of $Cu^{2+}$and Mn^{2+} had a significant effect on morpho-physiological characters and essential oils of Lemon balm such as trans-Piperitone epoxide percentage. The main components of the oil were Caryophyllene oxide, E-Caryophyllene, Geranial, Geraniol, Chavicol and Neral for all treatments. This indicates that foliar application of micronutrients has a significant effect on the composition of the essential oil. These results show that micronutrients applications can affect the growth and yield of Lemon balm, especially when it is grown in alkaline soils. This study provides some useful information about the effect of application of Cu^{2+} and Mn^{2+} on lemon balm production and in that way broadens our knowledge about the effect of micronutrients on crop production.

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