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Effects of Supplemental Light Quality at The End of Day on Herb Production and Some Phytochemical Properties of Lemon Balm (*Melissa officinalis* L.)

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ABSTRACT

Increasing day length during the short photoperiod in fall and winter is a beneficial method of increasing biomass production and altering plant morphology and phytochemistry. The objective of this study was to examine the effects of light quality at the end of the day (EoD) on the growth and phytochemical characteristics of lemon balm. During short-day photoperiods in autumn, lemon balm (*Melissa officinalis* L.) seedlings were exposed to red, blue, and combined red/blue light using light-emitting diodes for 2 hours at the EoD. The results showed that exposure to red light significantly increased biomass. Plants grown under blue light yielded the highest percentage of dry matter and their leaves had the highest chlorophyll content and flavonoids. The highest carotenoid content was found in plants irradiated with blue light and later with red+blue light. The highest levels of total phenols, anthocyanins, and antioxidant activity were found in plants grown under red light. In addition, light quality had a significant effect on essential oil content. The highest essential oil content was obtained in the red and red+blue light treatments. The light quality at the EoD significantly changed the essential oil composition. The blue light significantly increased the citronellal content but decreased the geranial and linalool content. This study provided insights into the effects of Eo D light quality on plant growth and metabolite accumulation in lemon balm with a short photoperiod. In conclusion, supplemental light at the EoD can effectively improve plant growth and secondary metabolite quality in medicinal plants.

Introduction

Lemon balm (*Melissa officinalis* L.) is a perennial herb in the Lamiaceae family that originated in the Mediterranean region and southern Europe (Sorensen, 2000). Lemon balm was traditionally interested by Avicenna (980-1037) who prescribed it for anxiety and nervousness (Castleman, 2010). *M. officinalis* consists of substances that prevent protein biosynthesis in cancer cells (Adjorjan and Buchbauer, 2010; De Sousa et al., 2004). These biological activities are attributed to the essential oil (Da Silva et al., 2005) and polyphenols in its extract (Mencherini et al., 2007; Carnat et al., 1998). Nowadays, lemon balm production is popular in the medicinal, cosmetic, and food industries due to its beneficial applications (Dousti et al., 2012).

Numerous environmental factors such as temperature, water, and light affect plant growth and secondary metabolite production (Lee et al., 2014). In this context, light is one of the most important environmental factors regulating photosynthesis and biosynthesis of phytochemicals (Huche-Thelier et al., 2016; Chen et al., 2016; Darko et al., 2014; Esmaeili et al., 2022). In plants, light activates many biosynthetic pathways for primary and secondary compounds depending on the quality, intensity, and duration

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(Wu et al., 2007; Rajapakse and Shahak, 2007; Yi et al., 2014; Heo et al., 2003; Zahedi and Sarikhani, 2017; Hosseini et al., 2018; Karimi et al., 2022). Light intensity and quality are also influential factors in plant morphogenesis and biochemical and physiological responses (Fan et al., 2013; Namdar et al., 2019). Light intensity stimulates fatty acid synthesis and chloroplast membrane composition (Wacker et al., 2016). Light quality affects enzyme activities in the production of secondary metabolites (Xu et al., 2014; Karimi et al., 2022). In addition, the duration and quality of light exposure can affect floral induction, morphology, and carbohydrate movement (Heo et al., 2003; Zahedi and Sarikhani, 2017; Javadi Asayesh et al., 2021).

Initially, light is received by an optical receptor and then triggers a biochemical response in plants. Typical light receptors include phytochromes, cryptochromes, phototropins, UVR8 and Zeitlupe. Phytochromes receive red, far-red, and blue light. In addition to blue light, cryptochromes, phototropins, and Zeitlupe receive ultraviolet light (Taiz et al., 2015). Phototropins are involved in the regulation of light-dependent processes such as phototropism, stomatal opening, and chloroplast movement (Batchauer et al., 2007; Hernandez, 2013). Artificial light exposure leads to significant changes in plant growth and functions, depending on the properties of light and the action of light receptors (Huche-Thelier et al., 2016).

In recent years, much attention has been paid to red and blue light and how they play their role as energy sources for photosynthetic carbon sequestration (Qian et al., 2016). Red and blue wavelengths of light-emitting diodes (LEDs) are widely used for enhancing photosynthetic products of plants (Choi et al., 2015). Red and blue light have satisfactory performances in photosynthesis, thereby effectively promoting growth traits associated with orthotropic growth habits (Ren et al., 2014).

The timing of light exposure also affects the primary and secondary metabolism of the plant. For example, light at the end of day (EoD) can cause the activation or closure of some biosynthetic pathways by altering the ratio of active optical receptors (Islam et al., 2014). In a study by Mulas et al. (2006), additional irradiation with red and far-red light at the EoD significantly affected the quantity and quality of rosemary essential oil. Moreover, red light reportedly increased the limonene content, while far-red light enhanced alpha-pinene, camphene and p-cymene in rosemary essential oil. However, there are few reports on the effect of daylight on plant growth and phytochemical properties.

In the last two decades, LEDs have been regarded as new light sources for plant production both in controlled environments and in plant physiological studies (Brown et al., 1995; Yanagi and Okamoto, 1997; Samuolienė et al., 2012; Bantis et al., 2016; Viršilė et al., 2017; Wu et al., 2020). The advantages of LED sources include high optical efficiency, considerable energy saving, low volume, long lifetime, low energy production, adjustable energy intensity and quality, followed by the absence of harmful UV rays. Its advantages are comparable to other conventional light sources, including fluorescent and halogen fog lights (Choi et al., 2015; Lee et al., 2014; Chen et al., 2014; Kozai et al., 2016; Wu et al., 2020). These characteristics of LEDs make them the perfect complementary light source for greenhouses, especially in areas with low light intensity (Chen et al., 2014; Wu et al., 2020) and short days (Wojciechowska et al., 2015).

Although using artificial light has been extensively studied in various plants, the effect of supplemental light has remained unclear. However, the timing and type of lighting could affect growth, yield, flowering, and secondary metabolite production. Using artificial light at the EoD may be economically justifiable due to the shorter exposure time. Since increasing biomass production and medicinal ingredients of medicinal plants is becoming increasingly important, the current research aimed to (1) evaluate the effects of light treatments on yield and quality of lemon balm and (2) select the spectral composition for evening supplemental lighting recommended for lemon balm cultivation to promote its growth and secondary metabolite content under short-day conditions.

Material and Methods

Plant material

Experiments were conducted in the research greenhouse, Department of Horticultural Science (34.8 N, 48.4 E; 1,800 m altitude), Bu-Ali Sina University, Hamedan, Iran. Lemon balm (*Melissa officinalis* L.) seedlings were purchased from the Ebne-Sina Medicinal Plants Garden, Hamedan, Iran, in July. Each plant was established in a 2 L pot containing an equal mixture of soil, sand, and leaf compost. It was transferred to the greenhouse under natural sunlight. All plants received fertilizers routinely, i.e. foliar application of 0.5 ml L^{-1} with a complete fertilizer (NPK 20:20:20 + micronutrients) every two weeks. Daily minimum and maximum temperatures, daylight duration (from sunrise to sunset), and natural sunlight intensity in the greenhouse are presented in Figure S1.

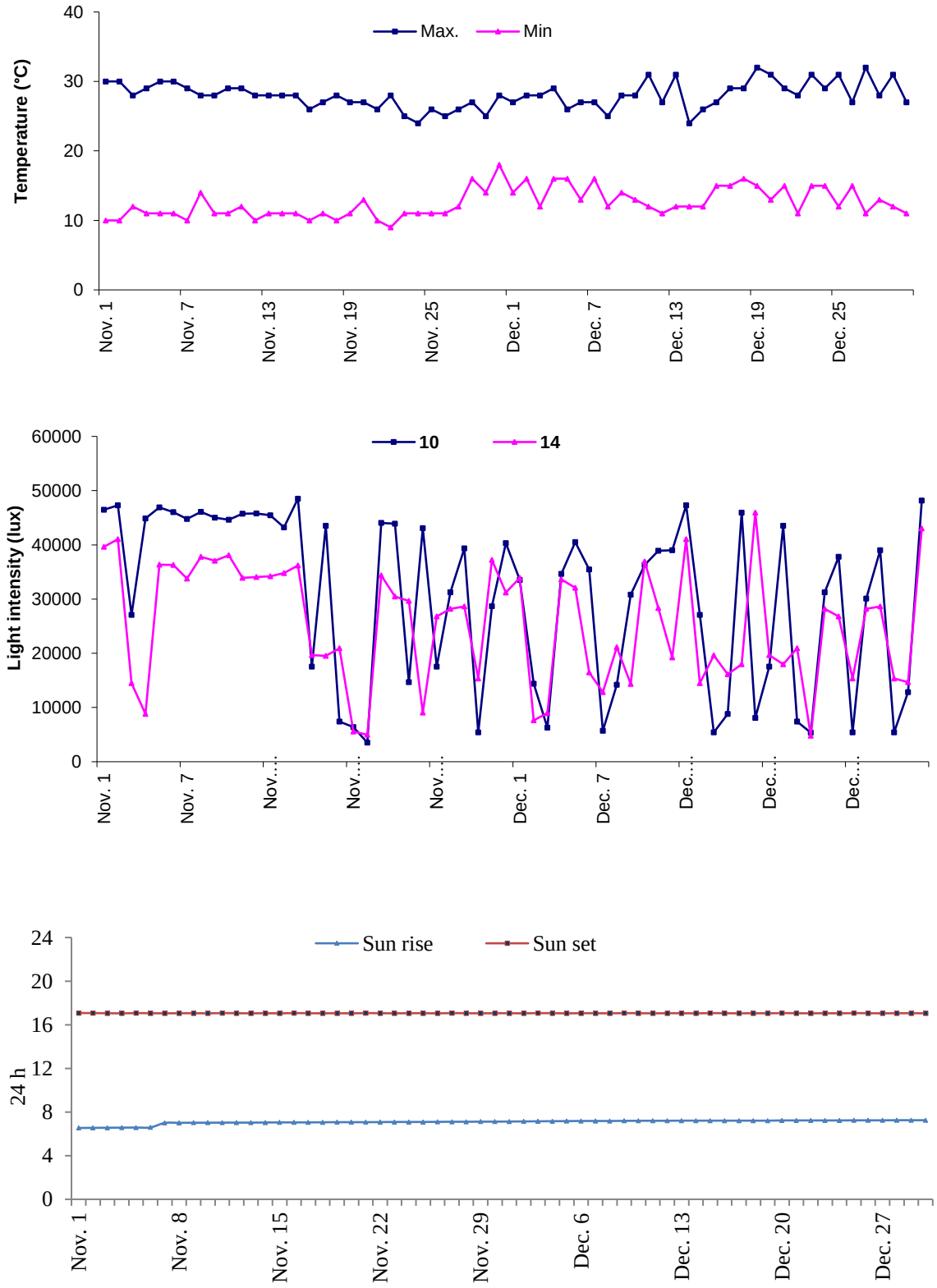


Fig. S1. Daily air temperatures (up), light intensity (middle), and natural sunlight duration (down) in the

greenhouse at the research site during the experiment.

Experimental design and light treatments

The experiment was planned and conducted based on a completely randomized design with three replications. Each replication included 6-test units (pot). The seedlings were grown under three light treatments, i.e. red light (100%), blue light (100%), and red+blue light (50% + 50%) provided by red (peak at 660 nm) and blue (peak at 450 nm) LEDs at the end of the day for 2 h immediately after sunset. Seedlings grown under natural light served as the control. Supplementary light intensity was 80 µmol m^{-2} s^{-1} at the pot surface using LED panels (Ledsazan, Tehran, Iran). The seedlings were cut 5 cm above the pot surface before starting the light treatment. The plants were treated by a light beam from a distance of 60 cm above the pot surface for five weeks starting from October 18.

Chemicals

Acetone, sulfuric acid, hydraulic acid, phosphoric acid, hexane, methanol, and ethanol were purchased from Amertat Shimi (Tehran, Iran). Folin Ciocalteu, gallic acid, Tris (hydroxymethyl) aminomethane (Tris), sodium carbonate, aluminum chloride, and potassium acetate were purchased from Merck (Darmstadt, Germany). Quercetin, Coomassie Brilliant Blue G-250, bovine serum albumin, anthrone, and 2, 2-diphenyl -1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Steinheim, Germany). All other chemicals were of analytical grade.

Measured features

The plant agro-morphological features included the number, length and diameter of the stem, node number, leaf number, internode length, leaf area (using Image-J software), fresh weight (using a balance with an accuracy of 0.01 g), dry weight, and leaf yield percentage. To obtain dry weights of the plants, roots and shoots (stems and leaves), samples were dried in the oven at 70 °C for 72 hours.

Chlorophyll and carotenoid concentration

A relevant method by Porra et al. (1989) was employed to measure chlorophyll concentration. Briefly, 200 mg leaf sample was crushed by 80% acetone and was centrifuged at 5,000 rpm for 5 min. The absorption of the extract was measured using a spectrophotometer (Carry 100, Varian, USA) at three wavelengths (664, 645, and 470 nm). Then, the concentrations of chlorophyll and carotenoid were calculated using the following equations:

[Chl a] = (12.25 × A664) - (2.55 × A645)

[Chl b] = (30.13 × A645) - (4.91 × A664)

[Chl a+b] = (17.76 A645) + (7.34 A664)

Carotenoids = (1000 A470 - 1.82Chl a - 85.02 Chl b)/198

Soluble carbohydrates concentration

The concentration of soluble carbohydrates was measured according to the Paquin and Lechasseur (1979) method. Briefly, 0.5 g of fresh leaf was ground with 5 mL of 96% ethanol. After centrifugation at 6,000 rpm for 15 min, the upper part was separated. In the next step, 5 ml of 70% ethanol was added to the remaining sediments and was centrifuged. After combining the extracts, 0.1 ml of the alcoholic extract was mixed with 3 ml of fresh anthrone. Then, it was placed in a bain-marie for 10 min at 95 °C. After cooling, the absorbance was read at 625 nm. By comparing it with the glucose standard curve, the carbohydrate concentration was expressed as mg g^{-1} FW.

Soluble protein concentration

The Bradford (1976) method was employed to determine plant soluble proteins. Briefly, 0.5 g of fresh leaf was mixed with 6.25 ml extraction buffer and placed in the refrigerator at 4 °C for 24 hours. The extraction buffer comprised of 121.14 g Tris dissolved in distilled water, brought to a volume of 1 L, while pH was adjusted to 6.8 via 1 N HCl. Then, the leaf sample was completely crushed and then centrifuged at 6000 rpm for 20 min. Subsequently, 5 mL of the Biuret reagent was added to 0.1 ml of the upper phase. The resultant mixture was shaken for a few seconds and the absorbance of the samples was recorded at 595 nm using a spectrophotometer. The absorbance was then compared with the bovine serum albumin standard curve and expressed as mg g^{-1} FW.

Extraction of polar antioxidant

To measure the phytochemical and antioxidant characteristics, an extract was preliminarily prepared according to Bedreag et al. (2014) with some changes. For this purpose, 500 mg of air-dried leaf sample was crushed in a Chinese mortar and mixed in 5 ml of 85% methanol. The samples were shaken at 120 rpm for 1 hour and then centrifuged at 4000 rpm for 15 min. The supernatant was separated and the procedure was repeated for the remaining phase.

Total phenolic concentration

The Singleton and Rossi (1965) method was employed to measure total phenol content. First, 1500 µl of the 10% Folin- Ciocalteu reagent was

added to the 300 µl of the extract and maintained for 5 min. Then, it was combined with the 1200 µl sodium carbonate (7.5%) and placed on a shaker for 1.5 hours at 120 rpm. Eventually, the solution absorbance was measured at 765 nm. Using the standard curve, the total phenol content was stated as mg of gallic acid in g extract.

Total flavonoid concentration

The aluminum chloride colorimetric assay, described by Chang et al. (2002), was used for total flavonoid measurement. At first, 1.5 ml of 85% methanol was added to the 0.5 ml of the solution of each extract. In the next step, 0.1 ml of 10% aluminum chloride and 0.1 ml of 1 M potassium acetate were added to the solutions. Finally, 8.2 ml of distilled water was added. The final mixture was kept at room temperature for 30 min. Then, the absorbance of the samples was determined at 415 nm. The total flavonoid concentration was expressed as mg of quercetin per g of extract weight by a standard curve.

Anthocyanin concentration

The anthocyanin concentration was determined by the Rapisarda et al. (2000) method. Briefly, 2.5 ml of the extract was diluted to 10 ml by an 80/20 (v/v) mixture of 95% methanol and 37% HCl. The absorbance was measured at 532 nm. The anthocyanin concentration was calculated using the following equation [C mg L^{-1} = A / 402.3 × 10000 × DF] in which C, DF, and A were the anthocyanin concentration, the dilution factor, and the absorption value.

Tannin concentration

The Folin-Deniz method, according to Bajaj and Devsharma (1977), was applied with a slight modification to measure the tannin content. First, 250 µl of methanol extract and 1375 µl of distilled water were mixed. Then, 125 µl of Folin-Deniz reagent was added to the mixture. After 3 min, 250 µl of sodium carbonate solution and 8 ml of distilled water were added to the solution and shaken for 1 hour. Then, the absorbance of the samples was measured at 725 nm. The total tannin content was calculated by comparing the data with the tannic acid standard curve.

Radical scavenging activity

The antioxidant activity was measured using DPPH free radical (Brand-Williams et al., 1995). Briefly, 500 µl of the extract was diluted with 500 µl of distilled water and centrifuged at 10,000 rpm for 5 min. Then, 2925 µl of 0.05 mM fresh methanolic DPPH solution was added to 75 µl of this sample. The absorbance of the sample was measured at 515 nm (At0). Then, the samples were placed in a dark environment for 30 min. The absorbance of the sample was measured (At30). The radical scavenging activity of extracts was calculated by [Activity (%) = (At0-At30)*100/ At0] equation.

Isolation of the essential oil

Considering the European Pharmacopoeia, about 40 g air-dried leaves were exposed to the hydro distillation for 3 hours with 600 ml distilled water by a Clevenger-type apparatus. Then, pentane was used as essential oil solvent to facilitate the collection. The essential oil was dried over anhydrous sodium sulfate, maintained in the dark, and stored in a refrigerator at 4 °C prior to further analysis. The essential oil yield was calculated based on the sample dry weight.

Analysis of essential oil composition

Hexane was applied to dilute the isolated essential oil of the lemon balm (dilution ratio 10:100), while a 0.1 ml sample was taken for the gas chromatographic analysis. For this purpose, a gas chromatograph (GC) (TRACE GC, ThermoQuest-Finnigan) was utilized with an HP-5MS non-polar fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). Operating conditions directed the oven temperature program from 60 °C (2 min) to 250 ºC at 10 °C/min. The final temperature was maintained for 5 min; 2 “split mode” ratio 1:100; carrier gas helium, flow rate 1 ml min-1; injector and detector temperature (flame ionization detector) fixed at 250 °C and 200 °C, respectively.

For volatile analysis, the mass spectrometer system (Trace GC, ThermoQuest-Finnigan) was equipped with a Thermo Fischer capillary gas chromatograph. An HP-5MS non-polar fused silica capillary column (30 m × 0.25 mm, 0.25 µm film thickness) was employed. The oven temperature was set at the initial temperature of 40 ºC for 2 min, and a programmed rate of 2 °C/min was used for increasing the temperature up to the final value of 460 °C with an isotherm for 10 min. The sample (1 ml of the diluted oil) was then injected at 250 °C splitless with the carrier gas of helium and a flow rate of 1 ml min-1. The operating condition included an ionization energy of 70 eV in the electronic ionization mode, an ion source temperature of 200 °C, a scan mass range of m/z 40–460, and an interface line temperature of 250 °C. The identification of components was made by determining their retention indices (KI) associated with those of a homologous series of n-alkanes (C8–C20) (Fluka, Buchs, SG, Switzerland) and by matching their recorded mass spectra with

those stored in the spectrometer database (NIST MS Library v. 2.0) and the bibliography (Fig. S2).

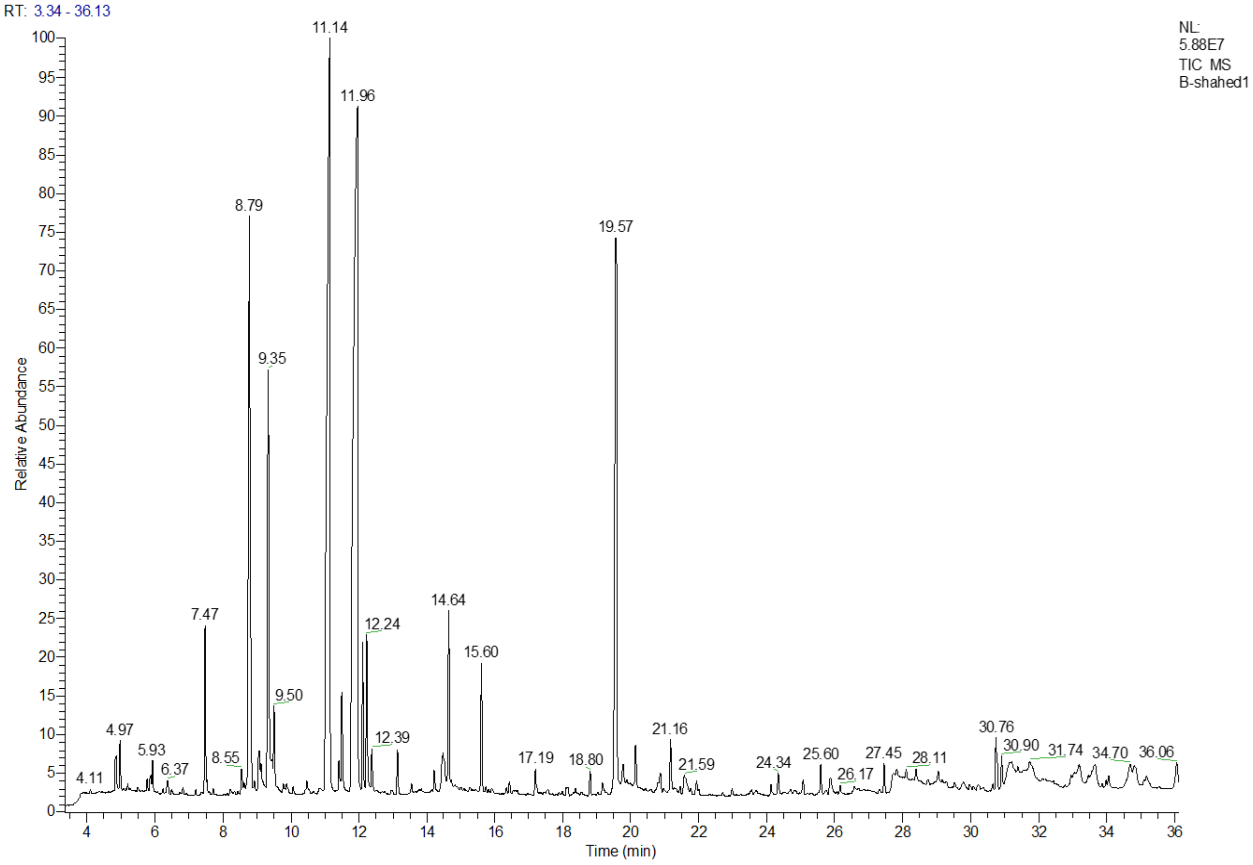


Fig. S2. Gas chromatography/mass spectrometry (GC/MS) chromatograms of lemon balm essential oil under controlled light.

Statistical analysis

All experimental data were analyzed for normal distribution and were statistically analyzed via one-way ANOVA analysis of variance using the PROC GLM procedure in Statistical Analysis System (SAS) software (Version 9.1; SAS Institute, 2003). Mean values were compared by performing Duncan's multiple range tests ($P \leq 0.05$).

Results

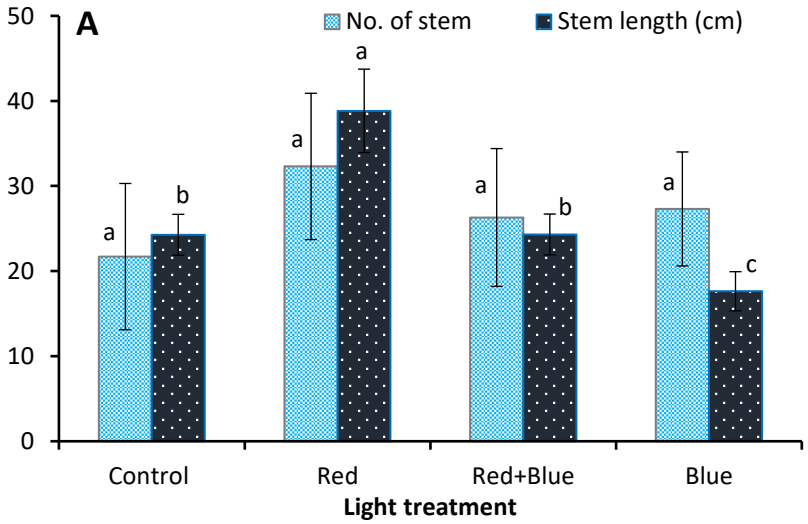
Morphological and vegetative characteristics

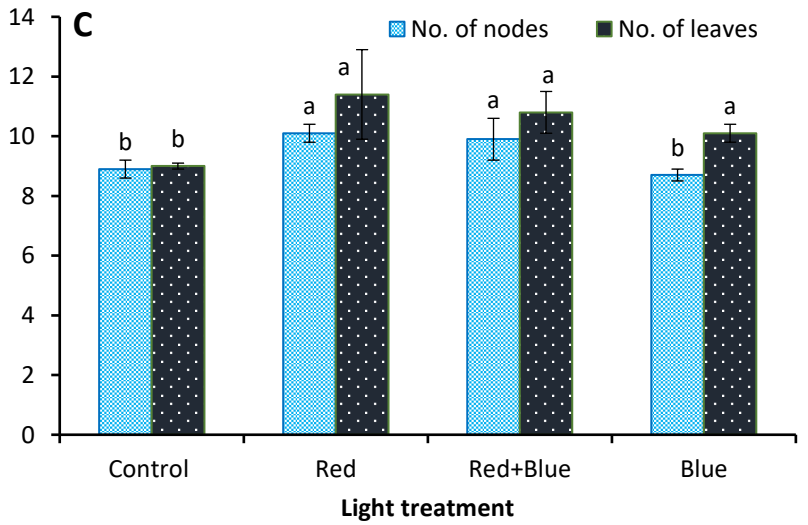
Analysis of variance showed that light quality had no significant effect on stem count, whereas it significantly affected stem length, stem diameter, internode length, and leaf area ($P \leq 0.01$). Also, it significantly affected the number of nodes and leaves ($P \leq 0.05$) (Table S1). In the red light treatment, the maximum stem length was obtained with an average of 38.8 cm, representing a significant difference between the red+blue light and the control. The shortest stem occurred in blue light-treated plants. The largest stem diameter (3.32 mm) was obtained in red light, followed by a combination of red and blue light. The red and combined light treatments resulted in the highest number of nodules in lemon balm plants, indicating a significant difference from the control and blue light treatments. The highest internode length was observed in red light treated plants, followed by the control and combined light treatment, with significant differences. Although the highest number of leaves was observed in the red light treatment, there was no significant difference between the blue and red+blue light treatments. The least number of leaves was observed in the control plants. The highest leaf area was observed in red light-treated plants, followed by the control and combined light treatments with significant differences. However, the least value was observed in blue light-treated plants (Fig. 1).

Table S1. Effect of the light quality at the end-of-the-day on morphological properties of the lemon balm.

Treatments	No. of stem	Stem length (cm)	Stem diameter (mm)	No. of nodes	Internode length (cm)	No. of leaves	Leaf area (mm^2)
Control (without light)	21.7± 8.6 [a]	24.27 ±2.4 [b]	2.94 ±0.12 [b]	8.9 ±0.3 [b]	3.11 ±0.41 [b]	9.0 ±0.1 [b]	2128 ±231 [b]
Red	32.3±8.6 [a]	38.84 ±4.9 [a]	3.32 ±0.06 [a]	10.1 ±0.3 [a]	4.28 ±0.33 [a]	11.4 ±1.5 [a]	3192 ±320 [a]
Red+Blue	26.3±8.1 [a]	24.31 ±2.4 [b]	3.13 ±0.02 [b]	9.9 ±0.7 [a]	2.81 ±0.16 [b]	10.8 ±0.7 [a]	1909 ±190 [b]
Blue	27.3±6.7 [a]	17.63 ±2.3 [c]	2.92 ±0.19 [b]	8.7 ±0.2 [b]	2.33 ±0.24 [c]	10.1 ±0.3 [a]	1465 ±243 [c]
Significance	ns	**	**	*	**	*	**

Values represent the means of three replications (n=6) ± standard deviation.
Means with the same letter are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.
**, * and ns means significant at 1%, 5% and not significant, respectively.





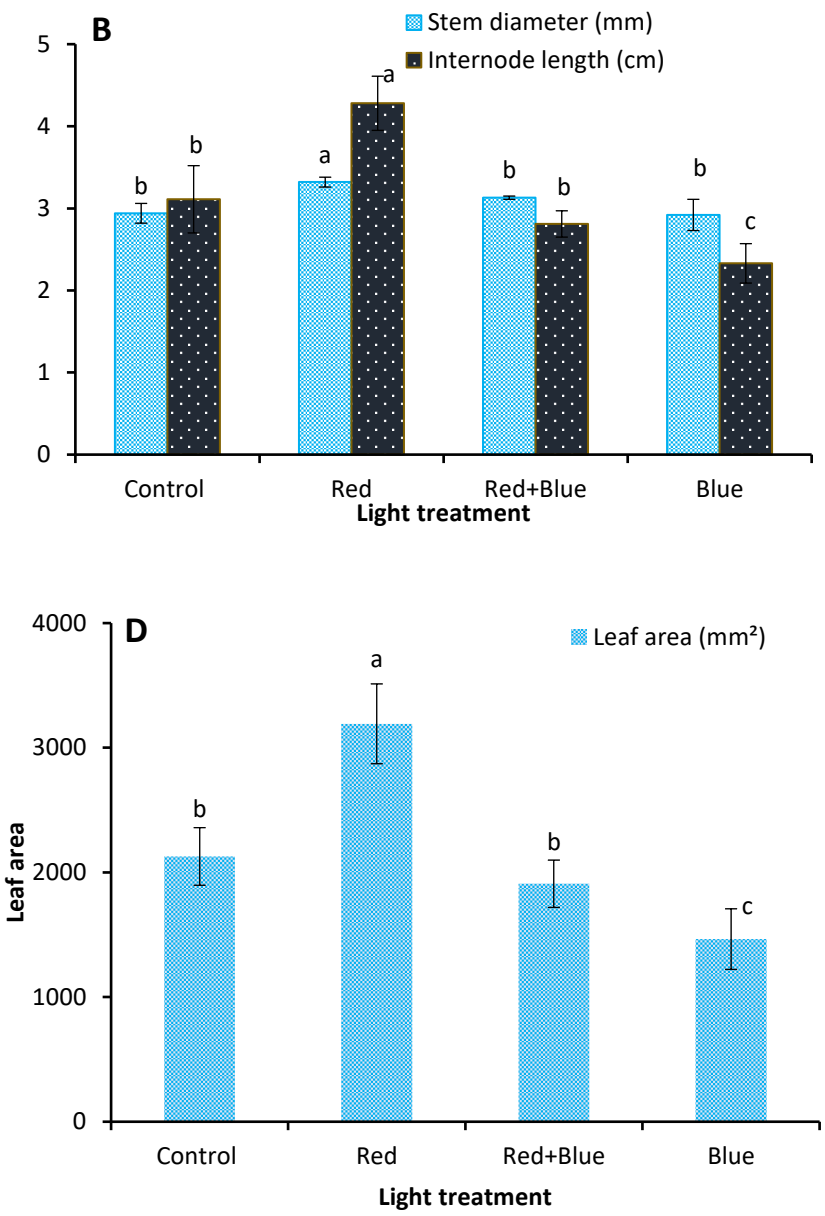


Fig. 1. Effect of light quality at the end of the day on morphological properties of the lemon balm. Values represent the means of three replications (n=6) ± standard deviation. In each trait, means with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

The effects of light quality on fresh weight and dry weight of the plant, its shoots and roots, were significant at 1% and 5% probability levels, respectively (Table S2). The highest mean values for plant fresh weight (394.2 g) and root (83.7 g) were obtained in plants treated with red light, with significant differences from the other treatments. The lowest whole plant and root fresh weights were observed in the control treatment. The red light resulted in plants with the highest shoot fresh weight, indicating a significant difference from the combined light treatment. However, the effect of the combined light showed no significant difference from that of the blue light. The lowest fresh weight of shoots was recorded in the control group, with no significant difference from the blue light treatment (Fig. 2). The highest dry weight of the plants was observed in the red light treatment, which showed a significant difference from the other treatments.

No significant difference was observed between the combined treatment and blue light treatment. Without a significant difference with blue light, plants treated with red light had the highest root dry weight. Moreover, the root dry weight was not significantly different from the values obtained under blue light, the combined light, and control. Maximum shoot dry weight was recorded in red light treatment followed by red and blue light-treated plants with significant differences. The minimum dry weight of shoots was recorded in control plants with no significant difference from the blue light treatment group (Fig. 2).

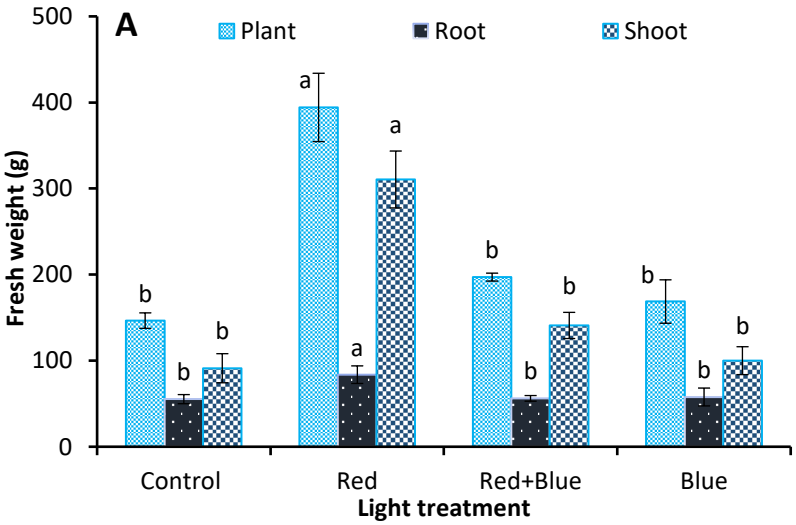
Leaf yield and dry weight percentage

The effect of light quality at the EoD on leaf yield was not significant, but it affected the percentage of dry weight ($P \leq 0.01$) (Table S2). The highest percentage of dry weight of plants was observed in blue light-treated plants, followed by red+blue and red light-treated plants with no significant differences. The lowest percentage of dry weight was observed in the control group (Fig. 2).

Table S2. Effect of the light quality at the end-of-the-day on the fresh and dry weight of the plant, root, stem, and leaf, leaf yield and dry matter percentage of lemon balm.

Treatments	Plant fresh weight (g)	Root fresh weight (g)	Shoot Fresh weight (g)	Plant dry weight (g)	Root dry weight (g)	Shoot dry weight (g)	Leaf yield (%)	Dry matter (%)
Control (without light)	146.5 ± 9.0 ^{b}	55.3 ± 5.3 ^{b}	91.2 ± 16.9 ^{c}	21.87 ± 0.68 ^{b}	8.55 ± 2.16 ^{b}	13.31 ± 2.65 ^{c}	30.94 ± 1.92 ^{a}	14.94 ± 0.37 ^{b}
Red	394.2 ± 39.7 ^{a}	83.7 ± 10.2 ^{a}	310.5 ± 33.1 ^{a}	69.54 ± 9.69 ^{a}	18.04 ± 4.08 ^{a}	51.50 ± 6.97 ^{a}	37.42 ± 0.57 ^{a}	17.66 ± 0.27 ^{a}
Red+Blue	197.1 ± 4.6 ^{b}	56.2 ± 3.26 ^{b}	140.9 ± 15.2 ^{b}	34.95 ± 1.28 ^{b}	11.65 ± 1.26 ^{b}	23.29 ± 2.53 ^{b}	38.44 ± 6.08 ^{a}	17.73 ± 0.66 ^{a}
Blue	168.7 ± 25.2 ^{b}	57.8 ± 10.4 ^{b}	100.0 ± 16.2 ^{bc}	31.46 ± 7.20 ^{b}	14.63 ± 4.40 ^{ab}	16.83 ± 3.18 ^{c}	33.92 ± 4.27 ^{a}	18.60 ± 0.38 ^{a}
Significance	**	*	**	**	*	**	ns	**

Values represent the means of three replications (n=6) ± standard deviation.
Means with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.
** and * means significant at 1% and 5%, respectively.



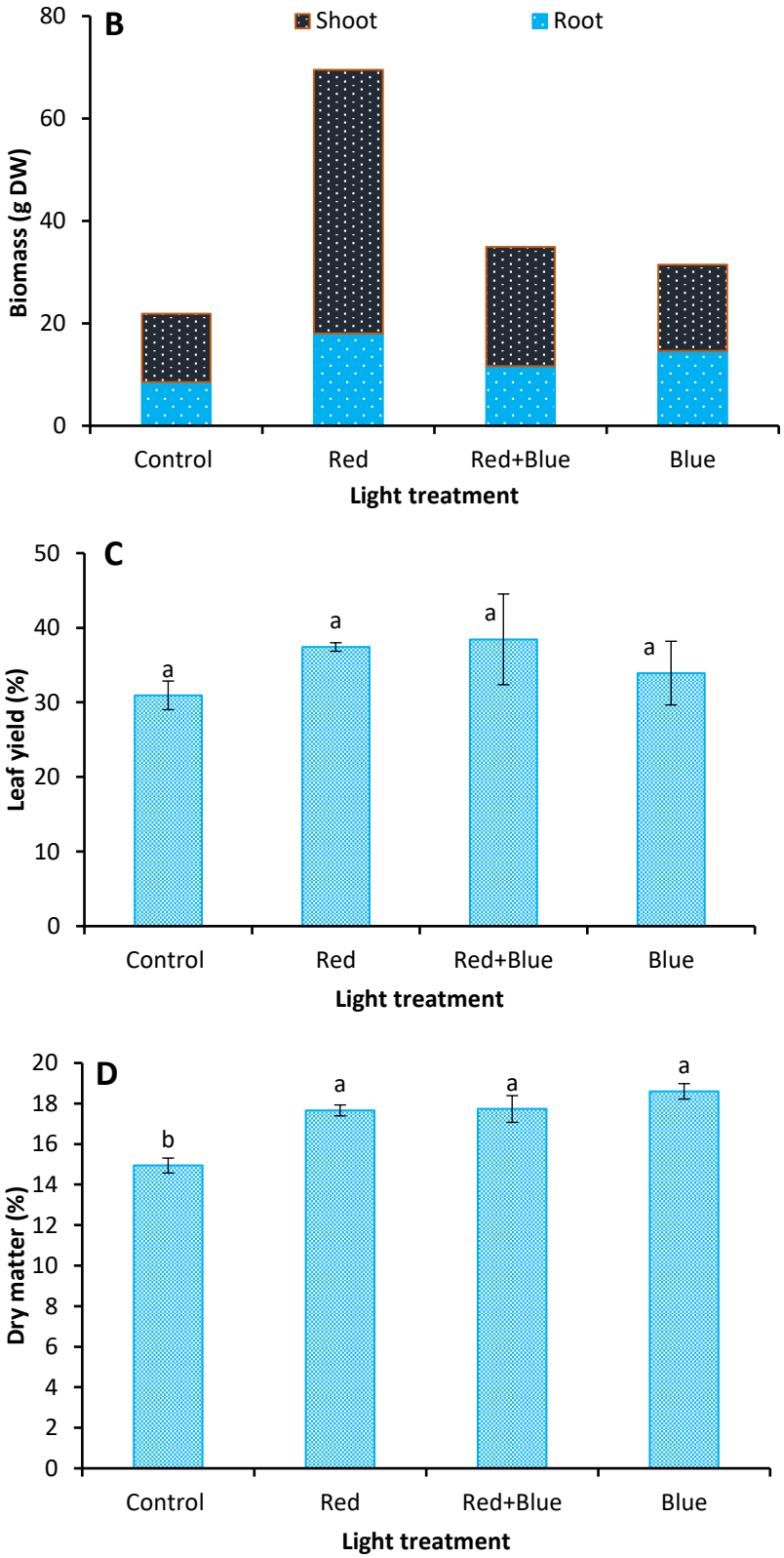


Fig. 2. Effect of the light quality at the end of the day on the plant, root and stem fresh (A), and dry weight (B), and leaf yield (C), and dry matter percentage (D) of the lemon balm. Values represent the means of three replications (n=6) ± standard deviation. In each trait, means with the same letters are not significantly different at $P \leq 0.05$ by Duncan's multiple range test.

Chlorophyll and carotenoids concentration

The effect of light quality on chlorophyll content, chlorophyll a, and carotenoids was significant (P≤0.01), but had no significant effect on chlorophyll b. Maximum total chlorophyll content was observed in the blue light treatment followed by red and combined light with significant differences. The lowest total chlorophyll content was observed in the control group which showed no significant difference with the red+blue light treatment. The highest levels of chlorophyll a were recorded in blue light treated plants followed by the combined light and red light-treated plants with significant differences. The lowest chlorophyll a was observed in the control treatment. The blue light treatment resulted in plants with the highest carotenoid content. The lowest carotenoid content was observed in control plants, with no significant difference from the red light-treated plants (Fig. 3).

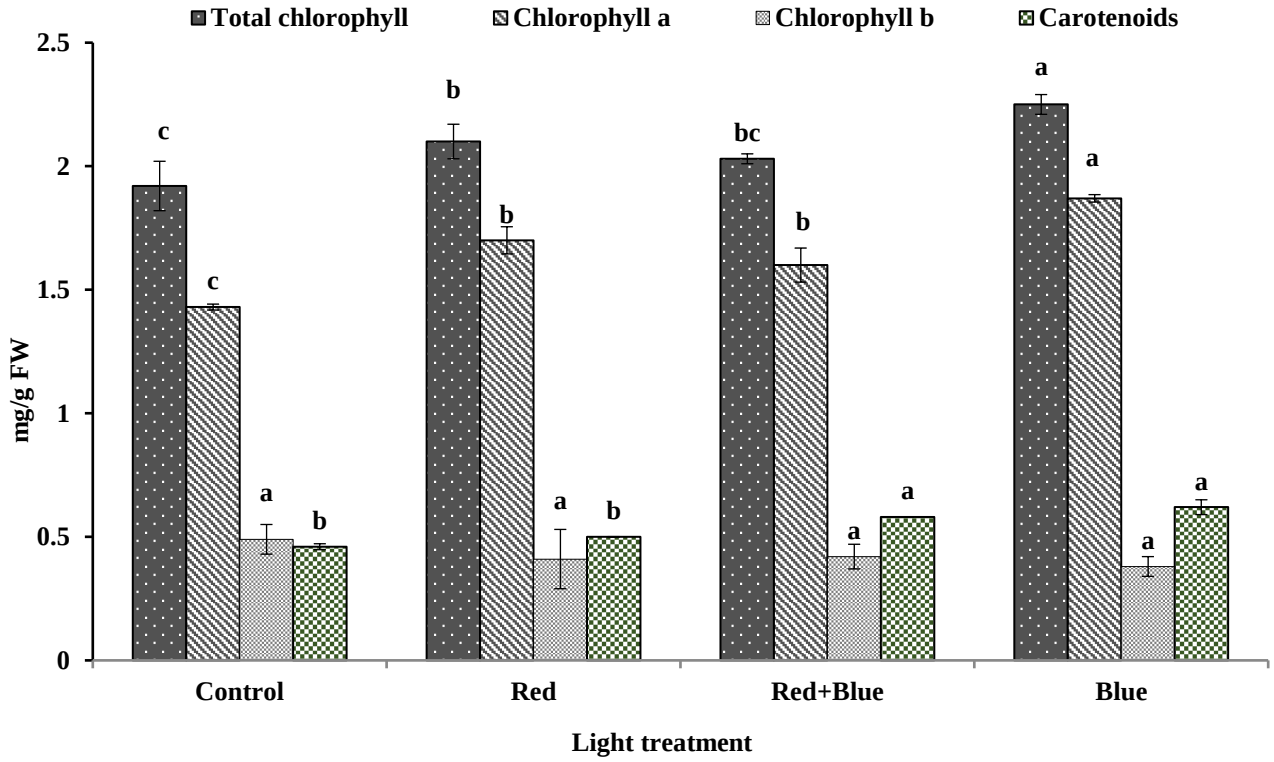


Fig. 3. Effect of the light quality at the end of the day on chlorophyll and carotenoid content of lemon balm. The values represent the means of three replications (n=6) ± standard deviation. In each trait, mean values with the same letters are not significantly different (P≤0.05) by Duncan's multiple range test.

Carbohydrates and soluble proteins

Light quality significantly affected the carbohydrate and protein content in lemon balm (P≤0.01) (Table S3). Total carbohydrate concentration was measured in the range of 11.54 to 8.25 mg g^{-1} FW. The highest amount of carbohydrate was observed in the red light treatment, which showed no significant difference from the red+blue light treated group and the control group (Fig. 4).

The soluble protein concentration ranged from 7.11 to 13.00 mg g^{-1} FW. The highest concentration was recorded in the blue light treated plants which were not significantly different from those of red+blue treated plants. The lowest concentration of soluble protein was measured in the control plants (Fig. 4).

Table S3. Effect of the light quality at the end-of-the-day on carbohydrate and protein content and antioxidant activity in lemon balm.

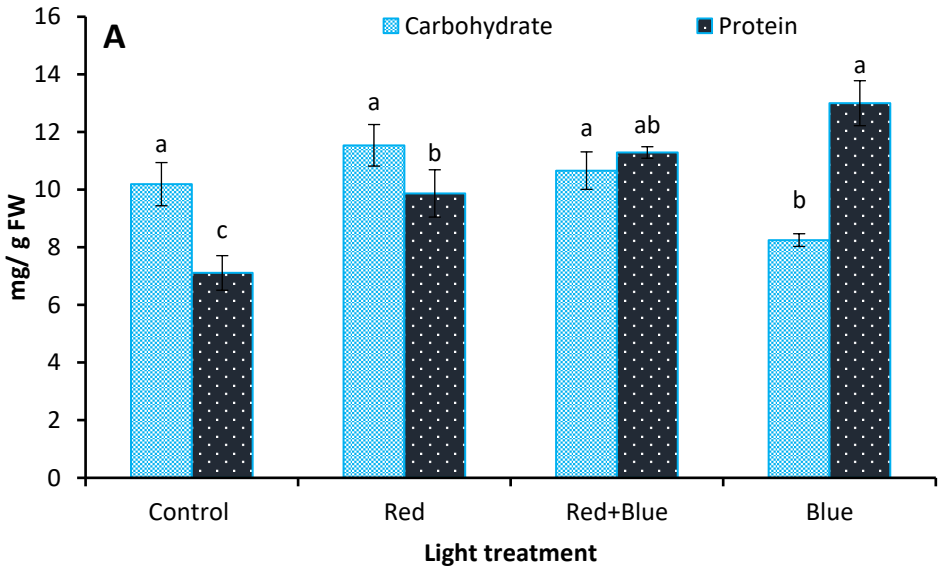
Treatments	Carbohydrate content (mg g^{-1} FW)	Protein content (mg g^{-1} FW)	Antioxidant activity (%)
Control (without light)	10.19 ± 0.75 ^{a}	7.11 ± 0.60 ^{c}	33.08 ± 0.77 ^{b}
Red	11.54 ± 0.72 ^{a}	9.87 ± 0.82 ^{b}	36.40 ± 0.53 ^{a}
Red+Blue	10.66 ± 0.65 ^{a}	11.29 ± 0.20 ^{ab}	32.19 ± 0.58 ^{b}
Blue	8.25 ± 0.22 ^{b}	13.00 ± 0.78 ^{a}	35.10 ± 0.27 ^{a}
Significance	**	**	**

Values represent the means of three replications (n=6) ± standard deviation.
Means with the same letters are not significantly different at P ≤ 0.05 by Duncan's multiple range test.
** means significant at 1%.

Phenolic properties and antioxidant capacity

Total phenolic, flavonoid and anthocyanin concentrations and antioxidant activity of lemon balm were significantly affected by light exposure with EoD (P≤0.01); however, tannin content was not significantly affected. The red light treatment had the highest total phenolic concentration at 48.7 mg g-1 extract weight, followed by blue light treatment, which were significantly different. The lowest total phenolic concentration was observed in the control plants (Fig. 5).

The total flavonoid concentration ranged from 17.95 to 24.81 mg g^{-1} of extract. The highest flavonoid content was observed in blue light treated plants which were significantly higher than the other values. The lowest flavonoid concentration was observed in the control group, with no significant differences from the red+blue and red light treatments. Total anthocyanin content ranged between 2.65 and 4.87 mg g^{-1} extract weight. Plants treated with red light had the highest anthocyanin concentration followed by red+blue light treatment with no significant differences. Moreover, no significant difference was observed between the anthocyanin levels of the combined light and blue light treatment groups. The lowest anthocyanin value was found in the control group (Fig. 5). Also, the antioxidant capacity ranged from 32.19 to 36.40% (Fig. 4). The highest percentage of inhibition was observed in the red light treatment with no significant difference from the results of the blue light treatment. The control and red+blue light treated plants showed the lowest antioxidant capacity.



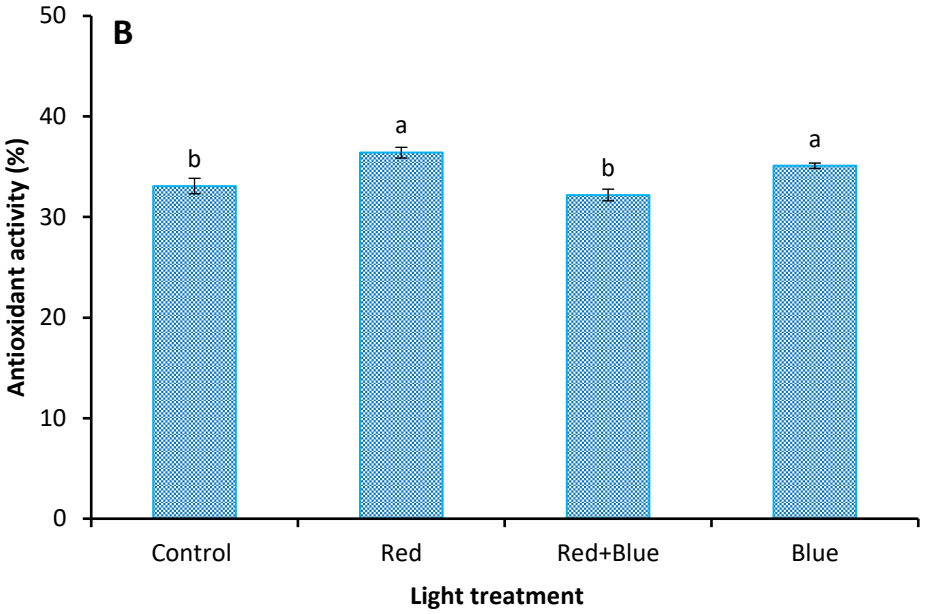


Fig. 4. Effect of the light quality at the end of the day on carbohydrate and protein concentration (A), and antioxidant activity (B) of the lemon balm. Values represent the means of three replications (n=6) ± standard deviation. In each trait, mean values with the same letters are not significantly different (P≤0.05) based on Duncan's multiple range test.

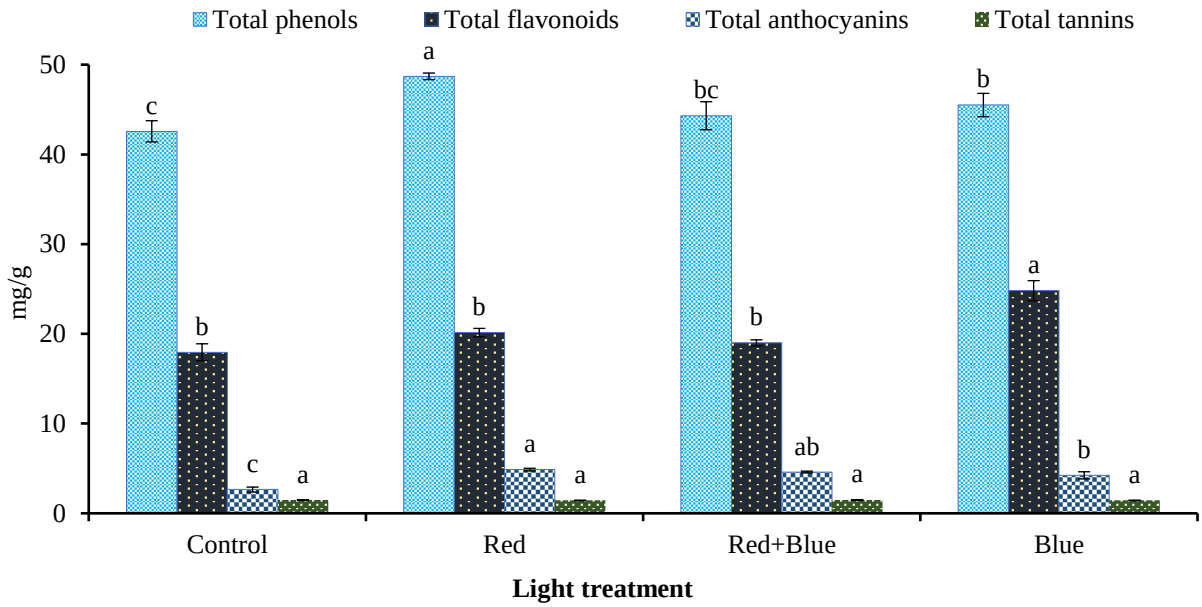


Fig. 5. Effect of light quality at the end of the day on phenolic properties of lemon balm. Values represent the means of three replications (n=6) ± standard deviation. In each trait, means with the same letters are not significantly different (P≤0.05) by Duncan's multiple range test.

Amount of essential oil and compounds

Light quality significantly affected the essential oil content ($P \leq 0.01$) which ranged from 0.39 to 0.73%. Lemon balm treated with red+blue light had the highest essential oil content (0.73%), with no significant difference compared to the red light treatment. The lowest essential oil value (0.39%) was measured in the control group, which was not significantly different from the blue and red light treatment (Table 1). The results showed that the quality of light affected the quality of essential oil. Geranial, neral, caryophyllene oxide, citronella and isocitral accounted for the highest percentages of essential oil composition (Table 1).

The effect of light quality on the compounds accounted for more than 1% in the essential oil, including para-cymene, citronella, isocitral, geranial, geranyl acetate and caryophyllene oxide content, whereas neral and linalool showed no significant difference from each other (Table 1).

The highest para-cymene content was observed in response to red light (6.63%), with a significant difference from plants treated with blue light. The control group had the lowest para-cymene content, with no significant difference from the red+blue light treatment. The blue light treatment resulted in maximum citronellal content (8.56%) in the EO, which was significantly different from the other treatments. This was followed by the red+blue light treatments, the control, and the red light, although no significant differences were observed among them. The highest isocitral ratio was found in the control treatment (5.08%), followed by the red and red+blue light treatments. The lowest amount was found in the blue light treatment (0.49%).

The highest amount of geranial was detected in the control (39.76%), which was not significantly different from the combined light treatment. The lowest amount of geranial was observed in the red light (32.00%), which showed no significant differences from the blue light treatment. The highest amount of geranyl acetate was observed in the control (1.78%), followed by the red, blue and combined light treatments, with significant differences among all treatment groups. The highest amount of caryophyllene oxide was observed in the red+blue light treatment, which showed no significant difference from the blue and red light treatments. The lowest amount of caryophyllene oxide was observed in the control (Table 1).

Discussion

This research confirms previous studies that light quality affects plant growth and morphology by influencing active light receptors such as phytochromes, as well as changes in the levels of plant hormones, particularly auxin and cytokinin (Taiz et al., 2015). In addition, the ratio of red to far-red light causes stem elongation. Similar results have been reported in the case of grapes (Kondo et al., 2014), strawberries (Samuolienė et al., 2010) and sorghum (Warrington and Mitchell, 1976). In another study, leaf area of lettuce increased under red light treatment (Johkan et al., 2010). In the current study, the combination of red and blue light increased the growth of lemon balm, which is consistent with previous results by Flourensia cernua (Estell et al., 2016), Gynura bicolor (Ren et al., 2014), and rapeseed (Li et al., 2013). Blue light often affects plant height by reducing apical dominance as a result of changes in hormonal balance (Li and Kubota, 2009; Huche-Thelier et al., 2016). Red LED radiation is the best light source for higher dry weight production achieved by photosynthesis in plants. It seems that the best light source for dry biomass production depends on the plant species. Red LED has different effects on different plant varieties. In artificial cultivation, plants may prefer red LEDs as a light source to achieve maximum dry weight and output (Islam et al., 2012; Yorio et al., 2001; Karimi et al., 2022).

Fresh and dry weights of plants under red light were higher than other treatments, similar to Chen et al. (2016) and Johkan et al. (2010). Piovene et al. (2015) reported that basil leaf fresh weight was consistently reduced by blue light; however, red light contributed to a 1.5-fold increase in fresh weight. This is consistent with the current research findings. In another study, orange light increased tomato dry matter at the EoD rather than blue light (Matsuda et al., 2016). Although different light regimes are required for different crops, the optimal ratio of blue to red light reportedly depended on crop yield (Piovene et al., 2015). Light quality usually affects photosynthesis and plant morphology, ultimately leading to changes in yield and dry weight percentages. The main findings of the current study showed that light quality does not affect the percentage of leaf yield. According to Huche-Thelier et al. (2016), blue light increased leaf thickness and epidermal cell number, which in turn led to an increase in plant dry weight. In addition, light quality affected the percentage of dry weight, similar to previous studies. Huche-Thelier et al. (2016) showed that blue light resulted in the highest percentage of dry weight, while the minimum stem length and internode length occurred in response to blue light.

Table 1. Effect of light quality at the end of the day on essential oil content (%) and EO composition (%) of lemon balm using gas chromatography mass spectrometry (GC-MS).

Component	RI	Light treatment			
		Control	Red	Red+Blue	Blue
Essential oil content (w/w %)	-	**0.39b**	**0.59ab**	**0.73a**	**0.43b**
p-Cymene	1025	0.99c	6.63a	1.77bc	3.20b
Limonene	1028	0.41	0.29	0.59	0.42
1,8-Cineol	1031	0.44	0.87	0.55	0.65
Melonal	1051	0.34	0.07	0.05	0.02
Linalool	1101	1.02	0.76	0.87	0.75
Isocitral<exo->	1145	0.13	0.18	0.12	0.14
Citronellal	1155	6.58b	6.20b	6.85b	8.56a
Menthone	1156	1.55	0.99	0.79	0.25
Isocitral<Z->	1165	0.26	0.18	0.22	0.10
Isomenthone	1166	0.38	0.23	0.55	0.98
Neomenthol	1168	0.38	1.01	0.35	0.28
Isocitral<E->	1184	5.08	2.37b	1.43c	0.49d
α-Terpineol	1195	0.17	0.11	0.21	0.24
Methyl salicylate	1200	0.05	0.05	0.05	0.03
Neral	1248	25.95	23.54	22.15	22.52
Piperitone	1259	0.57	0.83	0.40	0.53
Methyl citronellate	1261	0.34	0.31	0.30	0.33
Geranial	1279	39.76a	32.00b	38.50a	32.69b
Methyl acetate	1295	1.36	0.12	0.84	0.89
Methyl geranate	1325	0.34	0.34	1.56	0.57
α-Copaene	1378	0.77	0.35	0.44	0.77
Geranyl acetate	1384	1.78a	1.13b	0.36d	0.49c
trans-Caryophyllene	1423	0.33	1.31	0.73	0.41
trans-β-Ionone	1488	0.06	0.05	0.12	0.10
Caryophyllene oxide	1589	8.88b	16.62a	18.34a	17.27a
Viridiflorol	1598	0.10	0.01	0.01	0.02
Humulene epoxide II	1614	0.49	0.64	0.84	0.80
Murrolol<alpha->	1647	0.11	0.10	0.22	0.32
Cadinol<alpha->	1660	0.19	0.39	0.63	0.02
Total	-	98.81	97.68	99.84	93.84

Values represent the means of three replications.
Average values with the same letters in each row are not significantly different (P≤0.05) by Duncan's multiple range test.

Previous studies argued that the blue light-regulated the plant stomata conductance by increasing the stomata aperture. In addition, the stomatal conductance under blue light treatment happens due to the smaller size of the epidermal cells; when the blue light response is regulated by the carotenoids, zeaxanthin, phototropins and cryptochromes (Hernandez, 2013). Red light is of great importance in the stem formation, phytochromes response, and anatomy changes in the plants. Blue light usually benefits chlorophyll biosynthesis, stomatal opening, enzyme synthesis, chloroplast maturity, and photosynthesis. According to Samuoliene et al.

(2010), chlorophyll a and b contents increased in strawberries under red light treatment. However, Piovene et al. (2015) indicated that areas covered by the red light spectrum largely affect photosynthesis. Johkan et al. (2010) also observed that total chlorophyll in the lettuce was lowered by red and blue lights rather than fluorescent light. In response to blue LED, however, chlorophyll a to b ratio and carotenoid content increased.

Total chlorophyll content and carotenoid were affected by the light quality, so that blue light produced the highest levels of chlorophyll and carotenoids, consistent with Kondo et al. (2014). In research conducted by Hogewoning et al. (2010) on *Cucumis sativus*, increasing the blue light from zero to 50% improved photosynthetic capacity.

Carbohydrate and protein levels were affected by light quality. With respect to previous research, blue light increased amino acids, especially aspartic acid and glutamic acid, and soluble protein in the plants. In contrast, red light increased soluble sugars and starch (Warrington and Michel, 1976). Barro et al. (1989) observed that while red light caused a higher accumulation of the soluble carbohydrates and pigments, blue light resulted in more protein accumulation in soybeans. Besides, the activity of nitrate reductase in the leaves under red light was higher than those under blue light. Blue light also increased the synthesis of amino acids and soluble proteins. The increase in carbohydrate content and soluble protein, resulting from exposure to light and light quality, was in line with previous research by Wang et al. (2009) and Samuoliene et al. (2010).

The results of this study indicated that light quality was effective in the production of various compounds such as phenolic compounds, flavonoids, anthocyanins, and the antioxidant capacity of lemon balm. The effects of light quality on the production of phenolic compounds, flavonoids, anthocyanins and antioxidants were previously reported in pea (Wu et al., 2007), Flourensia cernua (Estel et al., 2016), strawberry (Choi et al., 2015) basil (Hosseini et al., 2018) and lettuce (Li and Kubota, 2009; Nagano et al., 2022). The presence of light is necessary for most primary and secondary metabolites. For example, the presence of light increases the production of antioxidants, total phenolic and secondary metabolites in *Artemisia absinthium* cell suspension. In addition, the presence of light stimulates the accumulation of secondary metabolites, including flavonoids, anthocyanins, artemisinin, and caffeic acid derivatives, while it can inhibit the accumulation of secondary metabolites such as nicotine and chiconin (Ali and Abbasi, 2014).

Based on the key results, red light had the greatest influence on the increase in phenolic and antioxidant compounds, which confirmed previous results by Wu et al. (2007) and Estel et al. (2016). The mechanism of red light on increasing phenolic compounds and antioxidant capacity is still unknown. One assumption is that ROS such as O_2- and H_2O_2 under monochromatic light may activate plant antioxidant systems (Cruces et al., 2017; Liu et al., 2018). Nevertheless, this needs further investigation. Meanwhile, contradictory results exist in the literature. For example, Qian et al. (2016) observed that in Chinese kale sprouts, blue light increased the antioxidant content rather than red light. These differences may be attributed to the dissimilarities in the type of phenolic compounds in the plants, as well as the light exposure procedures and conditions. In this study, the lemon balm plant was exposed to EoD light.

The flavonoid content was also increased in lemon balm exposed to the EoD light. This corresponded with findings by Piovene et al. (2015), where the antioxidant, phenol, and flavonoid activity of the basil increased, compared to the control treatment. Furthermore, by increasing the blue light intensity, there were increases in antioxidant capacity, anthocyanins accumulation, and flavonoids (Ren et al., 2014). Ouzounis et al. (2014) found that blue light increased phenol and flavonoid contents in roses, *Chrysanthemums* and *Campanulas*, suggesting that blue light improved resistance to stress.

It is revealed in various studies that blue light increases anthocyanins in plants (Seo et al., 2015; Kondo et al., 2014; Piovene et al., 2015; Baek, 2013; Liu et al., 2022). In contrast to the current research, Kondo et al. (2014) stated that maximum levels of anthocyanin production in grapes occurred by exposing them to blue and then red lights at night. In another study, long-wave radiation such as red light stimulated the biosynthesis of anthocyanins and flavonoids in tomatoes (Lange et al., 1971). Kondo et al. (2014) observed that red LED increased the anthocyanin content in grapes through gene expression, thereby increasing the amount of ABA and enhancing the positive association between anthocyanin and ABA values. Differences in previous research may be due to plant variety, treatment type, and time.

Lemon balm essential oil was influenced by light quality. In an experiment conducted on peppermint, red and combined lights, involving a combination of red and blue lights, significantly increased the percentage of essential oil in

peppermint. The combined light enhanced the functional properties of the peppermint essential oil by up to 2 times. The red light may affect the metabolic pathways, leading to an increase in the essential oil. It is also assumed that light influences hormonal balance in plants (Sabzalian et al., 2014). However, the exact mechanism of changes in the essential oils under various light treatments is still unknown and more research is needed to clarify the positive effect of light quality on essential oils.

So far, research has identified compounds in lemon balm essential oil. Different factors such as the location of plant growth, planting time and stage, type of planting, and other environmental factors influence the essential oil composition. Thus, different percentages of compounds were reportedly indicated in various cases of research (Sorensen, 2000; Son et al., 2021) .Several evaluations have revealed that geranial makes the bulk of the chemical composition in lemon balm, followed by neral, caryophyllin oxide, and citronella (Sorensen, 2000). In general, the findings of the current research revealed that light quality increased the amount of essential oil, although it reduced the quality of the EO because of an increase in caryophyllene oxide, an undesirable component.

Conclusion

Supplemental EoD lighting remarkably affected lemon balm in terms of plant growth, yield, stem length and diameter, internode length, and leaf area in short-day conditions. The highest growth rate, fresh, and dry weights were observed in plants irradiated with red light. It seems that red light radiation through EoD lighting can increase vegetative growth, although the underlying mechanism requires further evaluation. In addition, light quality in the EoD proved effective on phenolic compounds, flavonoids, anthocyanins, and antioxidant capacity. The highest antioxidant properties occurred in response to the red light. Flavonoids and soluble proteins reached maximum amounts under the blue light, whereas the red light treatment resulted in the maximum amount of soluble carbohydrates. Light quality through the EoD significantly affected the content and composition of essential oil. The maximum essential oil percentage was obtained in lemon balm plants grown under the combined red+blue, followed by red light. The main components of essential oils such as geranial and citronellal were influenced by the quality of the end-day light.

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Author contribution statement

Sarikhani and Azizi conceived the research idea. Aghakarim, Sarikhani and Azizi participated in the design. Aghakarim performed experiments and analysis. Sarikhani and Azizi encouraged Aghakarim to investigate morphological and biochemical analysis. All authors participated in the interpretation of data, writing the paper and participated in its revision. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors indicate no conflict of interest in this work.

Data availability

Data are available on request.

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