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Original article

Allometric Ratio of Menthol and Menthone to Pulegone in the Essential Oil of Peppermint (*Mentha piperita* L.) Affected by Bioregulators

Meisam Pourhadi[1], Hassanali Naghdi Badi[2], Ali Mehrafarin[2*], Heshmat Omidi[3], Reza Hajiaghaee[2]

[1]Department of Horticulture, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Medicinal Plants Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran.
[3]Agricultural College and Medicinal Plants Research Center, Shahed University, Tehran, Iran.

Abstract

Background and objectives: Menthol and menthone are two important components in the essential oil of peppermint (*Mentha piperita* L.) which are commercially used in the pharmaceutical industries. This study has presented the allometric ratio of menthol and menthone to pulegone affected by the induction of bioregulators. **Methods:** The experiment was conducted in controlled condition based on completely randomized design (CRD) in three replications. The plants were subjected to different bioregulator treatments including distilled water; 5 %v/v methanol; 40 ppm GA_3 (gibberellic acid) + 5%v/v methanol; 40 ppm IBA (indole butyric acid) + 5%v/v methanol, and 40 ppm GA_3 + 40 ppm IBA + 5%v/v methanol. Essential oils analysis were performed using different methods of gas chromatography and gas chromatography-mass spectroscopy. **Results:** The application of 40 ppm IBA + 5%v/v methanol increased the essential oil content. With the use of bioregulators, the content of menthol and menthone increased and the amount of pulegone was conversely reduced. The lowest content of pulegone was measured in the treatment with 40 ppm GA_3 + 40 ppm IBA + 5%v/v methanol. Allometric analysis of components showed that the ratios of menthol/pulegone, menthone/pulegone, and (menthone+menthol)/pulegone increased by bioregulators application, especially with 40 ppm GA_3 + 40 ppm IBA + 5%v/v methanol. **Conclusion:** Using plant bioregulators could be a suitable procedure to increase pro-health potential through increasing the amount of menthol with decreasing the content of pulegone in the essential oil. It is possible that the bioregulators were able to convert pulegone to menthol and menthone.

Keywords: allometric ratio, essential oil components, gibberellic acid, indole butyric acid, *Mentha piperita* L.

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Introduction

Peppermint (*Mentha piperita* L.) belongs to the Lamiaceae family and is native to the Mediterranean region [1]. It is one of the economically important medicinal herbs which is cultivated worldwide because of its therapeutic and economic values [2]. In the Iranian traditional medicine, it is used as a carminative, stimulant, tonic, antiviral, and antifungal agent and has drawn attention of consumers [3]. Peppermint oil is an important essential oil which possesses strong antibacterial and antifungal activities and has anti-inflammatory and anti-tumor effects [4]. Besides, it has some regulatory effects on central nerve system, reproductive, respiratory and digestive system [1]. Menthol and menthone are two cyclic monoterpene alcohols

*Corresponding author: Mehrafarin@imp.ac.ir; A.Mehrafarin@gmail.com

that compose the major and specific components of peppermint essential oil [5]. Menthol is the most abundant component of the essential oil of mature peppermint, but the overall quality of the essential oil, and thus its commercial value, is determined by the compositional balance of several constituents. Typically, superior essential oils of peppermint contain high quantities (35-60%) of menthol ($C_{10}H_{20}O$), moderate amounts (15-30%) of menthone ($C_{10}H_{18}O$), and low levels of pulegone ($C_{10}H_{16}O$) [3]. They are also included as an ingredient in a variety of consumer products including pharmaceuticals, cosmetics and pesticides, toothpastes, shampoos and soaps as a cooling and/or flavour enhancing ingredient [6]. Pulegone is an intermediate in the biosynthetic pathway of menthol, a major component of peppermint essential oil. Pulegone is recognized to be the other substance that plays a role in the aroma and flavour of mint [7]. Pulegone has been also reported as a renewable metabolite, starting material, a "bad agent" which causes neurotoxicity and hepatotoxicity in small amounts [1]. It is found in young peppermint leaves and is later metabolized to menthol; depending on the origin of the essential oil, however, there is a residual concentration of 1 to 4%. Because of its toxicity profile, the concentration of pulegone is limited to 1% or less for consumers [3]. This supplement is used in Indian folklore medicine to induce abortion and menses and to treat inflammatory conditions, and colic infants [8].

Bioregulators are natural or synthetic compounds and can influence the growth of plants and their secondary metabolites production when were appropriately applied [9,10]. Various bioregulators have been found to enhance the herb and essential oil yield in different species [10]. Among them auxin and gibberellic acid (GA_3), have been evaluated for improving the productivity of crops. Gibberellins and auxins especially GA_3 and indole butyric acid (IBA) are the most important valuable compounds for enhancing the productivity of medicinal plants. In spite of their beneficial effects on plant growth and development, their effects on essential oil content and composition have been widely considered recently. For instance, in *Mentha spicata*, GA_3 has been found to induce enhancement of whole herb and foliar spray of GA_3 and IBA have been found to increase the menthol essential oil content [11]. Bose et al. [12] have showed that application of gibberellic acid has influenced the chemical composition of *Mentha arvensis* L. essential oil. Another research has shown that α-bisabolol oxide A, has increased in chamomile by 100 ppm indole acetic acid (IAA) [13]. Methanol spray is an alternative method to affect the plant growth and productivity through increasing crop CO_2 fixation in unit area. Abundant carbon dioxide supply from methanol causes the photorespiration to be shifted from catabolism to anabolism [14]. Zbiec et al. [15] have reported that methanol foliar spray increased the concentration of carbon in the plant and thereby increased plant growth. Mehrabi et al. [16] have indicated that application of methanol and biostimulants improved the quantity and quality yield of savory. In medicinal herbs, assimilate allocation into medicinal parts is very important to produce high yield plants. Nowadays, it has been shown that allocated assimilates may be partitioned into low value metabolites. Different partitioning patterns reflect different strategies resulting from different selection pressures. Thus, analysis of partitioning patterns is the most appropriate available tool for considering the priorities of plants. Metabolites allometry is the study and measurement of relative metabolites. In this regard, the study of the allometric relationships between major components of the essential oil in understanding the effect of bioregulators on health potential of the plants is very vital. In the present study, our focus was to investigate the comparative effect of exogenous application of IBA, GA_3 and methanol on *Mentha piperita* L. in term of the major essential oil composition and the allometric analysis.

Material and Methods

Treatments and experimental design

To study the effects of plant bioregulators on essential oil traits of peppermint (*Mentha piperita* L.), a pot experiment was done under the same environmental and controlled conditions in a research greenhouse based on completely randomized design (CRD) at the Medicinal Plants Institute (MPI) in Karaj, Iran (35°, 90′ N and 50°, 88′ E, 1500 m above sea level) during 2015 and 2016. A voucher specimen (4580-MPIH) has been deposited at the Herbarium of MPI. Uniform transplants of peppermint were collected from gene bank and seed collection of MPI and then were transferred into each pot. Pots

with 10 L content were filled with 12 kg of farm soil. The soil texture was loam-silt, its physiochemical properties contained 0.08 % nitrogen, 48.9 mg/kg phosphorus, 33.6 mg/kg potassium, 7.9 pH, and electrical conductivity (EC) 2.4 dS/m. The plants were grown in a controlled research greenhouse with a photon flux density about 1450 µmol/m^2s^1, 16 h light and 8 h dark period and the average temperature of 25±1/18±2 °C for day/night temperatures, and 50-60% relative humidity. The pots were subjected to different bioregulator treatments listed in the table 1. All of the treatments were sprayed four times after the establishment during the growth stages with 15-day intervals on the aerial parts of the plants. Other operations were done regularly during the growing season as needed. The studied parameters were content of essential oil, menthol, menthone, pulegone, and also ratios of menthol/pulegone, menthone/pulegone, and (menthol+menthone)/pulegone as the most important traits in the essential oil of peppermint for the pharmaceutical industry.

Table 1. Treatments of plant bioregulator formulations on *Mentha piperita* L.

H1: Control treatment (distilled water)
H2: 5% v/v methanol
H3: 40 ppm GA_3 + 5% v/v methanol
H4: 40 ppm IBA + 5% v/v methanol
H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol

- Gibberellic acid (GA); Indole butyric acid (IBA)

Plant sampling and analysis

The plant materials were dried in the laboratory at room temperature (26±2 ºC) away from sunlight to prevent changes in the nature of the plants' constituents until the constant weight was gained. Essential oils of the above ground organs were isolated by hydrodistillation procedure for 4 h using Clevenger apparatus [17]. The extracted oils were dried over anhydrous sodium sulphate and kept at 3±1 °C until they were analyzed. GC analysis was conducted on a Younglin Instrument Acme 6000M gas chromatograph equipped with flame ionization detector (FID) and a HP-5MS capillary column (30 m×0.25 mm; 0.25 µm film thicknesses). The oven temperature was set at 50 ºC for 5 min, and then programmed at 3 ºC per min to 240 ºC and after that programmed at 15 ºC per min to 300 ºC (held for 3 min). Other operating conditions were: carrier gas, He with a flow rate of 0.8 ml/min; injector and detector temperatures were 290 ºC, and split ratio, 1:10. GC/MS analysis was performed on a GC mentioned above coupled with an Agilent Technologies 5973 Mass system. Other operating conditions were the same as described above, mass spectra were taken at 70 eV. Mass range was from m/z 35-375 amu. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of the essential oils were identified by comparison of their mass spectra and retention indices with those published in the literature [18] and presented in the MS computer library. Analysis of variance (ANOVA) appropriate to the experimental design was conducted using SAS software (version 9.1). Means of each trait were compared according to Duncan multiple range test at $p \leq 0.05$.

Results and Discussion

The results revealed that the foliar application of bioregulators and methanol significantly affected the content of essential oil (table 2). As the results show, the maximum (1%) and minimum (0.5%) contents of essential oil were obtained from the sprayed plants with 40 ppm IBA + 5% v/v methanol (H4), and 40 ppm GA_3 + 40 ppm IBA + 5%v/v methanol (H5), respectively. However, there were no statistically significant differences among the H5 and other treatments expect H4. Foliar application of 40 mg/L IBA + 5% methanol (H4), increased the content of essential oil two fold compared to the control treatment (figure 1). Overall, the exogenously application of GA_3 or IBA on peppermint increased the percentage of essential oil. Though, their effects were slightly antagonistic when were simultaneously applied.

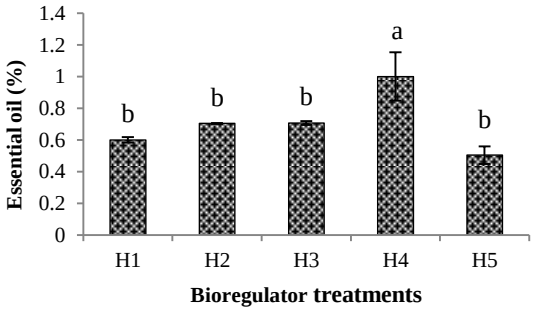

Figure 1. Effects of bioregulator formulations on essential oil content of *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at $p \leq 0.05$.

Table 2. Analysis of variance for effects of plant bioregulator formulations on essential oil traits of *Mentha piperita* L.

S.O.V	Df.	Mean square							
		Essential oil content (%)	**Menthol**	**Menthone**	**Pulegone**	**Menthol/ Pulegone**	**Menthone / Pulegone**	**Menthone / Menthol**	**(Menthol + Menthone) / Pulegone**
Rep. (Block)	2	0.714^{ns}	0.9744^{ns}	0.9835ns	0.2015^{ns}	0.1291^{ns}	0.0624^{ns}	0.9289^{ns}	0.0784^{ns}
Treatment	4	0.0187*	0.0052**	0.0218*	0.008**	0.0124*	0.0003**	0.0158*	0.0034**
Error	8	0.0184	5.956	4.1702	0.7652	0.018	0.4292	0.0034	7.3299
CV (%)		19.34	6.03	12.49	18.11	23.81	16.75	14.41	19.76

S.O.V: Source of variation; Rep: Replication; Df: Degree of freedom; C.V: Coefficient of variation
*,**, ns show significant in 5%, 1%, and insignificant, respectively.

Bioregulators can increase the content of essential oils by improving the growth and development of medicinal plants. In addition, it can be considered that part of the biosynthetic pathway of bioregulators such as GA_3 in plants is associated with the production of some components in essential oils by a similar pathway and common substrate. Thus, the percentage of essential oil and its components can be effectively impacted by application of these compounds. Methanol could affect the content of essential oil directly by influencing their biosynthetic pathways or indirectly by improving the plants organ growth. Naghdi Badi et al. [19] have shown that the methanol application on thyme (*Thymus vulgaris* L.) stimulated the biosynthesis of essential oil content. Alcohols and bioregulators affect the plants growth and their phytochemical contents by increasing the photosynthetic activity, cytokinins and nitric oxide production, reductase enzymes activity and reducing the plants respiration [14,20]. Naghdi Badi et al. [19] showed that the combined application of methanol, amino acids and phytohormones increased the essential oil content of thyme (*Thymus vulgaris* L.) based on an increase in the amount of its main components including thymol and carvacrol. Foliar application of gibberellic acid positively influenced the growth and quality of lavender (*Lavandula angustifolia* Mill.) through providing more assimilation in the biosynthetic pathway of essential oil [21]. Bose et al. [12] evaluated the effects of GA_3 on growth and essential oil content of *Mentha arvensis* L. They found that essential oil percentage and yield were positively improved by 1 µM of GA_3 [21]. The results of this experiment are consistent with the results of Yazdifar et al. [22] on *Calendula officinalis* L., Moradi and Ebadati Esfahani [23] on *Artemisia dracunculus* L. and Bagheri et al. [24] on *Lavandula angustifolia* Mill.

According to the GC and GC-MS analyses of essential oils, three major components including menthone (KI= 1153), menthol (KI= 1172), and pulegone (KI= 1237) were identified in the essential oils of peppermint (*Mentha piperita* L.). Based on the analysis of variance, foliar application of bioregulators and methanol biostimulant significantly influenced the biosynthesis of menthol, menthone, and pulegone in the essential oil and their ratios (table 2). A comparison of components in essential oils revealed that the foliar application of bioregulators and methanol affected the quantity of essential oil compositions. Figure 2 has shown the menthol content of treated samples by difference bioregulators and methanol.

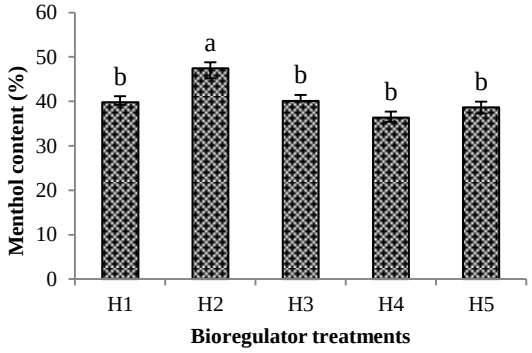

Figure 2. Effects of bioregulator formulations on menthol content of *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at p≤0.05.

The highest amount of menthol was observed when the plants were treated by methanol as the biostimulant. The content of menthol in the essential oil increased by foliar application of 5%

v/v methanol in comparison with control treatment up to 16.09%. Regarding the content of menthol as another important component of peppermint essential oil, foliar application of 5% methanol (H2), and 40 ppm IBA + 5% v/v methanol (H4) decreased its content by 25 and 26%, respectively. The highest content of menthone was observed when the plants were stimulated by 40 ppm GA_3 + 5%v/v methanol (H3). However, there was no significant difference with control treatment (figure 3). All bioregulators and methanol significantly induced a reduction in the content of pulegone in peppermint essential oil compared to the control treatment. The lowest level of pulegone was measured for treated plants by 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol (H5) which was about 60% lower than the control treatment (figure 4).

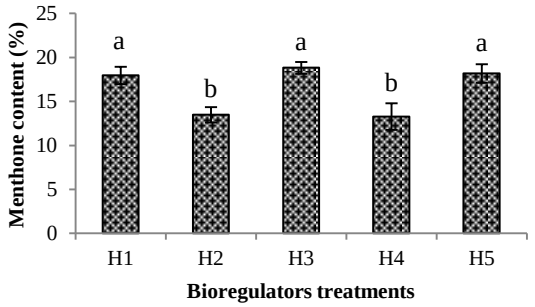

Figure 3. Effects of bioregulator formulations on menthone content of *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at $p \leq 0.05$.

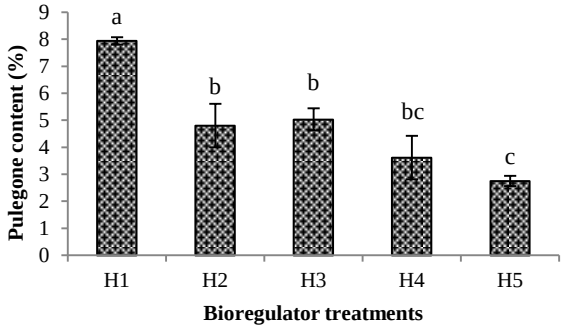

Figure 4. Effects of bioregulator formulations on pulegone content of *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at $p \leq 0.05$.

One of the best ways to decide on the selection of essential oil components is the application of the proportion and allometric properties of the essential oil components. Allometric analysis of the components showed that the ratios of menthol/pulegone and menthone/pulegone had increased by application of bioregulators. A much higher content of menthol/pulegone ratio was determined in the treated plants by 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol (H5) compared to other treatments, which caused a three-fold increase compared with control. It should be noticed that the treatment of H5 showed no significant difference with treatments of H2 and H4. So that, application of 5% v/v methanol (H2) was economically the best treatment for improving the menthol/pulegone ratio (figure 5). Compared to other bioregulator treatments, the greatest increase in menthone/pulegone ratio was found in the plants sprayed by 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol (figure 6). As shown in figure 7, the ratio of menthone/menthol in the samples was only significantly affected by application of 5% v/v methanol (H2), which was lower than other treatments. The foliar application of 40 ppm IBA + 5% v/v methanol also reduced the menthone/menthol ratio. But, the amount of this decline was not statistically significant compared to the control treatment. Analysis of variance (table 1) showed that the application of bioregulators significantly affected (menthol+menthone)/pulegone ratio. In general, all bioregulators treatments increased (menthol+menthone)/pulegone ratio, and its highest value was obtained when the plants were sprayed with treatment of 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol (figure 8).

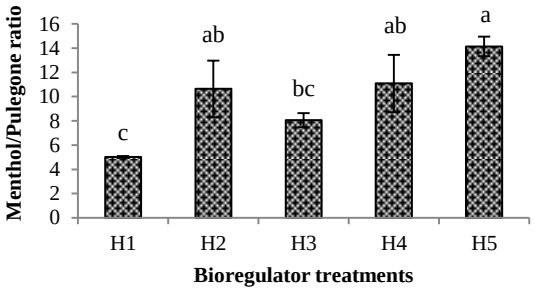

Figure 5. Effects of bioregulator formulations on menthol/pulegone ratio in *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at $p \leq 0.05$.

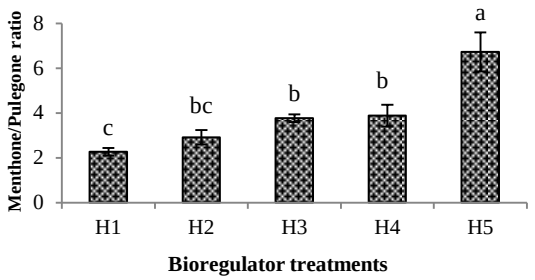

Figure 6. Effects of bioregulator formulations on menthone/pulegone ratio in *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at p≤0.05.

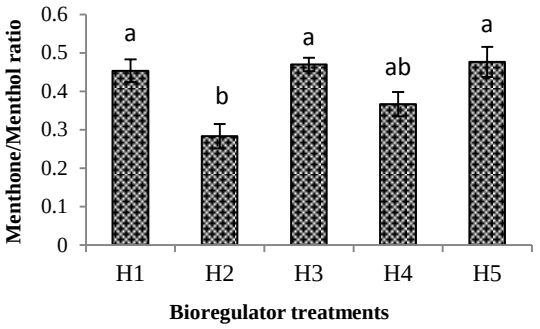

Figure 7. Effects of bioregulator formulations on menthone/menthol ratio in *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at p≤0.05.

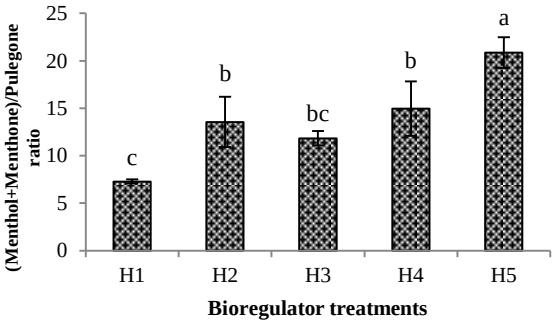

Figure 8. Effects of bioregulators formulations on menthol+menthone/pulegone ratio in *Mentha piperita* L. H1: distilled water; H2: 5% v/v methanol; H3: 40 ppm GA_3 + 5% v/v methanol; H4: 40 ppm IBA + 5% v/v methanol; H5: 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. Different letters indicate significant difference at p≤0.05.

Thus, examining patterns of allometry can be an effective method for assessing the changes in relative allocation of essential oil components across bioregulators.Biochemical allocation in plants has been conceptualized as a proportion or ratio-driven partitioning of a biochemical process. In the view of essential oil biosynthesis, a plant has a given amount of metabolic resources at any point in time, and it allocates these resources to different components of essential oil based on the allometric relationships. Allometry is the quantitative relationship between metabolic biosynthesis and allocation. Application of bioregulators not only affects essential oil content, but also its compositions. Silva et al. [25] have reported that the incorporation of auxin into the media increased neral and graniuel contents in lemon balm (*Melissa officinalis* L.).

Studying the effects of 1-naphthalene acetic acid (NAA), IAA and GA_3 on the essential oil of some species of eucalyptus (*Eucalyptus citriodora*, *E. urograndis*, and *E. urocan*) has shown that the application of IAA increased the essential oil yield and content of eucalyptol, pinene, and linalool. In addition, the use of GA_3 leaded to an increase in the percentage of eucalyptol, alpha-pinene, linalool, and reduction of spatulenol, glubbol, 1-dicotinone, 3-themine, alpha-terpinol, bronel, tetradecano [26]. Asrar stated that the gibberellic acid and terpenoids have a common biosynthetic pathway with a lot of influence on each other [27]. Plant growth regulators have a direct effect on the metabolism of monoterpenes due to the effects on activity of biosynthetic enzymes in the production of essential oils in plants. Therefore, if the plant growth regulators are applied on medicinal plants, then the quantity and quality of their essential oils will be affected [28].

The application of methanol as a biostimulant alone and in combination with other biostimulants, especially with the plant growth regulators can change the quantity and quality of oil constituents as the amount of menthol was increased in this experiment. This finding is similar to the results of Pilehvari et al. on *Thymus vulgaris* L. by methanol application [29]. Moradi and Ebadati found that in plant cells, methanol completely metabolizes to its components and converts to amino acids; so, it can be used as one of the satisfactory inputs used in organic or semi-organic farming systems [23]. It was reported that spraying of different levels of alcohols has increased the percentage and yield of essential oil, percentage and yield of carvacrol and thymol in thyme [30]. These results are in agreement with

results of Soad et al. on croton (*Codiaeum variegatum* (L.) A.Juss.) [31] and Baydar on safflower (*Carthamus tinctorius* L.) [32].

It seems that the bioregulators and methanol have crucial effect on primary and secondary plant metabolites. Yield of essential oil and menthol content were increased with leaf maturity and the lack of environmental stress, and against it, the presence of a range of stress conditions tended to promote the production of pulegone in the essential oil of peppermint [33]. It is possible that the bioregulators and methanol reduce the severity of environmental stresses and increase the rate of plant growth and subsequently, increase the amount of essential oil and menthol component. From an allometric perspective, plasticity in the allocation of essential oil components can be regarded as a change in a plant's allometric trajectory in response to the application of bioregulators and methanol. It is possible that the plant bioregulators and methanol can change the allometric ratios of essential components due to the changes in their allocation ratios. The biosynthesis and accumulation of essential oil positively responds to these molecules especially their synthetic ones at external applications [34]. Considering allometric relationships for essential oil components, it could reveal functional responses to application of bioregulators and methanol biostimulant. These findings have important applications in the commercial production of peppermint essential oils of high quality, and they may have broad implications for the control of natural products biosynthetic pathways. However, the physiological rationale for such complex regulation is presently unclear. As a result, exogenous application of plant bioregulators with methanol could be a best procedure to increase pro-health potential in essential oil of *M. piperita* L. due to the highest amount of menthol and menthone than the pulegone.

This study was conducted under uniform and completely controlled environmental conditions for all treatments in a research greenhouse, but its results will be generalized when the results would be once again reviewed in a separate experiment to replicate and validate them. Overall, foliar application of bioregulators had positive and significant effects on essential oil components of *Mentha piperita* L. With the use of bioregulators, the content of menthol and menthone increased and the amount of pulegone conversely reduced. Based on the findings, the treatment of 40 ppm IBA + 5%v/v methanol increased the content of essential oil to the highest value. Allometric analysis of components showed that the ratios of menthol/pulegone, menthone/pulegone, and (menthone+menthol)/pulegone increased by bioregulators application, especially with 40 ppm GA_3 + 40 ppm IBA + 5% v/v methanol. It is possible that the bioregulators can convert pulegone to menthol and menthone. These results suggested that the foliar application of bioregulators and methanol might influence the reduction of pulegone. The ability to reduce pulegone levels is of commercial significance in improving essential oil quality.

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Author contributions

Meisam Pourhadi carried out the experiment and collected available literature and prepared the first draft of the manuscript with support from Hassanali Naghdi Badi; 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; Heshmat Omidi and Reza Hajiaghaee 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 content of the paper.

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Abbreviations

ACECR: Academic center for education; culture and research; CRD: completely randomized design; IAA: Indole acetic acid; GA: Gibberellic acid; IBA: Indole butyric acid; MPI: Medicinal plants institute; NAA: Naphthalene acetic acid; GC/MS: Gas chromatography and mass spectrometry; FID: Flame ionization detector; KI: Kovats index