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Article

Optimizing Lemon Balm (*Melissa Officinalis* L.) Cultivation: Effects of Different Manures on Plant Growth and Essential Oil Production During Consecutive Harvests

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Abstract: This study examined the impact of organic manures from different sources (poultry, sheep, and cattle) on lemon balm (*Melissa officinalis* L., Lamiaceae) during different harvests. Manure application increased the photosynthetic pigments levels (chlorophyll-a, 9–41%; chlorophyll-b, 24–60%), biomass (41–60%), and essential oil yield (60–71%). Sheep manure treatment exhibited the highest antioxidant capacity among all the manures tested. Through GC-MS and GC-FID analysis, 10 chemical constituents were identified in the essential oil, accounting together for 91–95% of the total composition. The primary chemical component was geranial (39–46%), followed by neral (28–35%), (*E*)-caryophyllene (4.7–11%), geranyl acetate (2.7–5.9%), and caryophyllene oxide (1.7–4.8%). The utilization of livestock manures significantly improved the quality of the essential oil in terms of neral and geranial percentages compared to the control. Notably, during mid-August and early September, there was a substantial rise in these valuable compounds. However, a decrease in geranyl acetate and oxygenated monoterpenes resulted in a decline in the antioxidant capacity to 3%. Consequently, it is recommended to utilize essential oils from the second and third harvests for industrial purposes. Overall, the use of livestock manures, especially sheep manure, as a nutrient source for lemon balm cultivation proves to be a viable approach for producing high-quality essential oils.

Keywords: essential oil composition; biomass; photosynthesis; antioxidant capacity; lemon balm

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1. Introduction

Medicinal plant growers heavily rely on a variety of fertilizers to meet the demands of a rapidly growing global population [1]. Throughout history, manure has served as a crucial source of plant nutrition. Its ability to enhance and sustain soil productivity has been acknowledged since ancient times. The presence of fertilizer materials, humus, and other organic components makes manure a valuable tool for soil improvement [2]. Animal manure, including those deriving from poultry, sheep, and cattle, not only provide essential macro and micronutrients but also enhances soil properties, creating an optimal growth environment [3–5]. Consequently, this results in enhanced plant productivity while avoiding any negative impacts on human health and the environment. Whereas, inadequate handling of animal manure could potentially endanger human health and the environment.

In relation to medicinal plants, the application of organic manures becomes even more crucial as it not only enhances the growth and yield of plants but also improves the overall quality of the final products like essential oils [1,6,7]. Medicinal plants hold increasing importance in various industries worldwide, especially those with higher levels of secondary metabolites [8]. The quality of medicinal plants has garnered significant attention in research circles, with a particular focus on the effects of organic manures, which have been found to have a positive association with medicinal plant quality [6,9]. Various studies have demonstrated a boost in the yield of medicinal plants when organic fertilizers are applied [10–12]. However, it is highly recommended to use organic manures to enhance the quality and productivity of medicinal plants.

Melissa officinalis L., mostly known as lemon balm, is a fragrant herb belonging to the mint family (Lamiaceae). Throughout history, lemon balm has been utilized as a natural remedy to aid sleep, alleviate stress and anxiety, enhance digestion and appetite, and combat viral and other infections. In addition to its medicinal uses, lemon balm is frequently employed as a flavoring agent in food and beverages as well as in the production of health products and cosmetics [13]. Scientific research has revealed that lemon balm possesses sedative properties, acts as a muscle relaxant (antispasmodic), aids in digestion, relieves gas (carminative), exhibits antioxidant effects, and possesses antimicrobial activity [14]. The therapeutic effects of lemon balm can be ascribed to the existence of various chemical compounds, including flavonoids, tannins, phenolics like rosmarinic acid and caffeic acid, terpenes such as ursolic acid and oleanolic acid, and essential oils [15]. *M. officinalis* exhibits higher antioxidant activity and levels of total phenolic content compared to other Lamiaceae species, including *Salvia officinalis* L., *Lavandula angustifolia* L., and *Origanum vulgare* L. [16].

In their investigation, Sodré et al. [15] found that citral and citronellal are the primary constituents of the lemon balm essential oil (LEO) in organic and inorganic treatments. They observed that as the amount of cattle manure (CA) increased, the citronellal content in LEO obtained from dried leaves decreased. On the other hand, the content of geraniol showed a progressive increase with higher doses of cattle manure compared to the control treatment. According to de Assis et al. [17], the application of 900 kg ha^{-1} of cattle manure on lemon balm led to a remarkable improvement in plant height (17%), root biomass (237%), and shoot biomass (128%) in comparison to the control. Within natural antioxidants, citral plays a pivotal role. It effectively hampers the oxidation of linoleic acid and acts as a safeguard for IEC-6 cells, shielding them from the oxidative stress induced by aspirin [18]. The cultivation of plants rich in citral has become increasingly prevalent in recent times and citral, as a monoterpene, is capturing the attention of people globally [19].

In Iran, lemon balm is a perennial plant that is planted in the Spring and it has the capacity to produce more than one harvest annually. However, there is limited information available on the effects of different types of organic manures on the productivity and quality of lemon balm, especially during consecutive harvests. We hypothesize that (i) the use of manures can augment nutrient availability for lemon balm; (ii) the absorption of nutritional elements, along with the production of essential oil and its various compounds, fluctuates during different harvesting periods; additionally, (iii) an enhancement in the quality of essential oil is a beneficial sign for the preservation of plant productivity in agroecosystems. Based on these hypotheses, this study aims to provide the initial findings regarding the reaction of lemon balm to different manure sources across three harvests. The objective was to evaluate the impact of organic manures on biomass, nutrient content, yield of LEO, chemical compositions, and antioxidant activity in consecutive harvests.

2. Materials and Methods

2.1. Description of Study Site

This field experiment took place in Koohrang (50°16′ E; 32°29′ N), Chaharmahal, and Bakhtiari Province, Iran, in 2019, from April to October. The site is situated at an altitude of 2234 m above sea level. The study site exhibits a cold semiarid climate, known for its moderate winters and comparatively humid and hot summers. Table A1 shows the average temperature and monthly total precipitation for each month of the lemon balm's growing season.

2.2. Field Experiment and Cultivation Management

This research was carried out as a factorial experiment utilizing a randomized complete block design (RCBD) with 3 replications (Table 1). The initial factor consisted of four nutrient sources: poultry manure (PO) (3200 kg ha^{-1}), sheep manure (SH) (5400 kg ha^{-1}), cattle manure (CA) (6200 kg ha^{-1}), and control (CO) (without manure). The second factor was harvest, specifically HAR1, HAR2, and HAR3 (June 21, August 14, and September 6, 2019, respectively). To estimate the quantity of animal manure, the provision of 100 kg N ha^{-1} (equivalent to chemical fertilizer) throughout the lemon balm's growing season was considered. It was postulated that approximately 50% of the total organic nitrogen present in the livestock manures would be released through mineralization, as suggested by Fallah et al. [6] and Alizadeh et al. [20]. Matching the nitrogen release with the needs of the plant contributes to its superior efficiency compared to chemical fertilizer [20].

Table 1. Animal manures and harvest treatments tested on lemon balm in 2019.

Manure Treatment	**Harvest Treatment**		
	HAR1	**HAR2**	**HAR3**
Poultry (PO)	June 21	August 14	September 6
Sheep (SH)	June 21	August 14	September 6
Cattle (CA)	June 21	August 14	September 6
Control (CO)	June 21	August 14	September 6

On 23 April 2018, lemon balm transplants were planted into the experimental plots. The transplants selected for this research were derived from seeds sourced from the local genotype of lemon balm, which were nurtured in a greenhouse environment for a period of 6 weeks. By the end of the nursery stage, the transplants reached a height of 10 cm. On 24 April 2019, organic fertilizers were applied in close proximity to the root of the plants. Drip tape irrigation was used immediately after fertilization and manual weed control was performed multiple times throughout the growth season. Herbicides, fungicides, pesticides, and micronutrients were not applied in any of the treatments. The size of every plot was carefully determined to be 9 m^2, with measurements of 3 m by 3 m. The spacing between and within rows was consistently maintained at 50 cm. The plots were arranged with a distance of 1 m between them while the replications were spaced 2 m apart. Soil samples were taken randomly at a depth of 0–30 cm to assess the soil properties before seedling transplantation. Table 2 displays the characteristics of the soil and animal manure utilized. The soil in the area possessed a clay texture with a pH of 7.75, an electrical conductivity of 760 µS cm^{-1}, and an organic carbon content of up to 7.6 g kg^{-1}. There were no restrictions on the salinity levels of the animal manure employed. Poultry manure contained twice the amount of nitrogen compared to cattle and sheep manures, while sheep manure had a higher potassium content than cattle and poultry manures.

Table 2. Some chemical properties of the soil and livestock manures used in the experiment.

Parameter	**Unit**	**Soil**	**PO**	**SH**	**CA**
pH		7.8	6.7	7.9	7.9
EC	µS cm^{-1}	760	4750	4380	1980
OC	g kg^{-1}	7.6	312	175	195
Nitrogen	%	0.08	4.5	2.6	2.3
Phosphorus	%	8×10^{-4}	1.7	0.59	0.56
Potassium	%	328×10^{-4}	9.8	12.5	6.2
Iron	mg kg^{-1}	3.6	1475	3812	1718
Zinc	mg kg^{-1}	0.70	425	120	206
Copper	mg kg^{-1}	0.90	117	28.1	51.7
Manganese	mg kg^{-1}	8.1	493	331	220

EC: electrical conductivity; OC: organic carbon; PO: poultry manure; SH: sheep manure; CA: cattle manure.

2.3. Measurements

2.3.1. Chlorophyll

Simultaneously with each harvest, the quantification of the photosynthetic pigments was conducted using the method described by Rajput and Patil [21]. In summary, 1 g of three pairs of young leaves developed was crushed and blended in 10 mL of 80% acetone. The resulting mixture was then centrifuged at a speed of 5000 revolutions per minute for a duration of 5 min. The liquid portion, known as the supernatant, was carefully collected for further analysis. To determine the concentration of chlorophyll-a (Cha) and chlorophyll-b (Chb), the absorbance of the supernatant was measured at two specific wavelengths, namely 645 nm and 663 nm using a UV-Vis spectrophotometer (AE-UV 1606). These measurements were then used in the following equations to determine the levels of photosynthetic pigments.

$$\mathrm{Cha}(mg/g\ FW) = 12.7\times\mathrm{OD}_{663} - 2.69\times\mathrm{OD}_{645}\times\frac{V}{1000}\times\mathrm{W} \tag{1}$$

$$\mathrm{Chb}(mg/g\ FW) = 22.9\times\mathrm{OD}_{645} - 4.68\times\mathrm{OD}_{663}\times\frac{V}{1000}\times\mathrm{W} \tag{2}$$

The variables OD, V, and W represent the absorbance values obtained at particular wavelengths, the total volume of chlorophyll extracted using 80% acetone, and the fresh weight of the extracted tissue, respectively.

2.3.2. Macro- and Micronutrient Concentration

To determine the nutrient concentration at different harvests, samples (lemon aerial parts) were collected by randomly selecting plants (simultaneously with each harvest) from each plot to ensure a representative sample. The samples were then washed with distilled water to eliminate any surface contaminants, air-dried, and finely ground to a uniform powder. The ground samples were digested using the Kjeldahl method [22] to determine the N concentration. According to Ostadi et al. [23] and Jackson [24], the potassium and phosphorus concentration was determined utilizing the flame photometer (Fater Electronic Model 620G, Fater Electronic, Iran) and spectrophotometric method using a UV-Vis spectrophotometer (Model AE-UV 1606, A & E Laboratory Instruments Co., China). The concentrations of copper, manganese, zinc, and iron were determined using an atomic absorption spectrometer following the method outlined by Asadi et al. [25].

2.3.3. Air-Dried Biomass and Essential Oil (LEO) Isolation

The shoots (stem, leaf, and flower) of lemon balm plants were harvested at the initial flowering stage on June 21 (HAR1), August 14 (HAR2), and September 6 (HAR3). Harvesting was carried out manually by using a sickle to cut all the plants in each plot from a height of 5 cm above the soil surface. The plants were dried in a shaded environment post-cutting to ensure the moisture content reached a stabilized level of 8%. Subsequently, the aboveground air-dried herbal product was quantified in kilograms per hectare.

The LEO was isolated using the hydro-distillation method. A total of 50 g of crushed shoot samples were combined with 500 mL of distilled water and it then underwent hydro-distillation for 3.5 h utilizing a Clevenger apparatus following the recommendations of the British Pharmacopoeia [26]. The LEO that was gathered underwent dehydration with anhydrous sodium sulfate and was subsequently placed in opaque vials at a temperature of 4 °C for the purpose of chemical composition analysis. The calculation of the LEO yield involved the multiplication of air-dried shoot biomass (kg ha^{-1}) with the LEO content (g kg^{-1}) and expressed as kg ha^{-1}.

2.3.4. Qualitative and Semiquantitative Analysis of LEO Samples

Utilizing a Thermo-UFM gas chromatography system, gas chromatography (GC) analysis was carried out with a flame ionization detector (FID) and a Ph-5 column

(10 m × 0.10 mm i.d., film thickness 0.25 µm). The LEO samples from each treatment were introduced into the GC/FID system. Helium served as the carrier gas, flowing at a rate of 0.5 mL min^{-1}. The oven temperature was varied from 60 to 285 °C while both the injector and FID detector were held at a constant temperature of 280 °C.

Gas chromatography-mass spectrometry analysis was carried out employing an Agilent 7890A/5975C GC-MS system with a DB-5 fused silica column. The LEO samples from different treatments were injected into the GC/FID system for quantification of the compounds while one of the samples was injected into GC/MS for identification of the compositions. The oven temperature and other parameters were set according to the established protocol. Identification of the chemical constituents in LEO samples involved matching their mass spectra with those stored in a computer library or obtained from authentic components. Subsequently, retention indices (RI) were determined by comparing the retention times of *n*-alkanes (C_8–C_{24}) injected under identical conditions. The confirmation of chemical compositions was achieved through the use of relative retention indices and references from reputable literature sources [27–29]. The area normalization method was applied to ascertain the percentage content of the compounds present in the essential oil, without any corrections made [30].

2.3.5. Antioxidant Capacity

The assessment of antioxidant capacity was carried out by combining 20 µL of LEO from each treatment with 100 µL of 0.5 mM 2,2-diphenyl-1-picrylhydrazil (DPPH solution) in methanol. The resulting mixture was then adjusted to a final volume of 200 µL through microdilution. Afterward, the mixture was vigorously shaken and placed in a dark environment at room temperature for 15 min. The absorbance of the solutions, including a blank (without sample) and various concentrations, was measured using an Awareness Technology, Inc. (Palm City, FL, USA), Elisa reader at a wavelength of 517 nm. The inhibition percentage for the samples was calculated according to the formula proposed by Xiao et al. [31].

$$\text{Inhibition} = \left(\frac{AB - AA}{AB}\right) \times 100 \tag{3}$$

AB and AA were used to represent the absorbance values of the DPPH radical in the control and presence of the LEO sample, respectively. The resulting inhibition percentages were then plotted against the concentration of the samples. To determine the IC_{50} value, which signifies the concentration at which 50% inhibition occurs, linear regression analysis was utilized, as described by Rostaei et al. [32]. The antioxidant capacity of the samples was evaluated using the DDPH assay following the methodology outlined by Fallah et al. [6].

2.4. Statistical Analysis

The data were organized and subjected to analysis of variance (ANOVA) as an RCBD with the manure type (Mt) and harvest (H) as fixed effects. For the analysis, the PROC GLM procedure of SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) was used. The means were compared and significant differences were determined using the LSD test at a significance level of $p < 0.05$. Various parameters, including biomass, photosynthetic pigments, macro-micronutrients, antioxidant capacity, LEO content, and yield, were measured over three consecutive harvests with 3 repetitions in each treatment. The essential oil compounds (%) during the first and second harvests were expressed as the mean ± SD.

3. Results

3.1. Effect on Photosynthesis Pigments

Chlorophyll-a (Cha) and Chlorophyll-b (Chb) were affected by manure type and harvest (Table 3). In the HAR1 and HAR3, there were no significant differences observed in Cha levels between plants treated with livestock manures and the control ($p > 0.05$). However, in the HAR2, the Cha content in plants treated with poultry manure was higher compared to the CO treatment, measuring 6.6 and 4.5 µg mL^{-1}, respectively ($p < 0.05$;

Table 4). The Cha levels in plants treated with sheep manure did not show a significant variance from the CO treatment ($p > 0.05$; Table 4).

Table 3. Analysis of variance for photosynthesis pigments, nutrients concentration, biomass, essential oil, and antioxidant activity of different harvests of lemon balm cultivated in soil amended with livestock manures.

Source of Variance	Cha	Chb	N	P	K	Fe	Zn	Cu	Mn	Biomass	LEO Content	LEO Yield	AC
Block	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Manure type (Mt)	**	*	NS	*	**	NS	***	**	*	***	NS	***	**
Harvest (H)	***	**	NS	NS	***	**	***	***	NS	***	***	***	*
Mt × H	NS	NS	**	**	*	NS	*	**	NS	NS	**	NS	*

NS, *, ** and ***, nonsignificant, significant $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Cha: chlorophyll-a; Chb: chlorophyll-b. N: nitrogen; P: phosphorus; K: potassium, Fe: iron; Zn: zinc; Cu: copper; Mn: manganese; LEO: lemon balm's essential oils; AC: antioxidant activity.

Table 4. Mean comparison for photosynthesis, iron concentration, manganese concentration, biomass, and essential oil yield of different harvests of lemon balm cultivated in soil amended with livestock manures.

Treatments	Cha (μg mL^{-1})	Chb (μg mL^{-1})	Iron (mg kg^{-1})	Manganese (mg kg^{-1})	Biomass (kg ha^{-1})	LEO Yield (kg ha^{-1})
Harvest 1						
PO	5.72 ab	3.95 a	417 c	27.0 ab	1448 a	3.22 cd
SH	4.68 b–d	2.96 a–c	345 c	24.6 ab	1228 ab	3.84 b–d
CA	5.41 a–c	2.99 a–c	278 c	22.6 b	1282 ab	2.88 c–e
CO	4.29 b–f	2.40 b–d	327 c	21.6 b	895 cd	1.54 f
Harvest 2						
PO	6.61 a	3.15 ab	399 c	28.0 ab	1152 bc	5.43 a
SH	4.87 b–d	2.56 b–d	1129 a	30.9 a	1088 bc	3.89 bc
CA	5.35 a–c	2.71 bc	945 ab	31.6 a	1164 b	4.97 ab
CO	4.48 b–e	2.04 b–d	492 bc	24.8 ab	771 d	3.32 cd
Harvest 3						
PO	4.07 c–f	2.34 b–d	440 c	26.8 ab	702 d	2.64 d–f
SH	3.15 ef	1.82 cd	443 ab	26.5 ab	729 d	3.73 cd
CA	3.55 d–f	1.88 cd	480 bc	23.5 b	678 d	2.95 c–f
CO	2.90 f	1.47 d	388 c	27.0 ab	386 e	1.89 ef

Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); Cha: chlorophyll-a; Chb: chlorophyll-b.

In the HAR1, the Chb content in plants treated with poultry manure was found to be similar to those treated with sheep and cattle manure ($p > 0.05$) but significantly different from the unfertilized plants ($p < 0.05$; Table 4). Conversely, in the HAR2 and HAR3, there were no significant differences observed in Cha levels between plants treated with livestock manures and the CO treatment ($p > 0.05$; Table 4).

3.2. Effect on Nutrient Concentration

As shown in Table 3, the effect of manure treatments on the concentration of potassium, phosphorus, zinc, manganese, and copper in lemon balm leaves was statistically significant. The amount of potassium, iron, zinc, copper, and manganese was affected by harvest. The concentration of nitrogen ($p < 0.01$), phosphorus ($p < 0.01$), potassium ($p < 0.05$), copper ($p < 0.01$), and zinc ($p < 0.05$) was influenced by manure type and harvest interaction.

At each harvest, the concentration of manganese in plants treated with manures did not differ significantly (Table 4). In the HAR1, the iron concentration of plants treated with manure was found to be similar to the unfertilized plants ($p > 0.05$; Table 4). Conversely, in the HAR2 and HAR3, the iron concentration in plants treated with sheep manure (1129 and 443 mg kg^{-1}, respectively) was significantly higher than those treated with poultry manure and the control treatment ($p < 0.05$). However, there was no significant difference in iron concentration between the cattle manure treatment and the control treatment ($p > 0.05$; Table 4).

Figures 1 and 2 show the changes in the concentration of potassium, phosphorus, nitrogen, zinc, and copper in different treatments during consecutive harvests. In the HAR1, the levels of Cu, Zn, K, P, and N in the leaves of the manure-treated plants showed no significant variation compared to those in the CO treatment, except for the concentration of P and Zn in the CA and CO treatments, respectively ($p > 0.05$; Figures 1 and 2). In the HAR2, the nitrogen concentration in the SH and CA treatments and the phosphorus concentration in the CA treatment decreased compared to the PO treatment ($p < 0.05$; Figure 1) but they did not have significant differences with the CO treatment ($p > 0.05$). In the HAR2, the leaf potassium concentration in manure treatments was similar to the control ($p > 0.05$; Figure 1). In the HAR2, the concentration of zinc in the PO and SH treatments was significantly lower than in the CO treatment. The concentration of copper in the SH treatment was significantly lower than in the PO and CA treatments ($p < 0.05$; Figure 2).

In the HAR3, the highest concentrations of nitrogen, phosphorus, and zinc belonged to the SH treatment ($p < 0.05$) and there was no significant change between the PO and CA treatments compared with the CO ($p > 0.05$). In this harvest, the potassium concentration of manure treatments was considerably higher than the CO treatment ($p < 0.05$; Figures 1 and 2). However, the copper concentration of the CA treatment was higher compared to the PO and CO treatments ($p < 0.05$; Figure 2).

3.3. Effect on Biomass

The biomass of the lemon balm's aerial parts was altered by manure types ($p < 0.001$) and harvest ($p < 0.001$). The biomass in the manure treatments was in the range of 1015–1100 kg ha^{-1} ($p > 0.05$). The difference between the manure treatments and the CO treatment was 48–61% ($p < 0.05$; Table 3). Across all harvests, the amount of biomass from manure treatments exhibited a higher level compared to the CO treatment ($p < 0.05$; Table 4). There was an observable decline in biomass from the initial to the final harvest ($p < 0.05$). The variation in biomass levels for plants treated with manure in the HAR1, HAR2, and HAR3 ranged from 1282–1448, 1088–1164, and 678–729 kg ha^{-1}, respectively ($p > 0.05$; Table 4).

3.4. Effect on Lemon Balm's Essential Oils

As presented in Table 3, the LEO content was influenced by harvest ($p < 0.001$) and manure type × harvest interaction ($p < 0.01$), while the LEO yield was altered by manure type ($p < 0.001$) and harvest ($p < 0.001$). In the HAR2 and HAR3, the LEO content significantly increased compared to the HAR1 ($p < 0.05$; Figure 3a). In the HAR1, the LEO content of the plants treated with PO and CA treatments did not show a significant difference compared with the CO treatment ($p > 0.05$) but in plants treated with SH it was 83% higher than the CO treatment ($p < 0.05$). In the HAR2, the LEO content in manure treatments was not significantly different from that of the CO treatment ($p > 0.05$). In the HAR3, the LEO content in the CA and SH treatments was similar to the CO treatment ($p > 0.05$) but in the PO treatment, the LEO content was 24% lower than that of the CO treatment ($p < 0.05$; Figure 3a).

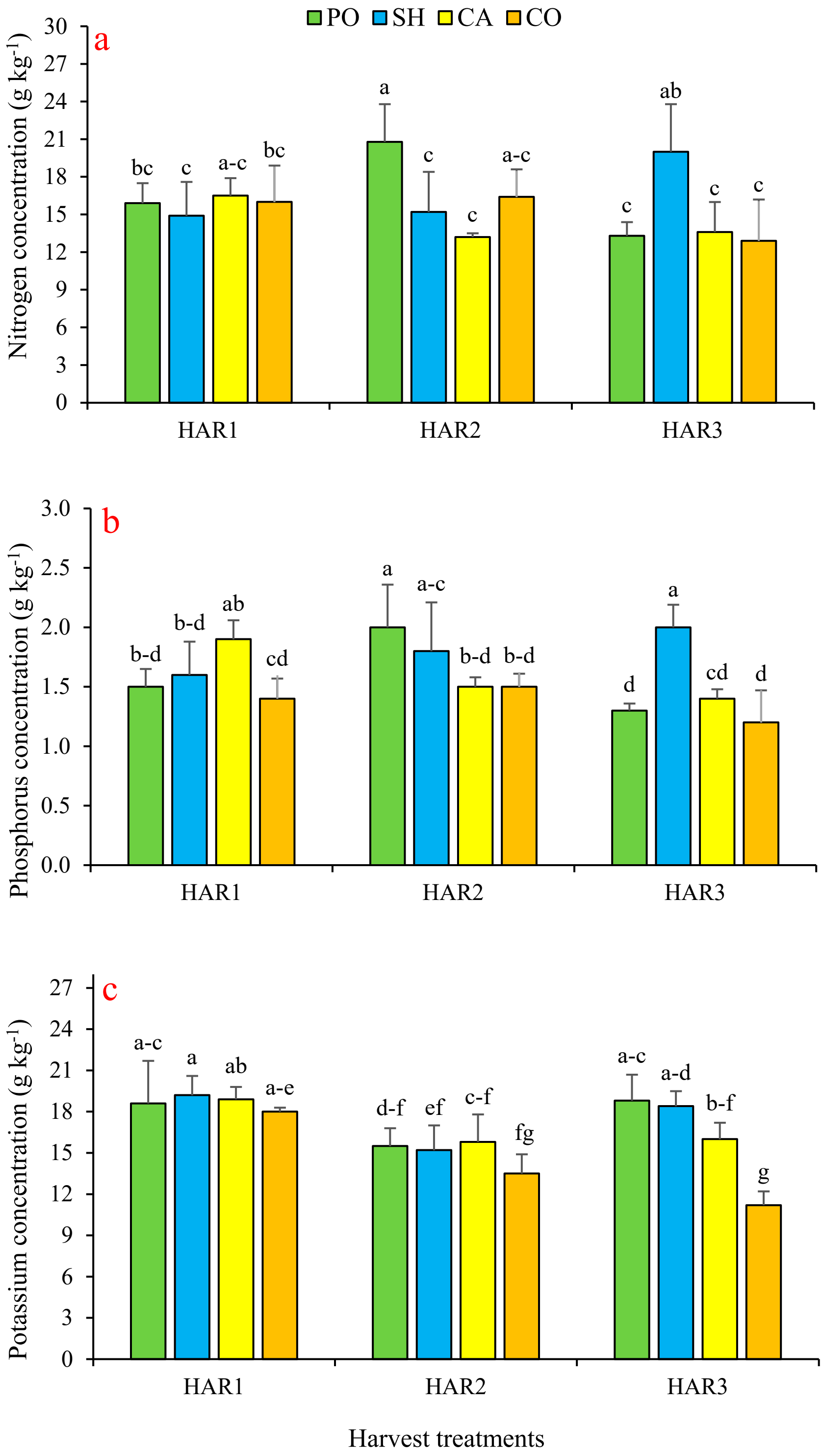

Figure 1. Concentration of nitrogen (**a**), phosphorus (**b**), and potassium (**c**) in different harvests of lemon balm cultivated in soil amended with livestock manures. Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). Bars represent standard deviation. PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); HAR1: first harvest; HAR2: second harvest; HAR3: third harvest.

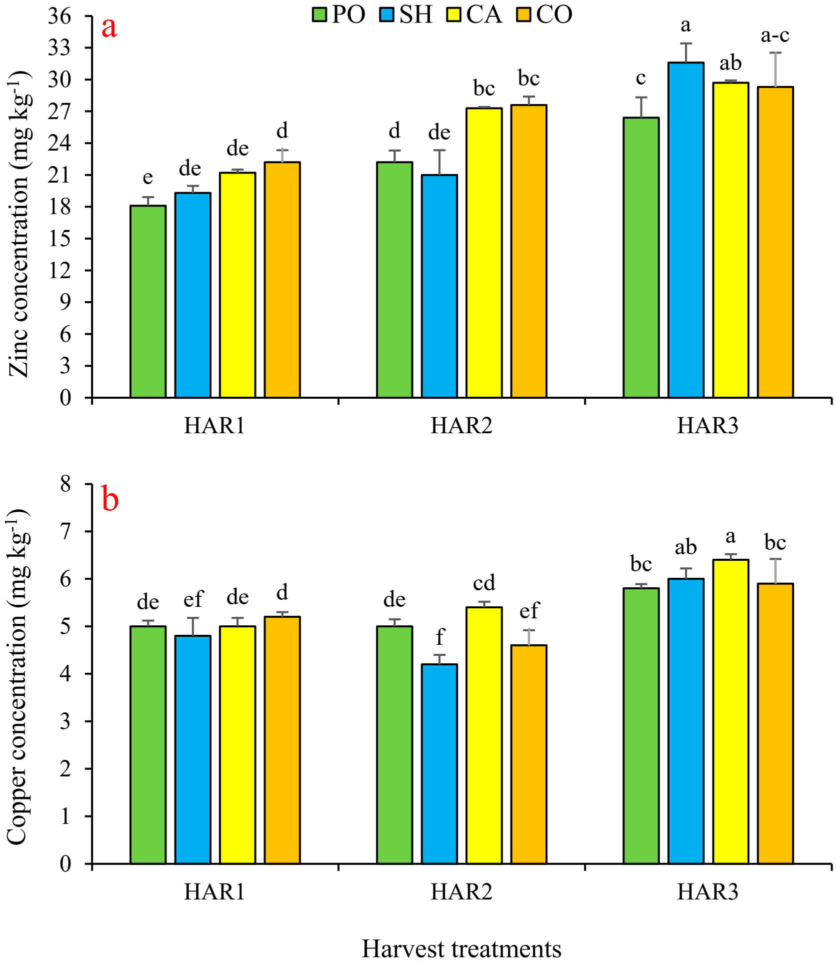

Figure 2. Concentration of zinc (**a**) and copper (**b**) in different harvests of lemon balm cultivated in soil amended with livestock manures. Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). Bars represent standard deviation. PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); HAR1: first harvest; HAR2: second harvest; HAR3: third harvest.

The LEO yield was changed by the manure type and harvest ($p < 0.001$; Table 3). In the HAR1 and HAR3, no significant difference among manure treatments was observed for the LEO yield ($p > 0.05$). Nevertheless, the LEO yield in the manure treatments showed a substantial increase of 87–109% and 44–97% compared to the CO treatment ($p < 0.05$; Table 4). In the HAR2, the greatest LEO yield was recorded in poultry manure at 5.43 kg ha^{-1}, which was statistically similar to the EO yield from cow manure at 4.97 kg ha^{-1} (Table 4). Poultry manure exhibited EO yield values that were 40 and 64% higher than those of sheep manure and the control treatment, respectively ($p < 0.05$; Table 4).

Based on the GC-MS and GC-FID analyses, 10 chemical compounds were identified in LEO with a total percentage ranging from 91 to 95% (Table 5, Figure A1). Table 5 shows the effects of manure type (Mt) and harvest (H) and their interaction effects for different chemical compositions. Compounds such as 1-octan-3-ol, 6-methyl-5-hepten-2-one, and nerol were found to be unaffected by the type of manure and harvest (Table 5). The type of fertilizer had a significant effect on the levels of linalool, citronellal, neral, geranial, geranyl acetate, (*E*)-caryophyllene, and caryophyllene oxide (Table 5). The main chemical component observed was geranial, accounting for 39–46% of the total composition. This was followed by neral (28–35%), (*E*)-caryophyllene (4.7–11%), geranyl acetate (2.7–5.9%), and caryophyllene oxide (1.7–4.8%) (Table 6; Figures 4 and 5). The lemon balm treated with livestock manures exhibited a decrease in the amount of (*E*)-caryophyllene compared to CO of HAR1 (Table 6). Additionally, the plants treated with PO and SH showed lower levels of geranyl acetate and citronellal, respectively, compared to the HAR1 control treatment (Table 6).

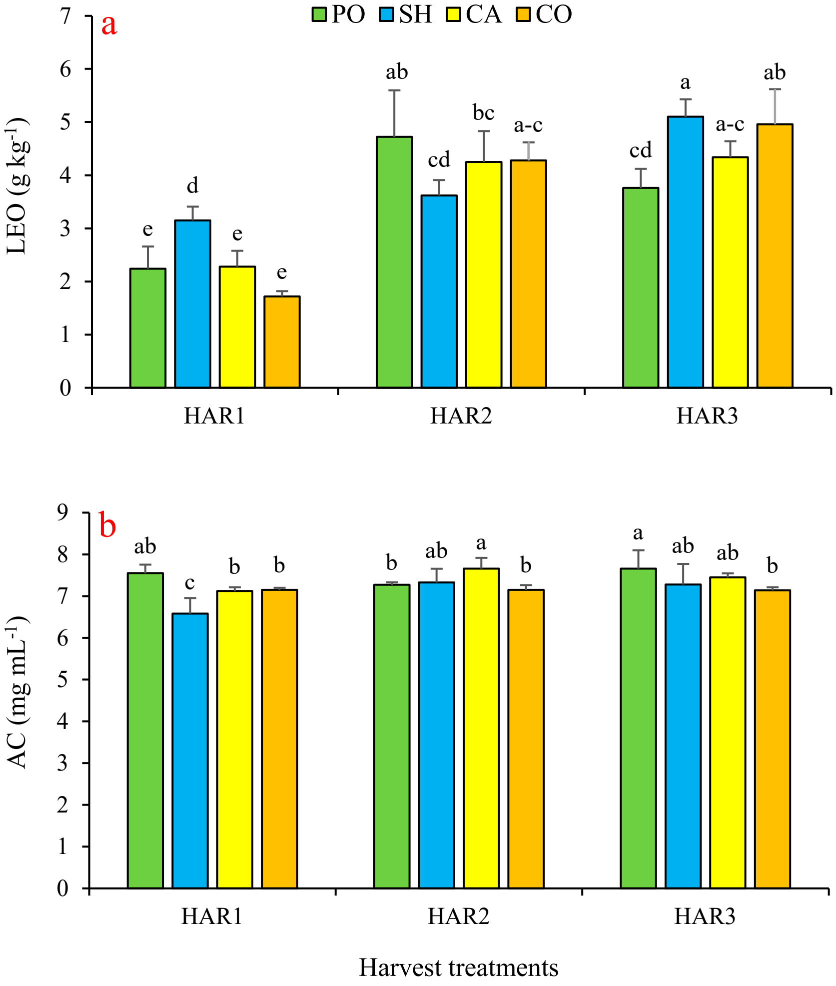

Figure 3. Essential oil content (LEO) (**a**) and antioxidant activity (AC) (**b**) in different harvests of lemon balm cultivated in soil amended with livestock manures. Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). Bars represent standard deviation. PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); HAR1: first harvest; HAR2: second harvest; HAR3: third harvest.

Table 5. Analysis of variance for the chemical compositions of different harvests of lemon balm cultivated in soil amended with livestock manures.

Source of Variation	**Retention Index (RI)**									
	975	**987**	**1100**	**1155**	**1230**	**1240**	**1265**	**1383**	**1415**	**1580**
Block	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Manure type (Mt)	NS	NS	***	**	NS	***	*	**	**	***
Harvest (H)	NS	NS	***	NS	NS	***	***	***	***	***
Mt × H	NS	NS	***	NS	NS	**	*	NS	NS	**

RI: Kovats index on the DB-5 column. NS, *, **, and ***, nonsignificant, significant $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. 975: 1-octan-3-ol; 987: 6-methyl-5-hepten-2-one; 1100: linalool; 1155: citronellal; 1230: nerol; 1240: neral; 1265: geranial; 1383: geranyl acetate; 1415: (*E*)-caryophyllene; 1580: caryophyllene oxide.

Table 6. Mean comparison for citronellal, geranyl acetate, and (*E*)-caryophyllene of different harvests of lemon balm cultivated in soil amended with livestock manures.

Treatments	Citronellal (%)	Geranyl Acetate (%)	(*E*)-Caryophyllene (%)
Harvest 1			
PO	1.22 a–d	3.82 cd	7.45 b
SH	0.92 d	5.90 a	7.56 b
CA	1.23 a–d	4.34 bc	7.22 bc
CO	1.38 ab	5.29 ab	10.7 a
Harvest 2			
PO	1.09 b–d	2.74 e	4.72 d
SH	1.04 cd	3.59 c–e	6.15 b–d
CA	1.50 a	3.12 de	5.72 cd
CO	1.30 a–c	3.29 c–e	6.41 bc

Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control.

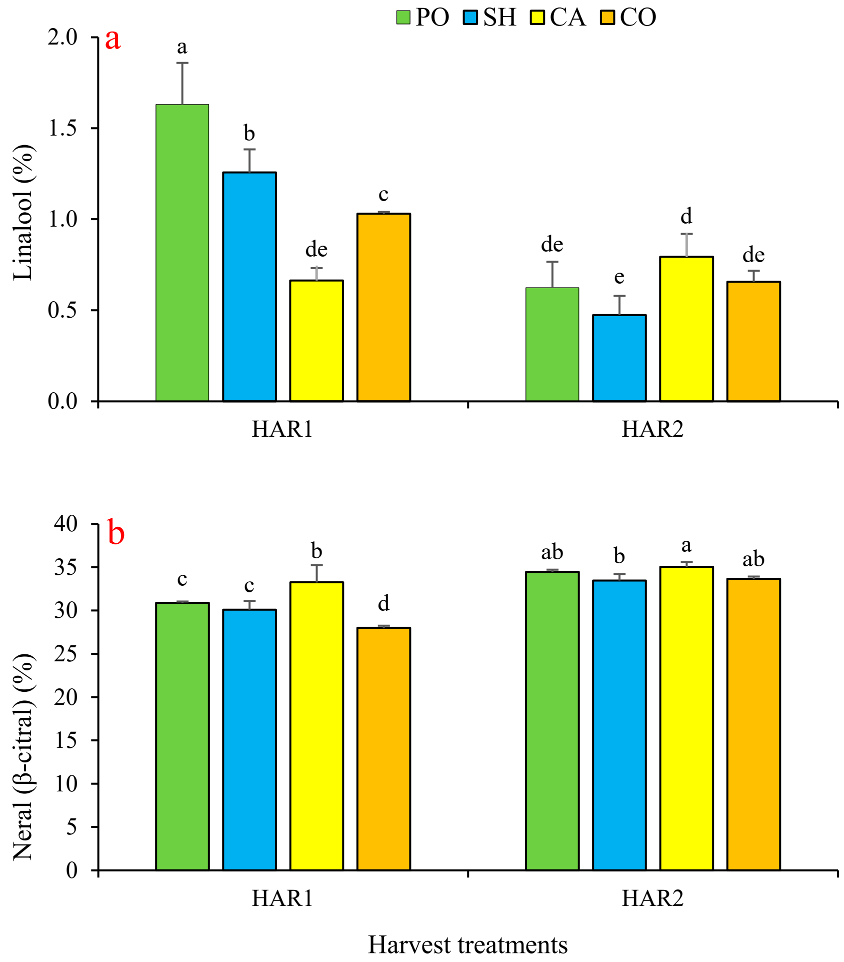

Figure 4. Linalool (**a**) and neral (**b**) in different harvests of lemon balm cultivated in soil amended with livestock manures. Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). Bars represent standard deviation. PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); HAR1: first harvest; HAR2: second harvest.

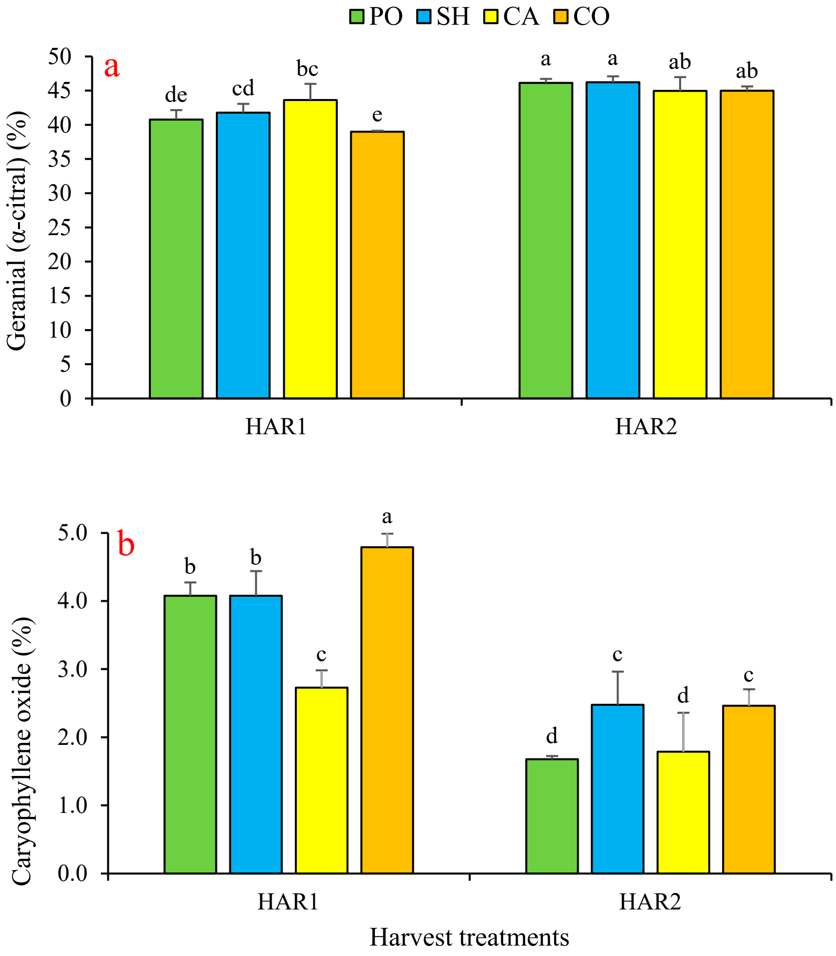

Figure 5. Geranial (**a**) and caryophyllene oxide (**b**) in different harvests of lemon balm cultivated in soil amended with livestock manures. Means with a similar letter are not significantly different according to LSD test ($p \leq 0.05$). Bars represent standard deviation. PO: poultry manure; SH: sheep manure; CA: cattle manure; CO: control (without manure); HAR1: first harvest; HAR2: second harvest.

The interaction effect of Mt × H was found to be significant for linalool ($p < 0.001$), neral ($p < 0.01$), geranial ($p < 0.05$), and caryophyllene oxide ($p < 0.01$) (Table 5). In the HAR1, the plants treated with PO and SH exhibited higher levels of linalool, neral, and geranial compared to the control, although no significant difference was observed in the HAR2 (Figures 4 and 5a). Moreover, in the HAR1, the plants treated with CA had lower levels of linalool and caryophyllene oxide but higher levels of geranial and neral compared to the control. In the HAR2 plants (Figures 4 and 5), the levels of linalool, neral, and caryophyllene oxide in the plants treated with CA were similar to the control (Figures 4 and 5b).

3.5. Effect on Antioxidant Capacity

The effect of Mt, H, and Mt × H on the LEO’s antioxidant capacity (AC) was significant (Table 3). In the HAR1, the AC of the SH treatment was 8% less than that of the CO treatment ($p < 0.05$). But the AC of the PO and CA treatments was similar to that of the CO treatment ($p > 0.05$; Figure 3b). In the HAR2, the AC of the CA treatment exhibited a higher value compared to the CO treatments ($p < 0.05$). Conversely, the PO and SH treatments did not show any significant difference when compared with the CO treatment ($p > 0.05$; Figure 3b). In the HAR3, the AC of the PO treatment was significantly higher than the CO treatment; other manures did not show a significant contrast when compared with the CO treatment (Figure 3b).

4. Discussion

4.1. Photosynthesis Pigments

The increase in chlorophyll content in the PO treatment and the subsequent decrease in chlorophyll levels during the third harvest can be attributed to the role of nitrogen in chlorophyll and protein molecules. Nitrogen is a crucial component for the formation of chloroplasts and the accumulation of chlorophyll within them [33]. According to Padilla et al. [34], there was a strong and positive relationship between chlorophyll a + b content and all chlorophyll measurements in sweet pepper with curvilinear relationships. Furthermore, there was a slight inclination toward stronger correlations with chlorophyll-a content compared to chlorophyll-b content for all chlorophyll measurements. In the PO treatment, the smaller particle size led to faster mineralization, resulting in higher nitrogen levels compared to other animal manure [32,35]. The continuous uptake of nitrogen across multiple harvests resulted in a decline in nitrogen concentrations within the rhizosphere, subsequently causing a reduction in chlorophyll content [36].

4.2. Nutrient Concentration

The elevated levels of nitrogen and phosphorus in lemon balm, supplied with PO and SH at HAR2, and HAR3, respectively, can be attributed to the availability of higher mineralized nitrogen during an extended period of plant growth. This was corroborated by the reports of Alizadeh et al. [20] and Baghdadi et al. [37], who also observed an improvement in soil conditions for nitrogenase activity in these specific treatments. In separate investigations conducted by Javanmard et al. [27] and Asadi et al. [25] on peppermint (*Mentha* × *piperita* L.), the application of vermicompost resulted in higher nitrogen levels compared to the control plants. Similarly, Bajeli et al. [38] found that the application of PO and CA treatments increased the nitrogen content in the leaves of mint (*Mentha arvensis* L.) compared to the CO treatment. Anwar et al. [39] described that the utilization of vermicompost also enhanced the nitrogen concentration in French basil (*Ocimum basilicum* L.) when compared to the control plants.

The phosphorus and nitrogen concentration patterns observed in the three harvests suggest that the peak mineralization of the PO and SH treatments aligns with the second and third harvests, respectively. However, in the case of the CA treatment, it is likely that after the mineralization of rapidly decomposing substances, the mineralization process of lignin materials has decreased. As a result, the release of phosphorus and nitrogen is restricted [26].

Zhu et al. [40] discovered that cattle manure (CA) had higher initial polysaccharide (cellulose and hemicellulose) concentrations compared to sheep manure across all sites. The order of initial cellulose concentrations was found to be cattle manure > sheep manure, while the opposite order was observed for initial crude protein concentrations: sheep manure > cattle manure. However, there were no significant changes in initial lignin concentrations among the different types of manure and the four sites. According to the findings of Markewich et al. [41], fresh manure exhibits a significant NH_4+ concentration resulting from the high decomposition rate of urea derived from urine. These processes include immobilization by the microbial decomposer population, volatilization as NH_3, nitrification followed by leaching, or further denitrification resulting in the emission of N_2, or NOx [42].

The potassium requirement of the plant has frequently been met through manure treatments, resulting in no significant alteration in potassium concentration. Nevertheless, the reduced potassium concentration observed in lemon balm harvested in the HAR3 of CO treatment can be attributed to an augmentation in plant biomass during the two preceding harvests, leading to a dilution of potassium content in the plants. Asadi et al. [25] found that the potassium concentration in peppermint harvested from HAR1 was higher than that in peppermint harvested from HAR2.

Iron and manganese levels in the HAR2 were found to be higher compared to the first and third harvests. This could be ascribed to the existence of substances like several secondary metabolites bearing an azetidine ring in the rhizosphere of some Lamiaceae species, which are more abundant in a larger volume of soil and modify the soil nutrient availability. Consequently, the plant absorbed a greater quantity of these two elements [43–45].

In the HAR3, the prevailing environmental conditions could have led to a decrease in the quantity and rate of recent carbon allocation during the belowground growth. Consequently, there has been an elevation in the allocation of this carbon toward respiratory use or sugar and starch reserves in roots, aiming to enhance winter hardiness [46,47]. The high levels of zinc and copper in plant tissue of the HAR3 in comparison to iron and manganese suggest that the expanded rhizosphere enhances the presence of these two elements (Zn and Cu). Additionally, the successive harvests resulted in a rise in their concentration in the plant, particularly in the CA and CO treatment, which exhibited lower biomass [32].

4.3. Lemon Balm Biomass

The utilization of manure led to significant improvements in various soil properties and biological activities [48]. These improvements included an increase in water-stable aggregation by 29%, soil organic carbon by 18%, available nitrogen by 16%, available phosphorus by 66%, and available potassium by 19% as well as the activities of urease by 25%, sucrase by 18%, and catalase by 16%. Additionally, the abundances of fungi, actinomyces, and bacteria were found to increase by 28, 60, and 38%, respectively. These findings suggest that the application of manure enhances the nutrient reservoir and decomposition capability of the soil, which in turn positively impacts crop yield. Abd-ElKader et al. [49] also demonstrated that the utilization of compost sharply raised peppermint biomass in three harvests in two consecutive seasons when compared to the control plants. Furthermore, another investigation revealed that the maximum biomass of *Thymus vulgaris* L. was achieved in thyme that received treatments of quail and cattle manure [50].

Typically, the decline in biomass over consecutive harvests can be ascribed to the plant's aging along with variations in temperature and lighting duration [23]. Likewise, several studies have indicated that peppermint harvested in the HAR2 exhibited lower biomass than in the HAR1 [23,51].

4.4. Lemon Balm's Essential Oils

The rise in LEO content in the HAR2 and HAR3, particularly in the CA and CO treatments, may be attributed to the limited nutrient availability (CO treatment), slow manure decomposition rate (CA treatment), and increased nutrient uptake by previous harvests (PO and SH treatments). Additionally, the leaves in the HAR3 were immature and the temperature was decreasing. These factors combined have resulted in the allocation of photosynthetic assimilates toward LEO production to enhance tolerance to unfavorable environmental conditions. Tripathi and Hazarika [52] found that the essential oil concentration of immature leaves (4.5%) of *Pogostemon cablin* (Blanco) Benth. was higher compared to vegetative and fully mature leaves (4.0, and 3.8%, respectively). This higher concentration in immature leaves can be attributed to the biogenetic activity of these leaves. Young leaves exhibit greater biogenetic activity compared to mature leaves, which means they have a higher rate of essential oil synthesis and accumulation [53].

In a separate study, Manjunatha et al. [54] observed a similar oil percentage for harvests conducted 6, 9, and 12 months after planting *P. cablin*. However, there was a significant decline in the essential oil yield for these harvests, with a more pronounced decrease noted from the initial harvest to the subsequent one. The LEO yield is dependent on both the biomass and the LEO content. As there is no significant variation in LEO content among manure treatments, it can be inferred that changes in biomass are the primary factor influencing the LEO yield. Consequently, the application of manure treatments, which provide essential nutrients and other advantageous effects [48], has resulted in a substantial increase in LEO yield in plants.

In terms of the plant's age (harvests), it is evident that the impact of the LEO content in determining the LEO yield is more significant when compared to the biomass. Thus, the LEO content not only offsets the reduction in biomass but also leads to a substantial increase in the LEO yield during the second harvest. This outcome highlights the importance of synthesizing primary and secondary metabolites under unfavorable conditions, as the temperature and daylight duration decrease in the second and third harvests along with a decline in nutrient availability.

Employing sequential harvesting can be an effective method for generating a superior end product that possesses enhanced bioactive properties [55]. Citral is a blend of geranial and neral that occurs naturally in various essential oils derived from citrus fruits, herbs, or spices [56]. The antimicrobial properties of citral have been proven effective against bacteria and fungi under various circumstances [57]. Given its widespread use across multiple industries, citral has gained approval from regulatory authorities in Europe and the US, earning the designation of being generally recognized as a safe (GRAS) substance [56]. Furthermore, it is a compound that finds extensive application in the pharmaceutical and cosmetic sectors [58]. Limonene, citral, and linalool are frequently found as nonphenolic terpenoid constituents in essential oils. These components have been associated with antioxidant properties, although there is some debate surrounding their effectiveness. Among these compounds, citral exhibits the highest reactivity due to the presence of the aldehyde group [59].

The presence of nutrients can impact the chemical compositions of the LEO. Phosphorus and nitrogen are crucial in improving plant photosynthesis and the synthesis of primary and secondary metabolites in plants, thereby supporting terpenoid production [60,61]. Terpenoid biosynthesis takes place via the mevalonic pathway and methylerythritol phosphate pathways, which do not conflict directly with nitrogen resource availability [61]. Additionally, the terpenoid precursors, such as isopentenyl diphosphate, farnesyl diphosphate, dimethylallyl pyrophosphate, and geranyl diphosphate, contain phosphorus as a structural component. Therefore, an increase in phosphorus availability could potentially lead to a rise in these pathways and subsequent terpene production [61,62]. Micronutrients in plants act as cofactors for important enzymes involved in secondary metabolism, thereby positively influencing the synthesis and accumulation of plant secondary metabolites [63]. Furthermore, potassium application has been found to enhance the content and quality of essential oils extracted from *Mentha spicata* L. according to Chrysargyris et al. [64]. Therefore, it is important for lemon balm plants to uptake sufficient nutrients to meet their growth and terpenoid synthesis requirements [65].

4.5. Antioxidant Capacity (AC)

The chemical components of LEO may contribute to plant antioxidant activity, particularly the main compounds [66]. It should be emphasized that this property is not solely dependent on the high concentration of a single compound [67]. This is because both the minor and major components can work together synergistically in the LEO, enhancing the overall antioxidant activity of plant essential oil [68]. Sheep and poultry manure gave a greater percentage in geranyl acetate and linalool compared to the control treatment. As a result, it exhibited the strongest antioxidant capacity. Moreover, it is evident that there is a direct correlation between the level of essential oil and antioxidant capacity. Hence, sheep and poultry manures demonstrated the highest antioxidant capacity in the HAR1 and HAR2, respectively. The enhanced antioxidant activity of this manure could be attributed to its ability to supply a significant amount of geranyl acetate and oxygenated monoterpenes, as suggested by Deba et al. [69], Fallah et al. [70], and Tohidi et al. [71]. Ozliman et al. [72] demonstrated that the utilization of various doses of farmyard manure not only enhances the EO content and components but also serves as a means to augment the antioxidant activity of *Anethum graveolens* L.

5. Conclusions

Our research findings indicate that lemon balm can thrive in cold semiarid climates and can be harvested three times a year. The application of animal manure resulted in a substantial increase in biomass (41–60%) and essential oil yield (60–71%) compared to the control. The use of livestock manure, particularly sheep and/or poultry manure, demonstrated a notable improvement in the essential oil's quality, as evidenced by the increase in its main components and enhanced antioxidant capacity. Notably, during mid-August and early September, there was a significant boost in the essential oil yield and citral levels. However, the decrease in geranyl acetate and oxygenated monoterpenes led to a decline in antioxidant capacity. Consequently, it is recommended to utilize essential oils from the second and third harvests for industrial purposes. Overall, employing animal manures, especially sheep manure, as a nutrient source for lemon balm cultivation proves to be a viable approach for producing high-quality essential oils and brings about the sustainability of the agricultural ecosystem.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Monthly average temperature and total monthly precipitation in 2019 and long-term (2016 –2019).

	Average Temperature (°C)		Average Minimum Temperature (°C)		Average Maximum Temperature (°C)		Precipitation (mm)	
Month	**2016–2019**	**2019**	**2016–2019**	**2019**	**2016–2019**	**2019**	**2016–2019**	**2019**
January	−0.06	0.1	−6.6	−5.5	5.5	5.7	262.6	615.2
February	0.2	−1.1	−6.3	−7.4	6.8	5.2	143.2	204.7
March	4.4	0.3	−1.7	−6.5	10.5	7.1	281.1	561.4
April	8.7	6.2	2.9	1.7	14.4	10.8	181.1	278.87
May	14.3	14.1	7.2	7.1	21.4	21.1	76.3	17.1
June	19.2	19.8	10.2	11.0	28.1	28.6	1.5	0.2
July	22.7	22.8	14.0	14.4	31.4	31.3	0.0	0.0
August	22.1	21.9	13.1	13.1	31.0	30.8	0.0	0.0
September	18.6	18.4	9.6	9.1	27.5	27.8	0.3	0.0
October	12.8	13.0	5.3	6.2	20.2	19.9	26.5	58.5
November	5.2	2.5	−1.6	−5.8	12.1	10.7	123.5	53.5
December	1.7	−2.3	−4.7	−9.2	8.0	4.6	235.8	229.8

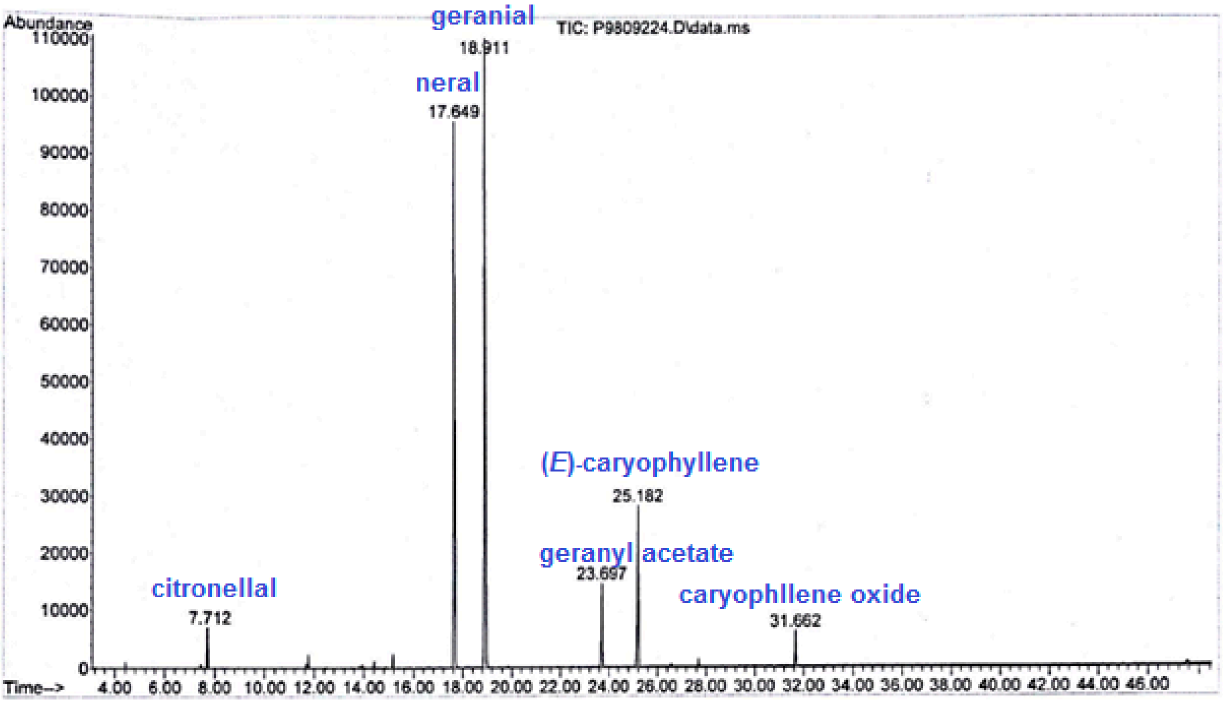

Figure A1. GC-MS profile of essential oil from aerial parts of *Melissa officinalis* treated with livestock manure.

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