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Original article

Evaluation of Phytochemical and Biochemical Patterns of Lemon Verbena (*Lippia citriodora* H.B.K.) at Different Temperatures

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Abstract

Background and objectives: Lemon verbena (*Lippia citriodora* H.B.K.) from Verbenaceae family, as an aromatic and medicinal plant, has attracted interests for its valuable essential oil (EO). This study was conducted to evaluate the effect of various temperatures on phytochemical, biochemical, and allometric traits of lemon verbena leaves. **Methods:** The experiment was designed on the basis of randomized complete block design (RCBD) with treatments of 5, 10, 15, 20, and 25 °C, and three replications. **Results:** The results showed that the EO content, main components, and chemical classes, except for oxygenated sesquiterpenes were enhanced by increasing the temperature from 5 to 25 ° C, while pigments, total soluble solid, proline, and soluble proteins were conversely decreased by increasing temperature. The highest fraction of variance among these variables was observed in the neral, EO, polyphenols and anthocyanins, respectively. According to cluster analysis (CA), the effect of temperature on the content of EO, main components, and chemical classes were classified into three groups (A: 5 and 10 °C, B: 15 and 20 °C, and C: 25 °C). Also, dendrogram cluster analysis showed three temperature groups (A: 5 °C, B: 10 °C, and C: 15-25 °C) on the basis of biochemical traits. **Conclusion:** The present study showed that the content of oxygenated sesquiterpenes and antioxidant pigments in contrast to the amount of EO were severely increased by decreasing the environmental temperature. These results clarify the quality and economic value of this plant at the time of harvesting and environmental conditions for the pharmaceuticals, health, and food industries.

Keywords: allometric traits; essential oil; geranial; neral; terpenes

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Introduction

Lemon verbena (*L. citriodora* H.B.K.) from Verbenaceae family is originated from South America and cultivated in northern Africa, southern Europe and some parts of Asia. Furthermore, it grows exclusively in the north of Iran, especially in Golestan province. As an aromatic and medicinal plant, it has been used in folk medicine for centuries. Extract of this perennial plant includes aromatic substances such as polyphenols, mainly flavonoids, which are responsible for the pharmacological properties of lemon verbena [1], such as analgesic, anti-inflammatory and anti-oxidant effects [2-5]. Monoterpenes and sesquiterpenes are the two main chemical classes of compounds found in the essential oil (EO) of this economically important plant. According to According to researches findings, these components act as fumigants, insect repellents or insecticides. The main EO components of this plant include geranial, neral,

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limonene, 1,8-cineole, spathulenol, nerol, geraniol, trans-β-caryophyllene and geranyl acetate [6-8]. Valuable pharmacological properties of lemon verbena EO has attracted scientific and health industries interest. The science of study and measurement of the metabolites relationships, known as allometry, is necessary for the comprehension of the effects of environmental stresses on the major EO components and the plant yield. High or low temperature influences the growth, yield and quality of plant production. The optimum range of temperature for the growth of lemon verbena is 12-25 °C. Lower temperature, as one of most serious environmental stresses, interrupts normal plant growth at the morphological, physiological and biochemical levels [9-14] and causes reversible or irreversible alterations depending on its duration and intensity [15]. The visible effects of low temperature are surface lesions, water loss [16], drying of the edges or tips of leaf blades [17] and in the severe and long lasting chilling stress leaf necrosis and plant death. About the physiological and biochemical effects, the soluble protein content has been declined in sensitive plants to low temperature [18-21]. Temperature plays an important role in the proportion of liberated phenols and flavonoids in lemon verbena [22]. Previous studies reported that the photosynthetic parameters were affected by low temperature [23].

In a chemotype of *Geranium*, excessive temperature rise reduced EO content but increased percentage of citronellol. Geraniol and citronellol showed a very negative correlation, probably due to their precursor and product relationship [24]. The content of monoterpenes in EO of *Mentha piperita* L. changed by high day temperature [25]. Considering these findings and this research results, the qualitative and economical value of such plants will be specified at the time of harvesting and under different environmental conditions for the pharmaceuticals, health, and food industries. The aim of the present study was to evaluate the effect of induction of different temperatures on the phytochemical, biochemical and allometric properties of lemon verbena (*L. citriodora*) leaves.

Materials and Methods

Plant materials

Rooted cuttings of *L. citriodora* H.B.K. were obtained from the research greenhouse of Institute of Medicinal Plants, ACECR (35°54´ N and 50°53´ E; 1235 m above sea level). Voucher specimen 101 (MPIH) has been deposited at the Institute of Medicinal Plants Herbarium (IMPH). Plants were grown in a greenhouse at day and night temperatures of 20 ± 2 °C and 16 ± 2 °C, respectively, a relative humidity of 55 ± 5 %, an average photosynthetically active radiation of about 650 mmol/m^2s^1 and a 16/8 h light/dark cycle. The propagules were planted in plastic pots (0.24 m height × 0.20 m width) filled with 5.5 kg soil. The soil was loam-silt with 0.071% nitrogen, 48.9 mg kg^{-1} phosphorous, 33.6 mg kg^{-1} potassium, EC 2.71 dS/m, and pH 8.3.

Treatment

In greenhouse, the experiment was conducted on the basis of randomized complete blocks design (RCBD) with five treatments and three replications. The factor was different temperatures of 5, 10, 15, 20 and 25°C. The different temperatures were induced on completely identical and annual verbena plants with approximately 80 cm height for 4 days. The temperature treatments were carried out in a Conviron PGV-36 chamber (Controlled Environments Ltd) with relative humidity of 70-80%, a photosynthetic photon flux density of 250 μmol/m^2s^1 provided by metal halide lamps, and a 12 h photoperiod. After temperature induction, the traits were measured. The studied traits were chlorophylls a, b and total chlorophyll (mg/g FW), carotenoids (mg/g FW), lycopene (mg/g FW), ß-carotene (mg/g FW), anthocyanins (mg/g FW), total soluble solid (TSS) (mg/g FW), polyphenols (mg/g DW), flavonoids (mg/g DW), soluble proteins (mg/g FW), proline (mg/g FW), EO content (%), EO main components (neral, geranial and spathulenol) (%), monoterpene hydrocarbons (%), oxygenated monoterpenes (%), sesquiterpene hydrocarbons (%), oxygenated sesquiterpenes (%), allometric traits (monoterpenes/sesquiterpenes, monoterpenes/EO, sesquiterpenes/EO). Antioxidant pigments chlorophylls a, b, total chlorophyll and carotenoids concentrations were determined using a spectrophotometer following the method of Arnon [26]. Fresh leaf sample of 0.2 g was extracted in 10 mL 80% acetone solution, then samples were centrifuged for ten minutes at a rate of 1600 rpm. The absorption spectrums (spectrophotometrically) were measured at 663,

645 and 470 nm. For determination of anthocyanins concentration, 0.2 g fresh leaves was extracted in 15 mL glass centrifuge tubes containing 10 mL of acidified methanol (methanol: HCl, 99:1, v:v) and kept overnight in the dark. The volume was adjusted to 10 mL with methanol and OD was measured at 550 nm. Anthocyanins concentration was calculated using an extinction coefficient of 33,000/mol cm[27].

Chlorophyll a mg/g FW) = 12.7(OD663)-2.69(OD645) × Volume/1000 × Weight (1)
Chlorophyll b (mg/g FW) = 22.9(OD645) - 2.69(OD663) × Volume/1000 × Weight (2)
Total chlorophyll (mg/g FW) = 20.2(OD645) + 8.02(OD663) × Volume/1000 × Weight (3)
Carotenoids (mg/g FW) = 100(OD470) - 3.27(Chlorophyll a) - 104(Chlorophyll b)/227 (4)
Lycopene 503 = (A×537×V) / (172×1000×V) (5)
ß-carotene 480= (A×537×V) / (139×1000×V) (6)

Proline and soluble protein measurement

Free proline was extracted from 0.5 g fresh leaf samples in 3% (w/w) aqueous sulphosalicylic acid, and was estimated using ninhydrin reagent. A spectrophotometer (RAY LEIGH UV-2601) at 520 nm wavelength was applied and the absorption was recorded. The appropriate proline standards were included in the calculation of its content in the samples tested [28]. Protein content measurement was performed by Bradford method [29].

Flavonoids measurement

The amount of flavonoids in the extracts was measured spectrophotometrically as previously reported [30]. Briefly, 500 µL of each extract was mixed with 1.50 mL of 95% ethanol, 0.10 mL of 10% aluminium chloride ($AlCl_3$.6H2O), 0.10 mL of sodium acetate ($NaC_2H_3O_2.3H_2O$) (1 M) and 2.80 mL of distilled water. After incubation for 40 min, absorbance was measured at 415 nm using a spectrophotometer. To calculate the concentration of flavonoids, a calibration curve was prepared using quercetin as the standard. The flavonoid concentration was expressed as quercetin equivalents in mg per gram of the extract. All assays were carried out in triplicate.

Polyphenols content determination

Polyphenols content was determined with the Folin-Ciocalteu reagent according to a procedure described by Singleton and Rossi [31]. Briefly, 0.50 mL of the diluted sample was reacted with 2.5 mL of 0.2 mol/L Folin-Ciocalteu reagent for 4 min, and then 2 mL saturated sodium carbonate solution (about 75 g/L) was added to the reaction mixture. The absorbance readings were taken at 760 nm after incubation at room temperature for 2 h. Gallic acid was used as a reference standard, and the results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of herbal material.

Total soluble solid (TSS) determination

For measurement of total carbohydrates and soluble sugars, a phenol-sulfuric acid assay was used [32]. A volume of 0.5 mL of 5% (v/v) phenol solution and 2.5 mL of concentrated sulfuric acid were added to 0.5 mL aliquots. The mixture was shaken, heated in a boiling water-bath for 20 min and cooled to room temperature. The absorption was then determined by spectrophotometry at 490 nm.

Isolation of the EO and GC/MS analysis

For EO isolation, the harvested leaves were dried 3 days at 40 °C in oven. Then 50 g of the ground leaves were subjected to hydro-distillation for 3 h using Clevenger-type apparatus, according to British Pharmacopoeia [33]. The oils were dried over anhydrous sodium sulphate and kept at 4 °C until analysis. The experiment was repeated three times and the mean was reported as the percentage of EO content on the dry plant.

The analysis was performed on an Agilent 6890 GC coupled with an Agilent 5973 Mass system equipped with a flame ionization detector (FID) and a BPX5 capillary column (30 m × 0.25 mm; 0.25 µm film thicknesses). Temperature program included oven temperature held for 2 min at 60 ºC, enhanced to 150 ºC with 3 ºC/min. Then, temperature enhancement was programmed up to 270 ºC as 5 ºC/min. This temperature was held for 15 min. The carrier gas was He with a flow rate of 1.1 mL/min; injector and detector temperatures were 300 ºC, and the split ratio was 1:50. Mass spectra were taken at 70 eV. Retention indices were calculated by using retention times of n-alkanes (C_8-C_{20}) that were injected at the same temperature and conditions. The mass spectra and retention indices of EO components were identified by comparison to published literature and presented by the MS computer library [34,35].

Statistical analysis

The analysis was done on the basis of

randomized complete blocks design using the SPSS software (ver. 24). Also, Duncan's multiple range tests was used to compare treatment means at a probability level of 0.05. Regression equations ($p \leq 0.05$) for traits of EO, monoterpenes and sesquiterpenes content as a function of temperature treatment were summarized by SigmaPlot 12.5 software for Windows (Systat Software (2), Inc., San Jose, CA, USA).

Results and Discussion

According to the variance analysis results, effect of different temperatures was significant ($p \leq 0.01$) on the biochemical traits of lemon verbena (*L. citriodora*.) leaves (table 1). The variance analysis on different phytochemical and allometric traits of *L. citriodora* showed significant effect of various temperatures on all traits except for two allometric properties of geranial/monoterpenes and spathulenol/sesquiterpenes (tables 2 and 3).

As the mean comparisons of the pigments assay in lemon verbena showed, the highest content of chlorophyll a was observed in plants at 10 °C, while the least was obtained at 25 °C. By increasing temperature from 5 to 10 °C, the amount of chlorophyll a was increased, while in higher temperatures the content was decreased. Furthermore, there was no significant difference of chlorophyll a content in plants at 5 and 20 °C. Chlorophyll b reached the maximum and minimum amount at 10 and 25 °C, respectively. According to the results, the amount of chlorophyll b raised when temperature increased from 5 to 10 °C, but higher temperatures had inverse effect and caused the least level at 25 °C. The total chlorophyll content reached the highest level at 10 °C, while the minimum amount attained at 25 °C. Increasing the temperature up to 10 °C raised the amount of total chlorophyll, while from 10 to 25 °C it was decreased. The total chlorophyll content in two temperatures of 5 and 20 °C were in the same statistical group without significant difference. The highest carotenoids content was observed in plants treated by 15 °C, while the least was obtained at 25 °C. By raise of the temperature from 5 to 15 °C, the carotenoids content increased; conversely, the higher temperatures declined the amount to 1.140 mg/g FW. The content of carotenoids did not show significant differences at 10 and 15 °C. The maximum content of lycopene was reached at 5°C and the minimum was reported at 25 °C. The amount of lycopene declined by raising the temperature from 5 to 25 °C. The plants in temperatures of 10 and 15 were in the same statistical groups for lycopene content without significant differences. ß-carotene gained the highest amount at 5 °C, unlike the least that was attained at 25 °C. Hence, the greatest content was obtained by decreasing the temperature to 5 °C. The plants exposed to 15, 20 and 25 °C showed almost similar content of ß-carotene without notable differences. Anthocyanins as pigments showed the same trend as ß-carotene and lycopene with the highest content in plants induced by 5 °C and the least at 25 °C. In other words, by increasing the temperature the amount of anthocyanins decreased (table 4).

Considering the results of mean comparisons for biochemical traits, the highest and the lowest amount of total soluble solid (TSS) was obtained in plants at 10 °C and 25 °C, respectively. The temperature increment from 5 to 10 °C raised the amount of TSS, while the higher degrees caused the lower TSS content. The plants treated by temperatures of 5, 20 and 25 °C were in the same statistical groups without significant differences. Polyphenols showed the highest content at cold stress of 5 °C, while the least was attained at 25 °C. This result showed that by increasing temperature from 5 to 25 °C, the content of polyphenols decreased and the plants in temperatures of 20 and 25 °C did not show significant differences. The highest value of flavonoids content was observed at 10 °C; the least was gained at 5, 20 and 25 °C without significant differences. An Arrhenius type response was observed in flavonoids content of plants induced by temperatures from 5 to 25 °C. The maximum value of proline was attained in plants exposed to 10 °C, while the least amount was observed at 25 °C and the higher temperatures of 15 and 20 °C caused decline in proline content without significant differences. The soluble protein content reached the maximum level in plants at 10 °C, while the minimum content was observed in plants at 5 °C. The soluble protein content showed a sharp increase from 5 to 10 °C and in higher temperatures it decreased. The plants in temperatures of 10, 15 and 20 °C showed similar content of soluble protein without significant statistical differences (table 5).

Table 1. Analysis of variance for the effects of different temperatures on the biochemical traits of lemon verbena (*Lippia citriodora*).

S.O.V	df	Mean square											
		Chl. a	Chl. b	Total Chl.	Caro	Lyco	ß-caro	ACNs	TSS	PPs	Flavo	Pro	SP
Block (rep)	2	0.012^{ns}	0.136^{ns}	0.018^{ns}	0.035^{ns}	0.00003^{ns}	0.001^{ns}	0.068^{**}	18.498^{ns}	0.900^{ns}	1.070^{ns}	18.618^{**}	3.805^{**}
Temp.	4	0.516^{**}	0.305^{**}	1.558^{**}	5.220^{**}	0.001^{**}	0.072^{**}	0.564^{**}	5093.286^{**}	873.502^{**}	15.563^{**}	448.723^{**}	9.223^{**}
Error	8	0.001	0.008	0.002	0.013	0.000023	0.00041	0.001	21.706	4.049	0.568	0.937	0.277
CV (%)		6.87	22.24	7.84	3.81	25.24	19.10	3.22	11.41	7.36	12.23	4.20	3.44

*,**, ^{ns} shows significant in 5%, 1%, and insignificant, respectively; S.O.V: source of variance; Temp: temperature; Chl. a: chlorophyll a; Chl. b: chlorophyll b; total Chl.: total chlorophyll; Caro: carotenoids; Lyco: Lycopene; ß-caro: ß-carotene; ACNs: anthocyanines; TSS: total soluble solids; PPs: poly phenols; Flavo: flavonoids; Pro: proline; SP: soluble protein

Table 2. Analysis of variance for the effects of different temperatures on the phytochemical traits of lemon verbena (*Lippia citriodora)*

S.O.V	df	Mean square								
		Essential oil	Monoterpene hydrocarbons	Oxygenated monoterpenes	Sesquiterpene hydrocarbons	Oxygenated Sesquiterpenes	Neral	Geranial	Spathulenol	Others
Block (rep)	2	0.002^{ns}	0.001^{ns}	0.787^{ns}	0.002^{ns}	0.550^{ns}	0.738^{ns}	0.459^{ns}	0.284^{ns}	0.001^{ns}
Temperature	4	0.012^{**}	0.015^{**}	6.374^{**}	0.861^{**}	8.956^{*}	9.704^{**}	2.635^{**}	0.593^{*}	0.173^{**}
Error	8	0.001	0.001	3.128	0.085	1.853	0.599	0.412	0.122	0.017
CV (%)		17.56	8.78	4.15	4.04	4.65	5.15	2.78	2.34	9.24

*,**, ^{ns} shows significant in 5%, 1%, and insignificant, respectively; S.O.V: Source of variance

Table 3. Analysis of variance for the effects of different temperatures on the phytochemical and allometric traits of lemon verbena (*Lippia. citriodora*)

S.O.V	df	Mean square					
		Monoterpenes/ sesquiterpenes	Monoterpenes	Sesquiterpenes	Geranial/ Monoterpene	Neral/ monoterpenes	Spathulenol/ Sesquiterpenes
Block (rep)	2	0.0004^{ns}	0.002	0.187	0.0002^{ns}	0.0003^{ns}	0.0001^{ns}
Temperature	4	0.019^{*}	6.999^{*}	4.382^{**}	0.00007^{ns}	0.003^{**}	0.0001^{ns}
Error	8	0.005	1.143	0.500	0.001	0.0003	0.0003
CV (%)		4.64	2.48	1.93	5.96	5.09	4.24

*,**, ^{ns} shows significant in 5%, 1%, and insignificant, respectively; S.O.V: Source of variance

Table 4. Mean comparisons for the effects of different temperatures on the biochemical traits of lemon verbena (*Lippia citriodora*).

Temp.	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	Total chlorophyll (mg/g FW)	Carotenoids (mg/g FW)	Lycopene (mg/g FW)	*ß*-carotene (mg g^{-1} FW)	Anthocyanins (mg g^{-1} FW)
5	0.325^{c}±0.16	0.296^{bc}±0.04	0.274^{c}±0.03	2.215^{c}±0.108	0.057^{a}±0.01	0.376^{a}±0.05	1.665^{a}±0.45
10	1.121^{a}±0.13	0.920^{a}±0.17	1.840^{a}±0.08	4.121^{a}±0.061	0.018^{b}±0.01	0.098^{b}±0.009	1.016^{b}±0.53
15	0.571^{b}±0.08	0.463^{b}±0.23	0.432^{b}±0.11	4.255^{a}±0.128	0.011^{b}±0.0001	0.043^{c}±0.01	0.983^{c}±0.49
20	0.316^{c}±0.15	0.248^{cd}±0.12	0.232^{c}±0.11	3.231^{b}±0.216	0.009^{bc}±0.001	0.007^{c}±0.001	0.634^{d}±0.31
25	0.012^{d}±0.006	0.086^{d}±0.02	0.077^{d}±0.01	1.140^{d}±0.096	0.002^{c}±0.001	0.007^{c}±0.001	0.584^{e}±0.29

*Means in each column followed by the same letter (a-d) are not significantly different according to Duncan's multiple range test at the 5% level of probability. Data represent the means of three replicates ± standard deviation (SD).

Table 5. Mean comparisons for the effects of different temperatures on the biochemical traits of lemon verbena (*Lippia citriodora*)

Temp.	TSS (mg/g FW)	Polyphenols (mg/g DW)	Flavonoids (mg/g DW)	Proline (mg/g FW)	Soluble protein (mg/g FW)
5	18.910^{c}±3.11	51.937^{a}±1.03	4.010^{c}±1.005	12.181^{b}±1.81	12.968^{b}±0.05
10	113.53^{a}±3.23	37.125^{b}±1.43	9.401^{a}±0.70	32.118^{a}±2.05	17.015^{a}±1.49
15	33.531^{b}±6.77	22.500^{c}±2.83	7.604^{b}±0.80	31.850^{a}±2.92	16.380^{a}±0.81
20	22.333^{c}±6.17	12.562^{d}±2.28	5.340^{c}±0.67	31.583^{a}±2.41	16.121^{a}±0.93
25	15.733^{c}±1.12	12.437^{d}±0.83	4.467^{c}±0.86	7.312^{c}±0.656	13.857^{b}±1.07

*Means in each column followed by the same letter (a-e) are not significantly different according to Duncan's multiple range test at the 5% level of probability. Data represent the means of three replicates ± standard deviation (SD).

In this research, the maximum EO content (0.83%) was obtained in plants at 25 °C, while the lowest (0.67%) was observed at 5 °C. Increasing temperature significantly raised the content of lemon verbena EO. The EO content from plants in temperatures of 5 and 10 showed no significant differences and they were almost similar. The trend line in figure 1 shows a polynomial quadratic regression as follows:

$Y=-0.0021x^3+0.025x^2-0.047x+0.6992$; $R^2=0.9851$

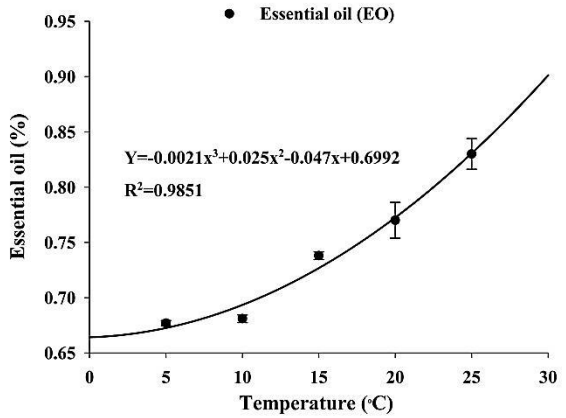

Figure 1. Changes of EO content (%) in lemon verbena (*Lippia citriodora*) leaves exposed to various temperatures (°C) for 4 days. A third order polynomial trend line and related regression equation ($Y=-0.0021x^3+0.025x^2-0.047x+0.6992$, $R^2=0.9851$) are shown. Error bars for all data represent standard deviation (±SD, n = 3) based on Duncan's multiple range tests ($p \leq 0.05$).

The main chemical classes of EO in lemon verbena leaves are monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and others. According to the obtained results, monoterpene hydrocarbons were increased to the highest level (0.45%) at 20 and 25°C without significant differences, while the least content (0.28%) was observed in plants exposed to 5 °C. The increasing temperature caused improvement in monoterpene hydrocarbons content (figure 2a). The maximum and minimum amount of oxygenated monoterpenes (44.21 and 40.59%) was obtained in plants at 25 and 5 °C, respectively. As the temperature increased to 25 °C, the oxygenated monoterpenes content improved simultaneously. The plants exposed to temperatures of 15 and 20 °C were in the similar statistical groups without significant differences (figure 2b). The highest amount of sesquiterpene hydrocarbons (7.89%) was resulted from plants treated by 25 °C and the least amount (6.48%) was attained at 5 °C. The content of sesquiterpene hydrocarbons was augmented in plants by increasing temperature from 5 to 25 °C. The plants treated by 15 and 20 °C showed no significant differences in sesquiterpene hydrocarbons content (figure 2c). Converse to all mentioned chemical classes, oxygenated sesquiterpenes content was decreased by increasing temperature in a way that the highest amount (31.34%) was observed at cold stress of 5 and 10 °C without significant differences and the least of that (26.67%) was obtained at 25 °C (figure 2d). In others chemical class the maximum value (1.71%) was attained in plants exposed to 25 °C, while the minimum value of that (1.10%) was reported at 5 °C and the increasing trend of others class was obtained by increasing temperature (figure 2e). The percent of monoterpenes/sesquiterpenes was raised in plants by increasing temperature from 5 to 25 °C. The highest (1.29) and the least (1.08) rate was attained at 25 and 5 °C, respectively. However, the mentioned rate at 15 and 20 °C did not have significant statistical differences (figure 2f).

By increasing temperature from 5 to 10 °C, the percent of monoterpenes in EO was increased to the highest level, but conversely the sesquiterpenes percent was decreased. The trend lines in figure 3 shows two linear regressions as follows:

$$Y(M)=0.9585x+40.086,\ R^2=0.9845;\ Y(S)=-0.1553x^3+1.2794x^2-3.679x+40.414,\ R^2=0.988.$$

This figure shows the higher amount of monoterpenes in comparison to sesquiterpenes in EO of lemon verbena leaves (figure 3).

According to figure 4, the highest content of neral (17.76%) as the main component of oxygenated monoterpenes was obtained in plants treated by 25 °C, while the least of that (13.01%) was observed at 5 °C. This figure shows that by increasing temperature the amount of neral was improved and the plants exposed to 15 and 20 °C do not have significant statistical differences (figure 4a).

The highest amount of geranial (24.98%) was attained at 20 and 25 °C and the least amount of that (21.20%) was gained at 5 and 10 °C without significant differences.

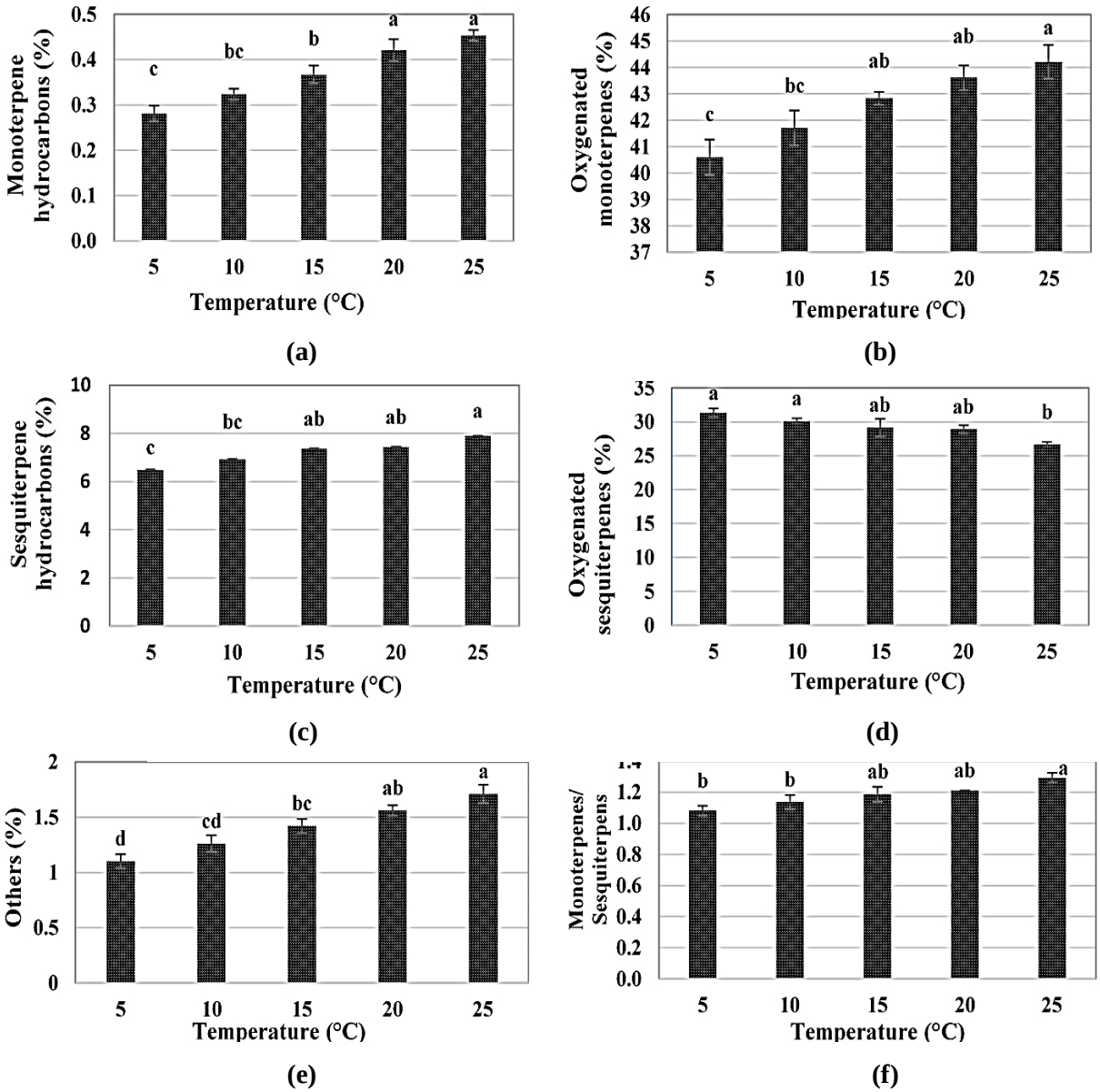

Figure 2. Effects of different temperatures on the percent of monoterpene hydrocarbons (**a**), oxygenated monoterpenes (**b**), sesquiterpene hydrocarbons (**c**), oxygenated sesquiterpenes (**d**), others (**e**) and rate of monoterpenes/sesquiterpenes (**f**) in the EO of lemon verbena (*Lippia citriodora* leaves for 4 days of exposure. Error bars for all data represent standard deviation (±SD, n =3) based on Duncan's multiple range tests ($p \leq 0.05$).

As it is shown by increasing temperature the content of geranial was raised in verbena plants (figure 4b). Neral and geranial are the main components of oxygenated monoterpenes. The content of spathulenol in verbena leaves showed the maximum level (15.28%) at 5 and 10 °C without significant differences, while the least content of that (14.28%) was observed at 25 °C. The rising of temperature to 25 °C caused decrease in spathulenol content (figure 4c). Spathulenol is the main component of oxygenated sesquiterpenes.

In spite of significant effect of temperatures on the content of geranial and spathulenol, the allometric traits of geranial/monoterpenes and spathulenol/sesquiterpenes showed insignificant effect of temperatures. Maybe the reason is the highest value of neral in monoterpenes by increasing the temperature. Neral/monoterpenes reached the highest rate (0.39) at 25 °C, while in the lower temperatures the rate was similar without significant statistical differences. By increasing temperatures the rate of neral/monoterpenes was raised at 25 °C (figure 5).

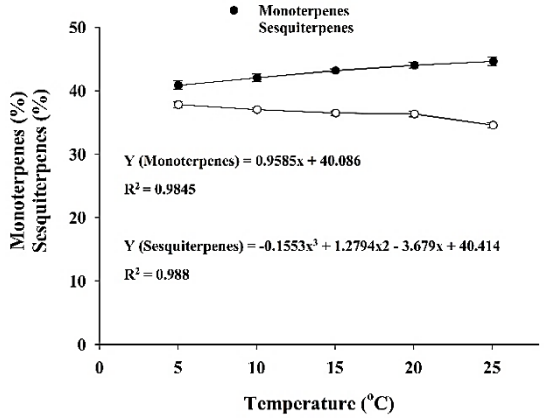

Figure 3. Chnages of monoterpenes/essential oil (M/EO) and sesquiterpenes/essential oil (S/EO) in lemon verbena (*Lippia citriodora*) leaves exposed to various temperatures (°C) for 4 days. The third order polynomial trend lines and related regression equations [Y(M)=0.9585x+40.086, R^2=0.9845; Y(S)=-0.1553x^3+1.2794x^2-3.679x+40.414, R^2=0.988] are shown. Error bars for all data represent standard deviation (±SD, n = 3) based on Duncan's multiple range tests ($p \leq 0.05$).

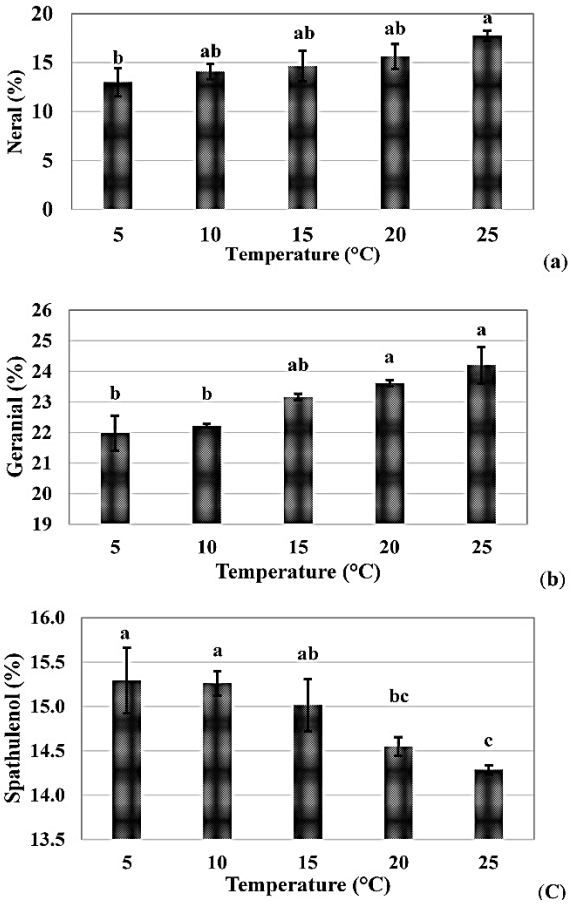

Figure 4. Effect of different temperatures on the percent of main components in EO of lemon verbena (*Lippia citriodora*) leaves for 4 days. (a) neral, (b) geranial, (c) spathulenol. Error bars for all data represent standard deviation (±SD, n = 3) based on Duncan's multiple range tests ($p \leq 0.05$).

Factor analysis provides a reduced dimension model to demonstrate the differences among traits on the basis of principal components. Principal components analysis (PCA) is used for multicollinear data evaluation and determining proper traits for classification. In fact it divides the total variance into different factors.

PCA resulted in five components indicating 90.504% of the total variance. The first three components (PC1-PC3) indicated 80.52% of the total variance. In PC1, anthocyanins, polyphenols, EO and neral had the highest variance. The significant negative correlation was observed between neral and polyphenols. In PC2, ß-carotene, soluble protein and EO and in PC3, monoterpenes/EO and geranial/monoterpenes showed the highest variance. These results indicated that different temperatures influenced significantly on EO content and its main components followed by antioxidants and biochemical traits, respectively (table 6).

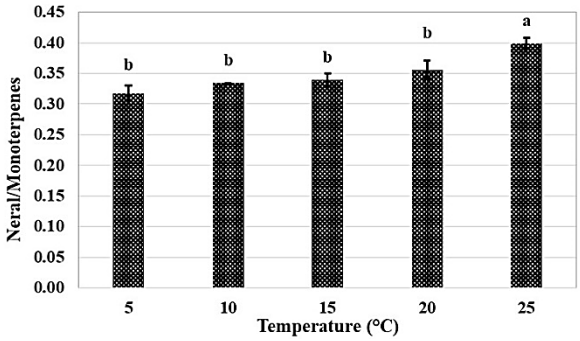

Figure 5. Changes of neral/monoterpenes in EO of lemon verbena (*Lippia citriodora*) leaves in response to different temperatures for 4 days. Error bars for all data represent standard deviation (±SD, n = 3) based on Duncan's multiple range tests ($p \leq 0.05$).

Dendrogram of Wards Cluster analysis (CA) at similarity coefficient of 10, divided the different temperatures into three main groups on the basis of EO content and other phytochemical traits.

The first group included two temperatures of 5 and 10 °C, while the second of that was divided into two temperatures of 15 and 20 °C. The third main group comprised the temperature of 25 °C. The vertical line represents the cut-off criterion for cluster partitioning on 5 (figure 6).

As illustrated in figure 7, the various temperatures were partitioned into three main groups based on biochemical characteristics. The first group included three temperatures of 15, 20 and 25 °C.

Table 6. Eigenvectors of the first three principal component axes from PCA analysis of phytochemical and biochemical properties in lemon verbena (*Lippia citriodora*.) under different temperatures.

Property	Component		
	1	2	3
Total chlorophyll	0.541	0.621	0.200
Carotenoids	0.492	0.787	-0.066
Lycopene 503	0.761	-0.609	0.117
ß-carotene	0.741	-0.655**	0.090
Anthocyanins	0.847**	-0.452	0.166
Soluble protein	0.083	0.908**	-0.126
Total soluble solid	0.478	0.661	0.173
Polyphenols	0.896**	-0.355	0.109
Flavonoids	0.355	0.845	0.264
Proline	0.272	0.832**	0.099
Essential oil	-0.911**	-0.090	0.288
Neral	-0.925**	0.036	0.116
Geranial	-0.836**	0.050	0.157
Spathulenol	0.811**	0.076	0.196
Monoterpene hydrocarbons	-0.889	0.157	0.117
Oxygenated monoterpenes	-0.865	0.209	-0.015
Sesquiterpene hydrocarbons	-0.833	0.225	-0.197
Oxygenated sesquiterpenes	0.809	-0.153	0.167
Monoterpenes/Sesquiterpenes	-0.837	0.151	-0.077
Monoterpenes/Essential oil	0.563	0.711	-0.289**
Sesquiterpenes/Essential oil	0.678	0.617	-0.245
Geranial/Monoterpenes	-0.278	0.060	0.861**
Neral/Monoterpenes	-0.830	-0.020	0.150
Spathulenol/Sesquiterpenes	0.094	0.347	0.365
Others	-0.889	0.170	0.005
Eigenvalue	12.589	6.023	1.519
% of variance	50.357	24.093	6.074
Cumulative (%)	50.357	74.450	80.524

** Eigenvalues are significant ≥ 0.50

The second and the third ones comprised 5 and 10 °C, respectively. The vertical line represents the cut-off criterion for cluster partitioning on 5 (figure 7). By comparison of the results, the highest content of photosynthetic pigments (chlorophylls a, b and total chlorophyll) was related to plants at 10 °C, while the maximum content of other leaf pigments was obtained in plants exposed to 5 °C.These results are according to Zhang et al. research [36]. They suggested that photoprotection increased in response to cold damage by genes coding for electron transporters of photosystem I (PSI) and photosystem II (PSII), PSI P700 chlorophyll a apoprotein A2 (PsaB) and PSII CP47 chlorophyll apoprotein (PabB) were induced by cold in *Santalum album* L. leaves. Low temperature stress for 7 h influenced chlorophyll content in *Chenopodium album* L. The first hours of stress showed the highest amount of chlorophyll content but at the end the lowest content of chlorophyll was observed. This effect on chlorophyll content is related to chlorophyll degradation and pigment photooxidation under environmental stresses [37,38]. The chlorophyll content at 5 °C could not resist the damaging effect of cold stress and antioxidant pigments increased at 10 °C for protective effects on chlorophyll content. Low temperature stress negatively influences the electron transport chain and limits the photosystem II (PSII) [37,39]. Over-accumulation of biochemicals such as proline, carbohydrates and polyphenols protects the photosynthetic pigments from cold stress [40]. Anthocyanins as vacuolar pigments have the role of red, purple, and blue coloration of plant tissues

in many plant species, and also photoprotection in photosynthetic tissues [41-43]. They have been observed in maximum content under high light in combination with cold stress. The obtained findings are in line with the mentioned researches due to protective properties of anthocyanins on photosynthetic pigments against cold stress [44-47].

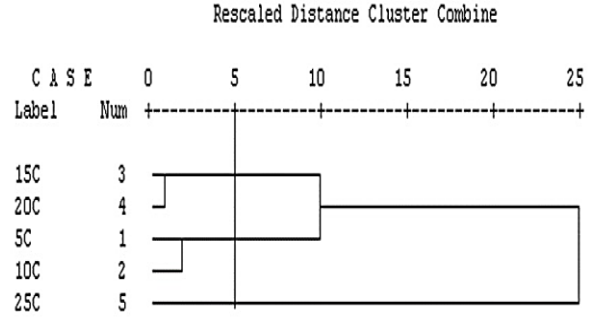

Figure 6. Wards cluster analysis of different temperatures exposed to lemon verbena (*Lippia citriodora*) based on EO and its main groups and components.

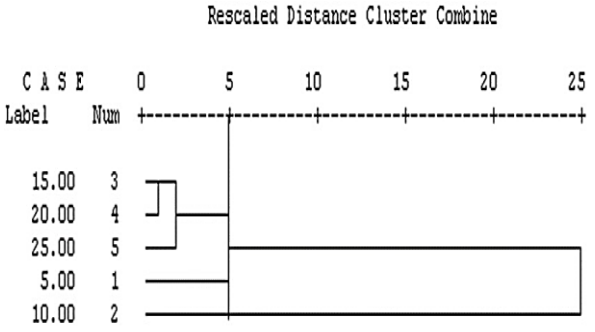

Figure 7. Wards cluster analysis of different temperatures exposed to lemon verbena (*Lippia citriodora*) based on biochemical traits.

According to the results, low temperature caused an increase in the rates of Chlorophylls. a and b accumulation in barely plants. ELIPs (early light-inducible protein) and the PSII-S protein, which are thylakoid polypeptides and are induced under high-light stress and related to the LHCB (PSII light-harvesting complex) family of light-harvesting polypeptides, may also bind carotenoids to protect the photochemical apparatus from potential oxidative damage under stress [48-51]. In addition to quenching the absorbed light energy when bound to antenna polypeptides, it has been proposed that unbound zeaxanthin and other carotenoids may also act to stabilize thylakoid membranes against stress conditions [48,52]**.** The biochemical traits of total soluble solid, flavonoids, proline and soluble protein showed the highest content at 10 °C. However, the greatest amount of polyphenols was attained at 5 °C. These results are in line with results of Shahryar and Maali-Amiri [53]. They reported that carbohydrate abundances increased in different intensities of oxidative stress in wheat plants. Low temperature changes protein and nucleic acid conformation [54] and photosynthesis [55]. In cold stress, protein content is usually declined mainly due to increase in the level of free amino acids especially proline and a sharp decrease in protein synthesis [21]. According to research results, cold stress induced the accumulation of proline and soluble carbohydrates in *Santalum album* L. leaves [36]. As the results showed, critical temperatures of 5 and 10 °C caused marked increase in all the biochemical traits. However, at cold stress of 5 °C as a shock, the plant could not show the defense response and the pigments content increased to protect the photosynthetic organisms form the damaging effect of stress. Biochemicals protect plant membranes and prevent cell disruption by stabilizing membrane lipids, maintaining ion homeostasis and scavenging reactive oxygen species (ROS) [56].

In this study, the amount of EO improved with raising temperature from 5 to 25 °C. By increasing temperature the content of EO chemical classes monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons and others increased except for oxygenated sesquiterpenes. The content of anthocyanins and polyphenols were declined by increasing temperature. Main chemical classes of EO in lemon verbena leaves are comprised of oxygenated monoterpenes with neral and geranial representing the main components followed by oxygenated sesquiterpenes with the main component as spathulenol. The results are similar to those of Catherine et al. on *L. citriodora* H.B.K. at two developmental stages of growth and full bloom [57]. The monoterpenes were the main group in both developmental stages. Furthermore, the content of oxygenated monoterpenes (neral and geranial) was prevailing in comparison to monoterpene hydrocarbons. A comparison of EO chemical classes and main components revealed significant differences in qualitative compositions under different temperatures. By increasing temperature, the main EO classes increased as well as their main components. Cold stress of 5 and 10 °C had negative effect on monoterpene hydrocarbons,

oxygenated monoterpenes (main components of neral and geranial), sesquiterpene hydrocarbons classes and also otherss. Vice versa, mentioned temperatures increased percentage of oxygenated sesquiterpenes (main component to be spathulenol). The maximum content of the four mentioned latter classes and components was related to 25 °C. In peppermint low temperature enhanced the formation of menthone and conversely depressed the accumulation of menthofuran and pulegone [58]. Researches on *Quercus coccifera* resulted that the biogenic volatile organic compound (BVOC) emission responses to temperature (15-55 °C) was mostly Arrhenius-type response curve. The decrease of emissions under high temperatures (from 40 °C to 55°C) was correlated to decreases in CO_2-assimilation and/or photosynthetic electron transport [59]. The rate of monoterpenes/sesquiterpenes was increased in higher temperatures and the maximum rate (1.29) was observed at 25 °C. This result was in line with increasing the oxygenated monoterpenes as the main class of EO. The higher content of monoterpenes in comparison to sesquiterpenes content and the main component of oxygenated monoterpenes (mainly neral as the most impressible component under different temperatures) resulted in improvement of mentioned rate simultaneous with increasing temperature. However, the rate of monoterpenes/EO and sesquiterpenes/EO reached the highest amount at 10 °C and by increasing the temperature the mentioned rate was declined. Maybe the reason for decreasing rate was increasing the components in others class of EO (figure 3). According to Sangwan et al. and Herath et al. the higher content of citronellol in EO of *Cymbopogon nardus* was obtained in plants raised in lower temperatures, while the maximum amount of monoterpene hydrocarbons was observed in plants propagated under high temperatures [60,61]. Furthermore, in accordance with researches on *Solanum lycopersicum*, cold stress resulted in higher sesquiterpene emissions at any given monoterpene emission level. In PCA, the highest variance in exposure to different temperatures was observed in neral, EO, anthocyanins and polyphenols content. As PCA showed the highest variance in exposure to different temperatures is related to anthocyanins, polyphenols, EO and its main components neral, geranial and spathulenol in PC1. EO and its components were the first and the most impressible factors under various temperatures followed by pigments and then biochemicals. The positive and negative correlations illustrated that in chilling stress the content of EO and its main components decreased, while photosynthetic and antioxidant pigments and also biochemicals were raised by decreasing temperatures. In main chemical classes the monoterpene hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons showed the same positive correlation, while the negative correlation of oxygenated sesquiterpenes was observed in PC1. It implies that by decreasing temperature the content of three main latter classes and their main components declined, but the amount of oxygenated sesquiterpenes and its main component (spathullenol) increased at cold temperatures. Dendrogram of Wards Cluster analysis (CA) showed three temperature groups 1) 5 and 10 °C, 2) 15 and 20 °C, and 3) 25 °C on the basis of EO components and allometric traits. The first group comprises the most phytochemical traits with the least content or rate at 5 and 10 °C. In these temperatures the plant is not able to produce oil and derivatives due to cold shock. This finding is similar to researches on *Solanum lycopersicum* [62]. They reported that chilling stress treatment at 3 °C and 6 °C did not significantly induce volatile compounds. The second group shows the phytochemical traits with the moderate content in temperatures of 15 and 20 °C and the third group with the highest content at 25°C [62]. Large emissions of volatile oil compounds can occur in exposure to high temperature in *Phragmites australi* [63]. According to the results, the biochemical traits were divided into three groups of temperatures 1) 5 °C, 2) 10 °C, 3)15, 20 and 25 °C. The first group was composed of the most biochemical traits with the same statistical group and the least content at 5 °C. The second group was related to the biochemicals and osmolytes with the highest content at 10 °C and the third included the traits of moderate concentration at 15, 20 and 25 °C. The plants exposed to 5 °C could not resist against the cold stress and they showed the least level of the biochemicals and osmolytes content. The highest amount of biochemicals was observed at 10 °C and the plants activated defense system under cold stress. To overcome chilling stress, plants can trigger chilling or cold acclimation that includes the activation of

antioxidative defense and the synthesis of protectants such as sugar and proline [64-66]. Increasing content of soluble sugar in plants causes osmoregulation, cryoprotection or signaling molecules [65,67]. Funnekotter et al. reported that the increase in soluble sugar in cell membrane composition during low temperature stress enhanced the chilling tolerance in *Grevillea scapigera* [68,69]. Osmolytes help in the stabilization of photosynthetic machineries and thylakoid membranes, thereby, stabilizing photosynthesis, mitochondria, and metabolisms [70,71].

Among the different traits in exposure to raising temperatures from 5 to 25°C, the EO content and its components were the first traits to change with increasing trend followed by pigments and antioxidant biochemicals with conversely decreasing trend. Numerous researches on various plants for essential oil content and composition under stress conditions species have been performed [72]. Some research showed that essential oil content decreased under environmental stresses [73-75]. However, it is worthy to note that the essential oil alterations in response to the drought are both dependent on species and severity and duration of the stress period [76].

Overall, induction of various temperatures as one of the environmental factors showed significant effects on phytochemical and biochemical traits in a way that the EO content increased in higher temperatures and the maximum content of EO was obtained at 25 °C. By increasing temperature, the content of oxygenated monoterpenes (neral and geranial) showed an increase. Conversely, the amount of oxygenated sesquiterpenes (spathulenol) decreased. The biochemical pigments showed a significant increase at 10 °C (except for lycopene, ß-carotene, anthocyanins, and polyphenols at 5 °C) and then they were declined by increasing temperature. In general, the highest variance based on PCA was observed in neral, EO, polyphenols, and anthocyanins, respectively. Furthermore, the obtained results clarify the quality and economic value of this plant at the time of harvesting and environmental conditions for the pharmaceuticals, health, and food industries.

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Author contributions

Hanieh Rafiee carried out the experiment and collected available literature and prepared the first draft of the manuscript with support from Ali Mehrafarin; Heshmat Omidi and Hassanali Naghdi Badi analyzed the statistical data and verified the accuracy of the tests; Ali Mehrafarin designed the model and the computational framework and he was also responsible for the correspondence; Hassanali Naghdi Badi and Farahnaz Khalighi-Sigaroodi edited the manuscript as physiological and phytochemical consultants, respectively.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

EO: Essential oil; df: degree of freedom; FW: Fresh weight; OD: Optical density; PCA: Principal components analysis; S.O.V: Source of variance; TSS: Total soluble solids; Chl. a: Chlorophyll a; Chl. b: chlorophyll b; Total Chl.: total chlorophyll; Caro: carotenoids; Lyco: lycopene; ß-caro: ß-carotene; ACNs: anthocyanines; TSS: total soluble solids; PPs: poly phenols; Flavo: flavonoids; Pro: proline; SP: soluble protein