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# The Effect of Melatonin on Essential Oil Production in Mentha Spicata

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## ABSTRACT

*Mentha Spicata* (spearmint), an herbaceous perennial aromatic plant, is the most common herb in tropical and subtropical countries. The essential oil (EO) of *Mentha spicata* is a valuable source of antioxidants in nutraceuticals and cosmetic industries. In-vitro culturing of Mentha Spicata was done via the micro-propagation technique, and exogenous Mel was used as a plant growth regulator. The field trials involved the plant's foliar spray of different concentrations of Mel at various time points. The EO of leaves of control and Mel-treated plants (S1-S6) was steam-distilled using a Clavenger-type apparatus. The UV, FTIR, and GC analyses of the EO of control and S1-S6 were recorded. The antioxidant capacity of the EO of control and S1-S6 was measured using a DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay. The nodal explants of Mentha Spicata showed different responses to the shoot proliferation process in the presence of exogenous Mel. The EO yield percentage of control and S1-S6 were in the range of 0.92±0.05% and 0.74±0.03%-0.39±0.04%%, respectively. The UV, FTIR, and GC spectra of the EO of control and S1-S6 presented different numbers of peaks and compounds in the selected range. The EO of control and Mel-treated plants could reduce the free radical DPPH to DPPHH with varying values of IC50. Melatonin could regulate the plant's growth and development, change the EO yield, improve the EO's phytochemical profile qualitatively, and enhance the free-radical scavenging capacity of the plant's EO in a concentration and time-dependent mode. So, it may act as a promising molecule to increase aromatic crop yield with the desired quality.

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## Introduction

The genus *Mentha*, belonging to the family Lamiaceae, is one of those genera that includes a considerable number of medicinally important economic aromatic plants. This sub-cosmopolitan genus is distributed across Asia, Africa, Europe, and North America. These

fragrant plants (APs) are perennial herbs, in most cases, and are widely cultivated for their essential oil (EO) content (Tafrihi *et al.*, 2021).

Mentha spicata L. (spearmint), an herbaceous perennial AP growing 10-100cm high, is the most common herb in tropical and subtropical countries. Mentha Spicata's EO is a valuable source of antioxidants in the nutraceutical and cosmetic industries. Mentha Spicata grows best in moist, slightly acidic soil and light sunlight to partial shade (Snoussi, *et al.*, 2015).

Infections, wounding, or herbivory induce the production of several secondary metabolites (SMs), including EOs. Due to genetic variation, resistance and susceptibility of plant varieties speed up the extent of such induction. In addition, phytohormones are the other molecules that control specific SMs' production. Although, the role of salicylates. Jasmonates and melatonin are not fully understood as signaling molecules, but they are implicated as signals in these responses and many other physiological processes (Sarrou *et al.*, 2015; Martínez-Medina *et al.*, 2017).

Melatonin (Mel) as an antioxidant and growth regulator could enhance plant resistance to various biotic and abiotic stress factors (Arnao & Hernández-Ruiz, 2014; Tan, *et al.*, 2015; Arnao & Hernandez-Ruiz, 2018). Scientists are trying to discover the mechanism by which Mel regulates these responses.

As stated previously, the genus *Mentha* is one of the main genera, and it includes several medicinal plants with extensive use in different industrial sectors. One of the species belonging to this genus is *Mentha spicata*. This plant's EO and herbal preparations are widely used in the pharmaceutical, cosmetic, and nutrition industries. Despite various usages, the plant contains a minimal amount of EO, so one should harvest a large number of plant biomass to extract a notable amount of EO. Moreover, cultivating plants with good quality and quantity of SMs, including EO, requires pre-determined and specific conditions, which are highly geoclimatic dependent. In addition to other measures already established for improving SM production by different plant species, the application of plant growth regulators has recently attracted much attention in this context. So, in the present study, we investigated the effect of exogenously applied Mel on SM production, especially EOs by Mentha Spicata, and, indirectly, plants' growth and development process. The aims of this study are as follows:

1. To evaluate the effect of Mel on the growth and development process of Mentha Spicata.
2. To evaluate the effect of Mel on the EO yield of Mentha Spicata.
3. To evaluate the effect of Mel on the chemical profile of Mentha Spicata *'s* EO
4. To evaluate the effect of Mel on the free-radical scavenging capacity of Mentha Spicata 's EO

## Methods and Material

Mentha spicata was selected as the test plant. To investigate the effect of exogenously applied Mel on plant growth and development, SMs, including EO yield, phytochemical profile, and free-radical scavenging capacity, were used at different time points. The details of each method used are mentioned as follows:

### *Sample Collection*

Healthy plants were collected from the medicinal and herbal garden of the Pharmacy Department of Lovely Professional University, Phagwara, Punjab, India, in March 2019. The collected plant material was identified as Mentha *Spicata* by Prof. Devendra Kumar Pandey, the Botany department faculty staff, LPU, Phagwara, Punjab, India. The voucher specimens (No. LPU18032017–LPU19032017–LPU20032017) were placed as references in the plant biotechnology lab herbarium at LPU, Phagwara, Punjab, India, together with the herbarium sheets of the collected plant. After being thoroughly cleaned and freed of stem fragments, the freshly harvested plant biomass was cut into small pieces and split in half. While the second portion was labeled and stored in a refrigerator (-200C) for later use, the first portion was utilized immediately to extract the EO.

### *In-vitro Culture of Mentha spicata*

In-vitro culturing of Mentha Spicata was done via the previously described propagation technique and contained five stages: preparatory, initiation, multiplication, rooting, and acclimatization/hardening or transplantation.

In the preparatory stage, all the requirements were prepared, including culture media, growth regulators' stock solutions, surface sterilizing agents, and nodal explants. Full-strength Murashige and Skoog (MS) medium was prepared by dissolving an accurately weighed amount of ready-made powder of MS medium (HiMedia company) in distilled water and supplemented by 8 grams of agar as a gelling agent. Before adding agar, pH was adjusted to 5.8± 0.01 with 1N NaOH/ HCl. The medium was sterilized at 121°C and 15 psi.

A stock solution of growth regulators, BAP, kinetin, 2, 4-D, IBA, and Mel (PCT0840, neoLab company), was prepared by dissolving accurately weighed amount of growth regulators in a few drops of appropriate solvents (0.1N NaOH for BAP, kinetin, 2.4-D, and IBA, and, DMSO for Mel). The strength of stock solutions was 1mg/ml for BAP, kinetin, 2.4-D, and IBA, and 1 mM for Mel—prepared stock solutions after filter sterilization and stored at 4°C in the refrigerator. Mercuric chloride was selected as a surface sterilizing agent and was prepared by dissolving 0.1 grams of $HgCl_2$ powder in 100 ml of distilled water.

Collected young shoot tips and nodal explants were thoroughly washed under running tap water, followed by 1% Tween-20 for 15-20 minutes, and rinsed 3-5 times with distilled water. Disinfection was done using 70% ethanol for 30-60 seconds, and the selected shoots were then surface sterilized with 0.1% $HgCl_2$ for 5 minutes and rinsed with autoclaved distilled water three to four times. After surface sterilization, Mentha Spicata shoots were excised in

small parts to get nodal explant. The nodal explant was prepared in laminar air flow (Fejéra *et al*., 2018).

The primary purpose of the initiation stage was the induction of multiple shoots. In this stage, prepared nodal segments were inoculated vertically in 50 ml culture tubes containing 5-10 ml of MS medium fortified with different concentrations of growth regulators, alone or in combination. Six culture tubes including MS medium without any growth regulators as negative control; MS medium supplemented with 0.5 mg/L BAP as positive control; MS medium supplemented with 1µM Mel, combination of 0.5 mg/L BAP and 1µM Mel, 10µM Mel, and combination of 0.5 mg/L BAP and 10µM Mel were prepared. Inoculated culture tubes were maintained in a culture room under controlled conditions of temperature (22± 2°C), light (photoperiod of 16 hrs. light/ 8hrs. dark), white fluorescent light (having 2500-3000 Lux light intensity) and 50- 80% of relative humidity for three weeks. During this period, cultured tubes were checked every 3-5 days for any observable changes or contamination. Data were recorded after 3-4 weeks.

The main purpose of the multiplication stage was to increase the yield (shoots and leaves) for consequent processes. In this stage, the initiated mass of regenerated shoots was subcultured in a fresh MS medium containing the same concentrations of BAP (0.5mg/L) and Mel ($1^{-1}$0µM) as the initiation stage for shoot proliferation and elongation. Three-week intervals are required to record the data for the multiplication stage.

Rooting of proliferated shoots was done by transferring the excised elongated shootlets into a rooting medium (MS medium containing auxins). IBA was used for rooting purposes. In total, six culture tubes, including MS medium without any growth regulators as negative control; MS medium supplemented with 2 mg/L IBA, 1µM Mel, a combination of 2 mg/L IBA and 1µM Mel, 10 µM Mel, and a combination of 2 mg/L IBA and 10µM Mel were prepared.

Newly rooted plantlets were transferred in pots containing sterile soil and coco peat/vermiculite (1:1) and covered with plastic bags to maintain sufficient humidity. During this period, plantlets were irrigated with nutrients like MS medium containing Mel (1-10 µM). After one week, pots were kept in the greenhouse for about two weeks. Finally, plantlets were acclimatized in the field for hardening (Ghanti *et al*., 2004; Islam *et al*., 2017; Fejéra *et al*., 2018).

***In-vivo Studies of* Mentha Spicata *(Mel Foliar Spray)***

The field trials involved applying a growth regulator, Mel, and control treatment. Mel stock solution was prepared by dissolving an accurately weighed amount of Mel in a few drops of DMSO. The final strength of the stock solution was 1mM. The working solutions at three different concentrations of 0.05mM, 0.1mM, and 0.2mM were diluted in distilled water, and 20 ml per plant was sprayed. Control plants were sprayed only with distilled water. The treatment was carried out twice, and the leaves of plants were collected after two different time points, two and five days, and stored in the refrigerator until further analysis (Nafea & Abdulfatah, 2014).

### *Essential Oil Extraction of* Mentha Spicata

EO of Mentha Spicata was obtained using a Clavenger's-type apparatus. About 100 grams of a sample containing leaves and young shoots of control and Mel-treated plants were weighed and chopped off after washing in tap water. Sliced leaves were immersed with distilled water in a 1000 ml round bottom flask. Distillation time was set for 2 hrs. at 95-100°C until no EO got distilled. The condensed EO was collected and dried over anhydrous sodium sulfate at the end of the hydro-distillation process. The obtained EO was then stored in the dark refrigerator at 4°C until further analysis (Abdel-Hameed *et al.*, 2018).

### *UV Analysis of Essential Oil*

The molecular absorption UV spectrum of the EO was recorded in the region of 190- 400 nm. A computer-operated spectrophotometer was used with a quartz cuvette of a 1 cm optical path. The data were acquired and processed using the software package (UV Probe, Ver. 2.61) (Hussein *et al.*, 2019).

### *FTIR Analysis of Essential Oil*

The FTIR spectra of the EO were acquired on Shimadzu FTIR spectrometer 8400S. The environmental conditions were maintained unchanged (25°C temperature and 30 percent humidity). The signal-to-noise ratio was 20000:1. Ten percent of the EO and reference chemicals and their specific solvents were deposited directly on the ATR sampling device without any treatment. The related spectra were recorded in the 4000-500 cm–1 range with a resolution of 4 $cm^{-1}$ and 12 scans for sample and background. The velocity of the scan was 10 kHz with an interferogram size of 14220 points. Before scanning each sample, the device was cleaned with 70% ethanol, and the air background was taken. Shimadzu's IR solution software was used to acquire spectra of samples and spectral manipulation (Socaciu *et al.*, 2017).

### *GC Analysis of Essential Oil*

GC analysis of EO was done using a Shimadzu -QP2010 Ultra mass spectrometer equipped with a flame ionization detector (FID) and a DB-5 (30m* 0.25mm internal diameter, 0.25µm film thickness) fused silica capillary column. The temperature of the GC oven was maintained at 40°C for 5 minutes and increased to 300°C at 5°C/min. The temperature of the injector was kept at 250°C, and Helium gas was used as the carrier with a flow rate of 1.24 ml/min and 66.7 kph. The injection volume was 0.03 µL neat with a split ratio 1:10, ionization energy of 70eV, and mass scan range of 40-500amu. EO's constituents were identified based on their retention MS Library search (NIST and WILEY) and by comparing RI and mass spectral data with the literature (Chaturvedi *et al.*, 2018).

### *Antioxidant activity (DPPH assay) of Essential Oil*

The antioxidant activity of EO of control and Mel-treated plants was measured by the method described previously based on bleaching of the purple-colored methanolic solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl). A volume of 1 mL of EO of control and Mel-treated plants diluted at different concentrations (2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, 10 µg/mL, and 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, and 25 µg/mL) were mixed with 0.27 ml of 90

µM DPPH methanolic solution. The volume of the mixtures was completed to 3 mL with methanol. After vigorous shaking, the mixtures were incubated for 30-60 minutes at room temperature in the dark. The conversion of free radical DPPH to DPPHH and the disappearance of its purple color was measured at 517 nm with a spectrophotometer (Shimadzu $UV^{-1}240$, Kyoto, Japan). The following equation was used to calculate the inhibition percentage of the DPPH radical by the tested EO.

$$\text{Scavenging value \%} = (A_{control}-A_{sample}/A_{control})*100$$

In this equation, $A_{control}$ shows the absorbance of the negative control (mixture of DPPH and methanol), and $A_{sample}$ is the absorbance of the test sample (EO). $EC_{50}$/$IC_{50}$ was calculated as the effective/inhibitory concentration of EO at which it could scavenge 50% of DPPH radical. Ascorbic acid was used as a positive control (Ammar *et al*., 2018).

### *Analysis of the collected data*

The experiments were repeated in triplicates, and the results were recorded as mean± SD. Microsoft Excel 2010 was used in data analysis. Microsoft Excel 2010 tool-pack was used to calculate mean and standard deviations.

## Findings

The effect of exogenously applied Mel on plant growth and development, the EO yield, the EO's phytochemical profile, and the EO's free-radical scavenging capacity obtained from Mentha Spicata was explored. The result of the study showed this endogenic indoleamine could regulate plant growth and development, change the *Menthta spicate*'s EO yield percentage, improve the phytochemical profile of the plant's EO qualitatively, and enhance the free-radical scavenging capacity of plant's EO in a concentration and time-dependent manner.

### *In-vitro Culture of Mentha spicata*

The nodal explants of Mentha Spicata, which were inoculated on MS medium supplemented with BAP (0.5 mg/L), Mel (0.001, 0.01, 0.5, and 1mM), and a combination of BAP and Mel, showed a different response to the shoot proliferation process. The shoot proliferation response was high in the nodal segments cultured on MS medium in the presence of BAP (Figure 1). In contrast, the shoot regeneration was not seen for the explants inoculated on MS medium containing different concentrations of Mel alone or in combination with BAP. Incorporation of kinetin as an alternative cytokinin did not improve bud proliferation and shoot induction. Proliferated shoots from BAP-containing medium were further sub-cultured on fresh MS medium with the same (0.5 mg/mL or 1.0 mg/mL) concentration of BAP every three weeks. In addition, the full-strength MS medium supplemented with Mel at different concentrations failed to induce the rooting of newly regenerated shoots.

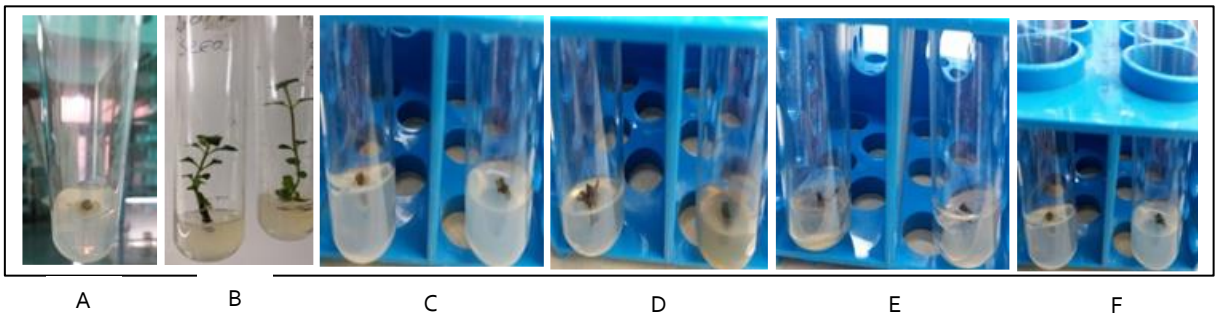


*Figure 1. Cultured nodal segments of Mentha spicata on MS medium containing BAP, Mel, and a combination of BAP+ Mel. A: control; B: 0.5 mg/L BAP; C: 0.5mM Mel; D: 1mM Mel; E: combination of 0.5 mg/L BAP+ 0.5mM Mel; F: combination of 0.5 mg/L BAP+ 1mM Mel*

Production of plant SMs is a complicated process, which is influenced by several factors, including physiological variability, nutritional priorities, taxonomical specificity, environmental adaptability, biogeography, application of exogenous plant growth regulators and their effectiveness, etc. (Figueiredo *et al.*, 2008). Although SMs are not directly involved in plant metabolic processes such as growth and development as primary metabolites, their role as a part of the plant defensive system and signaling molecules in ecological aspects is still vital (Pagare *et al.*, 2015; Wallace, 2004). Different SMs synthesized by plants differ in function, quantity, effectiveness, and toxicity. EOs are a group of such compounds representing the source plant's essence and odor.

The effect of exogenously applied plant growth regulators on improving EO production in plants has been investigated. One of these phytohormones is Mel, which significantly influences the biosynthesis of SMs. There is not much information in the literature about the effect of the exogenous application of Mel on the volatile content and phytochemical profile of APs, including Mentha Spicata. Foliar application of Mel as a plant growth regulator/elicitor at concentrations of 0.05, 0.1, 0.2, 0.4, and 0.5mM examined in this study positively affected chemical composition, especially on the amount of most abundant components of EOs of Mentha Spicata. There were differences observed in the phytochemical profile of EO obtained from control and Mel-treated plants in terms of their appearance or disappearance, as well as the increase or decrease in each component in total EO. In most of the cases, these changes were time and dose-dependent.

Consideration of Mel as a phytohormone and its application for callus induction or shoot proliferation requires dose standardization. In our experiment, Mel negatively affected the shoot proliferation in Mentha Spicata at concentrations of 0.5 and 1 mM. There was no response of the plant to the shooting process in nodal explants inoculated on MS medium containing melatonin as a growth regulator at concentrations of 0.5 and 1mM, singly or in combination with other shoot-inducing phytohormone (cytokinin). In contrast, shoot induction was positive in the control group, inoculated on MS media in the presence of BAP (Figure 1). These data are in accordance with other studies done to prove the efficiency of Mel as a plant growth regulator with an optimum dose. Coskun *et al*. (2019) reported that high concentrations of Mel in the range of 100- 200mM in MS medium decreased the callus

production of leaf explant. This inhibitory effect is probably the effect of Mel on increasing phenolic and aromatic compounds, which reduces shoot induction and callus formation in experimental plants. According to Riaz *et al*. (2018), sub-culturing of calli of *Catharanthus roseus* var. *alba* with different concentrations (0.5-20 µM) of Mel-induced callus biomass accumulation with the best result obtained at a concentration of 1µM. Kachhap *et al*. (2018) reported that 2, 4- D increased the callus initiation in *Ocimum sanctum* at lesser concentrations (0.1mglL) and decreased or even totally arrested the callusing at higher concentrations (0.2- 0.8 mg/L and 1.2- 1.4mg/L, respectively).

Experimental data suggest a critical role of Mel in the regulation of senescence, activation of defense responses, plant productivity, and developmental phase transitions, which may be related to antioxidant and growth-promoting properties. Mel has a protective role in the senescence process. The primary mechanism underlying the regulation of the senescence process seems to be via an Ascorbate-glutathione cycle, in which the accumulation of $H_2O_2$ is inhibited, and ascorbate peroxidase activity is enhanced. Simultaneously, it led to higher ascorbic acid and glutathione contents, lower dehydroascorbate, and oxidized glutathione (Wang *et al*., 2012). A decline in EO yield can be due to leaf senescence. Considering the role of Mel in regulating the senescence process, it could be stated that one of the possible reasons to justify the positive effects of Mel on increasing the EO of Mentha Spicata is its regulatory effect on leaf senescence. The auxin-like effect of Mel may also contribute to its positive impact on the production of SMs. Mel, a well-known indoleamine, shares the same precursor, tryptophan amino acid, with IAA. Due to its structural similarity with indole-3-acetic acid (IAA), it has been suggested that Mel may play a role in the plant growth and development process (Sun *et al*., 2016).

### *Essential Oil Extraction of* Mentha Spicata

The EO extracted from control and Mel-treated plants was a clear pale yellow colored liquid with a specific odor. The EO yield percentage of control and Mel-treated plants is presented below (Table 1). The yield percentages of EO for control and S1-S6 were 0.92±0.05%, 0.74±0.03%, 0.43±0.02%, 0.41±0.04%, 0.36±0.05%, 0.42±0.03%, 0.39±0.04%%, respectively.

*Table 1. Yield percentage of* Mentha Spicata.

| Timepoint (days) | Concentration of applied melatonin (mM) | EO yield percentage (%) |
|---|---|---|
| 0 | 0 | 0.92±0.05 |
| | 0.05 | 0.74±0.03 |
| 2 | 0.1 | 0.43±0.02 |
| | 0.2 | 0.41±0.04 |
| | 0.05 | 0.36±0.05 |
| 5 | 0.1 | 0.42±0.03 |
| | 0.2 | 0.39±0.04 |

The results revealed changes in yield, content, and quantity of main components of EOs obtained from Mel-treated experimental Mentha Spicata. Mel caused a significant decrease in the EO yield of Mentha Spicata compared to the control group at two different time points. The amount of distilled EO was different for control (0.92±0.05%). Mel-treated Mentha Spicata leaves in the following order: 0.74±0.03% (0.05mM Mel treatment, collected after 2 days) > 0.43±0.02% (0.1mM Mel treatment, collected after 2 days) > 0.42±0.03% (0.1mM Mel treatment, collected after 5 days) > 0.41±0.04% (0.2mM Mel treatment, collected after 2 days) > 0.39±0.04% (0.2mM Mel treatment, collected after 5 days) > 0.36±0.05% (0.05mM Mel treatment, collected after 5 days. Although the EO yield percentage was lower in Mel-treated plants than in control, Mel still positively affects the content of EOs, especially the main components. The results of GC proved this enhancement of main compounds of EOs in response to Mel treatment.

***UV Analysis of Essential Oil***

The UV absorption spectra of the EO extracted from control and Mel-treated Mentha Spicata were recoded and presented below (

Figure 2). The UV spectra of the EO of control and Mel-treated plant samples (S1-S6) included 17, 8, 12, 17, 17, 16, and 11 peaks in the selected range (190-500 nm), respectively.

The UV spectrum of the EO extracted from control showed peaks at λ= 368.60 nm, 341.20 nm, 337.20 nm, 327.20 nm, 323.60 nm, 310.40 nm, 295.40 nm, 291.20 nm, 283.60 nm, 278.80 nm, 274.20 nm, 260.60 nm, 244.80 nm, 240 nm, 228.40 nm, 221.60 nm, 212.40 nm, which in some cases either were missing for the Mel-treated oil samples or present at different wavelength range (Table 2). For example, there were no peaks observed at λ= 368.60 nm and 244.8 nm for S1-S6, λ= 341.20 nm for S2, λ=337.20 nm for S1, S2 and S6, λ=327.20 nm for S1, S2, S5 and S6, λ=323.60 nm for S1-S4 and S6, λ=310.40 nm for S5, λ=295.40 nm for S2- S6, λ=291.20 nm for S1 and S6, λ=283.60 nm for S1 and S2, λ=278.80 nm for S1, S3 and S4, λ=274.20 nm for S1-S3, λ=240 nm for S1, S3, S5 and S6, λ=228.40 nm for S2, S3 and S6, λ=221.60 nm for S1, S5 and S6, λ=212.40 nm for S1, S2 and S6. Similarly, some peaks, which were present in the spectra of Mel-treated EO samples, are absent in the UV spectrum of the control EO sample. For instance, peaks at λ=472 nm and 413.20 nm of S4, λ=389.40 nm and 371.40 nm of S3, λ0=368.40 nm of S6, λ=353.40 nm of S3, λ=334.80 nm and 331.60 nm of S5, λ=301.60 nm of S2, λ=307.20 nm of S3, λ=280.60 nm of S6, λ=254 nm of S1, λ=258.80 nm of S3, λ=251 nm of S5, λ=253 nm of S6, λ=230.60 nm-232.20 nm of S1- S3 and S6, λ=208.60 nm -212 nm of S2, S4 and S5, λ=203.20 nm- 209.40 nm of S2, S3, S5 and S6, λ=200.60 nm- 201.80 nm of S1, S2 and S5, λ=192.60 $nm^{-1}$98 nm of S2- S4, and λ=193.80 nm of S4, were absent in the spectrum of control EO sample. According to the obtained spectra, there is a significant difference in the number of observed peaks and correlated wavelengths for the control and Mel-treated tested EOs.

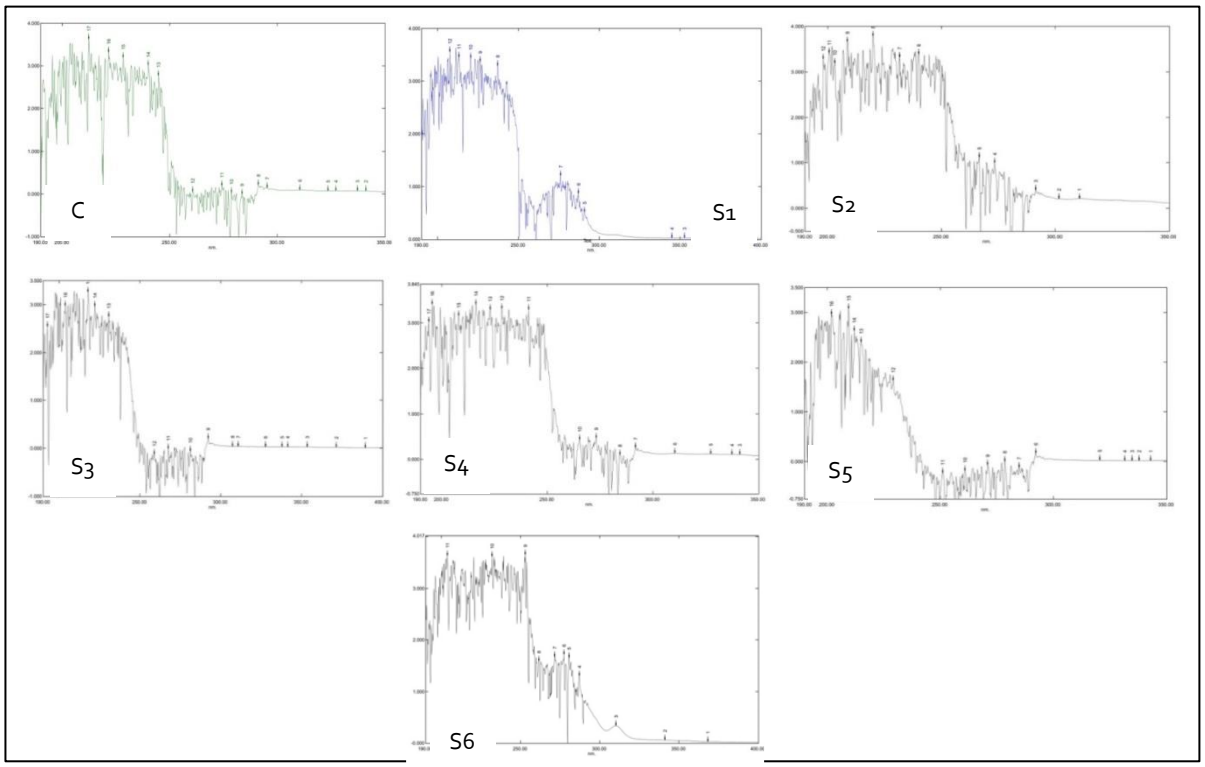


**Figure 2.** UV spectra of the EO extracted from control and Mel-treated Mentha Spicata leave

S1:0.05mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days; S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days; S5: 0.2 mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

*Table 2. The wavelength and OD profile of the EO of control and Mel-treated* Mentha Spicata *leaves*

| Chromophore | C | | S1 | | S2 | | S3 | | S4 | | S5 | | S6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | λ | OD | λ | OD | λ | OD | λ | OD | λ | OD | λ | OD | λ | OD |
| Colored compounds | - | - | - | - | - | - | - | - | 472.00 | 0.010 | - | - | - | - |
| | - | - | - | - | - | - | - | - | 413.20 | 0.012 | - | - | - | - |
| Polyunsaturated and aromatic compound | - | - | - | - | - | - | 389.40 | 0.012 | - | - | - | - | - | - |
| | - | - | - | - | - | - | 371.40 | 0.019 | - | - | - | - | - | - |
| | - | - | - | - | - | - | - | - | - | - | - | - | 368.40 | 0.028 |
| | - | - | - | - | - | - | 353.40 | 0.027 | - | - | - | - | - | - |
| | 368.60 | 0.027 | - | - | - | - | - | - | - | - | - | - | - | - |
| | 341.20 | 0.068 | 341.20 | 0.052 | - | - | 341.40 | 0.031 | 341.20 | 0.109 | 343.00 | 0.019 | 341.20 | 0.056 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 337.20 | 0.070 | - | - | - | - | 338.00 | 0.031 | 337.60 | 0.111 | 338.00 | 0.019 | - | - |
| | - | - | - | - | - | - | - | - | - | - | 334.80 | 0.019 | - | - |
| | - | - | - | - | - | - | - | - | - | - | 331.60 | 0.019 | - | - |
| | 327.20 | 0.075 | - | - | - | - | 327.80 | 0.032 | 327.20 | 0.120 | - | - | - | - |
| | 323.60 | 0.075 | - | - | - | - | - | - | - | - | 320.40 | 0.022 | - | - |
| | 310.40 | 0.092 | 310.40 | 0.578 | 310.60 | 0.213 | 310.80 | 0.043 | 310.40 | 0.136 | - | - | 310.20 | 0.334 |
| | - | - | - | - | 301.60 | 0.211 | 307.20 | 0.042 | - | - | - | - | - | - |
| | 295.40 | 0.147 | 294.60 | 0.784 | - | - | - | - | - | - | - | - | - | - |
| | 291.20 | 0.205 | - | - | 291.40 | 0.396 | 292.20 | 0.199 | 291.80 | 0.253 | 292.20 | 0.160 | - | - |
| | 283.60 | -0.010 | - | - | - | - | 281.20 | -0.061 | 284.40 | 0.102 | 284.80 | -0.122 | 287.00 | 1.303 |
| | - | - | - | - | - | - | - | - | - | - | - | - | 280.60 | 1.655 |
| | 278.80 | 0.032 | - | - | 273.40 | 0.989 | - | - | - | - | 278.60 | -0.002 | 277.40 | 1.710 |
| C=O, H-CH=O | 274.20 | 0.190 | - | - | - | - | - | - | 273.20 | -0.469 | 271.00 | -0.072 | 271.60 | 1.675 |
| | 260.60 | 0.051 | 268.00 | 2.122 | 266.60 | 1.115 | 267.40 | -0.021 | 265.40 | 0.431 | 260.80 | -0.185 | 261.60 | 1.587 |
| C-X, $CH_3$-OH, $CH_3NH_2$, $CH_3I$ | - | - | 254.00 | 3.812 | - | - | 258.80 | -0.134 | - | - | 251.00 | -0.231 | 253.00 | 3.649 |
| | 244.80 | 2.773 | - | - | - | - | - | - | - | - | - | - | - | - |
| | 240.00 | 3.017 | - | - | 240.00 | 3.385 | - | - | 241.20 | 3.287 | - | - | - | - |
| | - | - | 232.20 | 3.552 | 231.60 | 3.315 | 230.60 | 2.764 | - | - | - | - | 232.00 | 3.614 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 228.40 | 3-.192 | 227.20 | 3.704 | - | - | - | - | 228.60 | 3.300 | 229.20 | 1-.622 | - | - |
| 221.60 | 3.322 | - | - | 220.00 | 3.779 | 222.00 | 2.964 | 223.00 | 3.284 | - | - | - | - |
| 212.40 | 3.621 | - | - | - | - | 217.80 | 3.259 | 216.20 | 3.403 | 215.00 | 2.405 | - | - |
| - | - | - | - | 208.60 | 3.657 | - | - | 208.00 | 3.154 | 212.00 | 2.622 | - | - |
| - | - | - | - | 203.20 | 3.187 | 203.80 | 2.974 | - | - | 209.40 | 3.070 | 203.60 | 3.626 |
| - | - | 200.60 | 3.565 | 200.60 | 3.413 | - | - | - | - | 201.80 | 2.956 | - | - |
| - | - | - | - | 198.00 | 3.280 | 192.60 | 2.538 | 195.60 | 3.399 | - | - | - | - |
| - | - | - | - | - | - | - | - | 193.80 | 3.014 | - | - | - | - |

S1:0.0 5mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days.

S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days.

S5: 0.2mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

***FTIR Analysis of Essential Oil***

The FTIR spectra of the EO extracted from control and Mel-treated Mentha Spicata were recorded and presented below (

Figure *3*). The FTIR spectra of the EO of control and Mel-treated plants showed a slight variation for S1-S3 and S6 and a significant difference for S4 and S5 compared to control. This variation can be explained by the presence or absence of different bands and their absorbance wavelengths. As each band corresponds to specific functional groups, which indirectly correlate phytochemicals present in the composition of EOs, so different spectra may indicate a different chemical profile for control and Mel-treated plants.

There was an H-bonded OH band (3329.25 $cm^{-1}$) in the FTIR spectrum of the S5, whereas no bands were observed for the rest. The C-H band (3078.49 $cm^{-1}$) for S6, $CH_3$ group (2922.25; 2924.18; 2928.04; 2924.19; 2924.18 and 2360.95; 2353.23; 2351.3; 2355.16; 2357.09 $cm^{-1}$) for control, S1, S2, S3 and S6, a weak methyl band (1367.58; 1369.5; 1369.5; 1369.5; 1369.5; 1369.5 and 1437.02; 1440.87; 1438.94; 1440.87; 1440. 37 and 1440.87 $cm^{-1}$) for control, S1, S2, S3, S5 and S6, respectively, which show the presence of saturated aliphatic structures, were absent in spectra of control, S1-S5 in case of C-H stretch, in spectra of S4-S5 in case of $CH_3$ band and spectrum of S4 in case of a weak methyl stretch. Similarly, the =C-H-out of-plane

bend in FTIR spectra, which correspond to more than one aromatic substitution observed for control, S1, S2, S3, S5, and S6 in the range of 704.0-893.07 $cm^{-1}$), but was absent in the spectrum of S4. In addition, bands appeared in 1055.1-1053.17$cm^{-1}$ for control, S1, S2, S3, & S6 and 1109.11$cm^{-1}$ for control, S1, S2, S3, S5, and S6, a characteristic for mono-substitutes aromatic compounds, were not seen in the spectrum of S4. The C-O stretching (1246.06 and 1247.99 $cm^{-1}$) for control, S1, S2, S3, and S6, which allows for the differentiation of ethers or compounds having C-O- group from alkanes was not observed in spectra of S4 and S5. The C- X bond (565.18 and 563.23 $cm^{-1}$) for control and S6, respectively, which most probably corresponds to the halogen-carbon stretching, was missing in the spectra of S1-S5. In these spectra, the bands appeared at 484.15-495.72 $cm^{-1}$ for control, S1, S2, S3, S5, and S6 and missing in the spectrum of S4, which indicates the presence of aliphatic amines. There was a peak at 439.78$cm^{-1}$ only for S6, showing the occurrence of aromatic and secondary aryl amines in the EO samples. The FTIR spectra and their detailed explanation are listed below (Table 3).

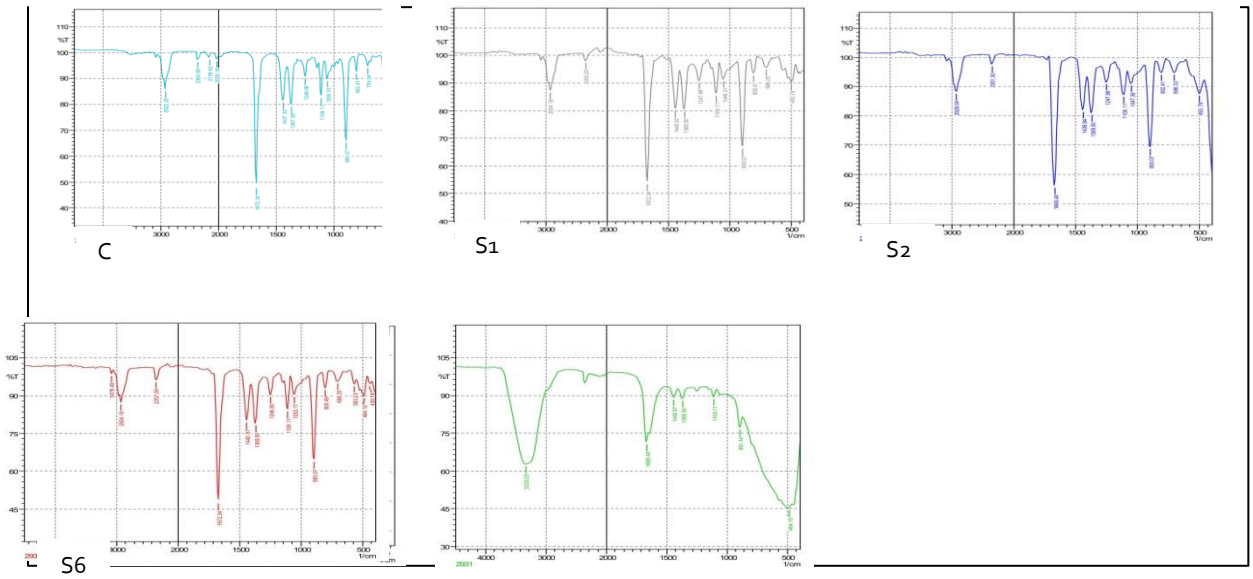


*Figure 3. FTIR spectra of the EO extracted from control and Mel-treated* Mentha Spicata *leaves*

S1:0.05mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days; S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days; S5: 0.2 mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

*Table 3. The FTIR profile of the EO extracted from control and Mel-treated* Mentha Spicata *leaves.*

| Bond assignment | Control | S1 | S2 | S3 | S4 | S5 | S6 |
|---|---|---|---|---|---|---|---|
| Aromatic and secondary aryl amines | - | - | - | - | - | - | 439.78 |
| Aliphatic amines | 486.08 | 493.79 | 493.79 | 495.72 | - | 484.15 | 484.15 |
| C-X stretch | 565.18 | - | - | - | - | - | 563.23 |
| =C-H-out of-plane bending (aromatic substitution) | 704.04 | 698.25 | 696.33 | 698.25 | - | - | 698.25 |
| | 802.41 | 802.41 | 802.41 | 800.49 | - | - | 800.49 |
| | 893.07 | 893.07 | 893.07 | 893.07 | - | 891.14 | 893.07 |
| C-H-in-plane bending (aromatic) | 1055.1 | 1049.31 | 1047.38 | 1051.24 | - | - | 1053.17 |
| | 1109.11 | 1109.11 | 1109.11 | 1109.11 | - | 1109.11 | 1109.11 |
| C-O stretching | 1246.06 | 1247.99 | 1247.99 | 1247.99 | - | - | 1246.06 |
| Aliphatic $CH_3$ bending | 1367.58 | 1369.5 | 1369.5 | 1369.5 | - | 1369.5 | 1369.5 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Aliphatic $CH_2$ bending | 1437.02 | 1440.87 | 1438.94 | 1440.87 | - | 1440.37 | 1440.87 |
| Non-conjugated C= C stretching | 1672.34 | 1672.34 | 1668.48 | 1672.34 | - | 1668.48 | 1672.34 |
| -C≡- stretch | 2029.18 | - | - | - | - | - | - |
| | 2158.42 | - | - | - | - | - | - |
| $CH_3$ stretching | 2360.95 | 2353.23 | 2351.3 | 2355.16 | - | - | 2357.09 |
| | 2922.25 | 2924.18 | 2928.04 | 2924.19 | - | - | 2924.18 |
| C-H stretching | - | - | - | - | - | - | 3078.49 |
| H-bonded –OH stretch | - | - | - | - | - | 3329.25 | - |

S1:0.0 5mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days.

S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days.

S5: 0.2mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

### *GC Analysis of Essential Oil*

The GC chromatogram, chemical composition, and retention indices of the EO of Mentha Spicata extracted from control and Mel-treated plants are presented below (

Figure **4,** Table 4). The phytoconstituents of the EO are listed based on their elution order on the DB-5 column. In total of fifty, fifty-six, sixty-two, forty-three, fifty-seven, thirty-four and fifty-seven compounds, which were identified from the EO of control and Mel-treated plants (S1-S6), respectively, represent 100% of the total EO and the most abundant compounds were (-)-carvone (66.89 %), followed by d-menthol (3.44 %), isocaryophyllene (2.47 %), germacrene-D (2.17 %), α-bourbonene (0.87 %), trans-carveol (0.85 %), β-ocimene (0.64 %), α/β-pinene (0.45 %) and endo-borneol (0.42%) in respect to the EO of control plant. The chemical profile of the EO of Mentha Spicata showed a significant variation in Mel concentration and the time points, after which the plant materials were collected. This variation can be explained in terms of the presence or absence of novel phytoconstituents and their percentage in total EO (Table 4).

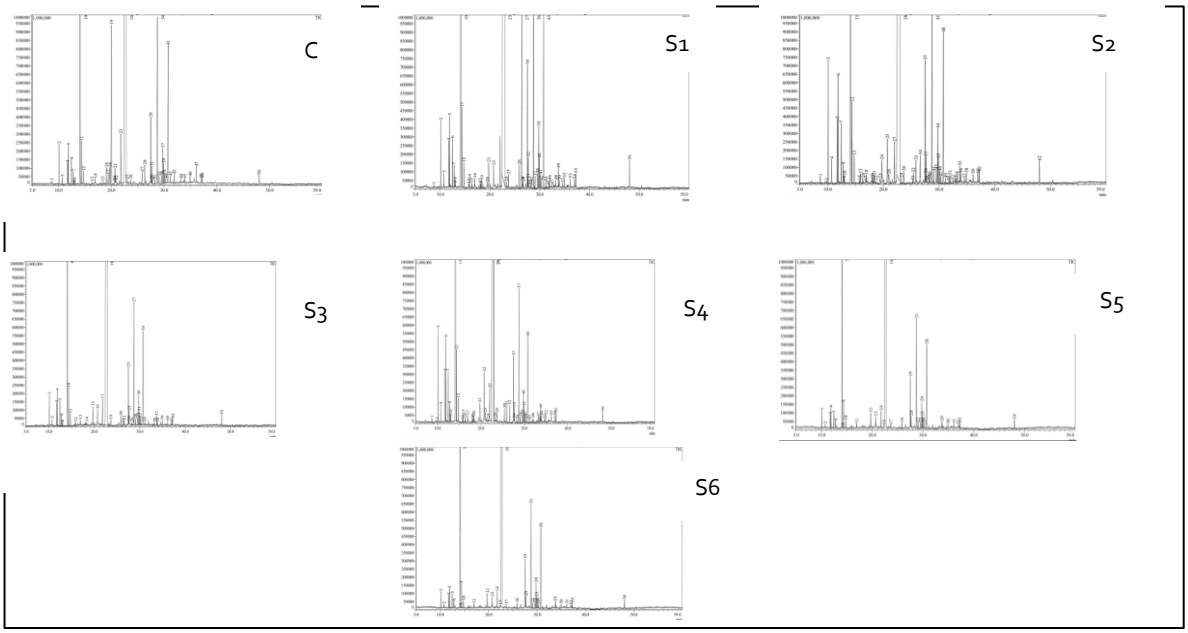

*Figure 4. GC chromatogram of the EO extracted from control and Mel-treated* Mentha Spicata *leave*

S1:0.05mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days; S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days; S5: 0.2mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

*Table 4. The percentage of different phytoconstituents in the composition of the EOs of control and Mel-treated* Mentha Spicata *leaves*

| No. | Compound name | Percentage of different phytoconstituents in total EO | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Control | S1 | S2 | S3 | S4 | S5 | S6 |
| 1 | α-Pinene | 0.45 | 0.35 | 0.68 | 0.38 | 0.6 | 0.33 | 0.78 |
| 2 | Camphene | 0 | 0 | 0.13 | 0 | 0.11 | 0 | 0.14 |
| 3 | Sabinen | 0.25 | 0.25 | 0.38 | 0.29 | 0.36 | 0.27 | 0.4 |
| 4 | β-Pinene | 0.45 | 0.38 | 0.6 | 0.44 | 0.56 | 0.4 | 0.63 |
| 5 | β-Myrcene | 0.28 | 0.27 | 0.35 | 0.31 | 0.34 | 0.28 | 0.36 |
| 6 | 3-Octanol | 0.15 | 0.14 | 0.12 | 0.14 | 0.15 | 0.14 | 0.14 |
| 7 | 1, 2-Diisopropenyl cyclobutane | 16.74 | 17.04 | 21.95 | 18.63 | 21.8 | 15.63 | 21.89 |
| 8 | β-Ocimene | 0.64 | 0.56 | 0.61 | 0.65 | 0.65 | 0.62 | 0.68 |
| 9 | Isomenthone | 0.21 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | p-Menthan-3-one | 0.14 | 0 | 0 | 0 | 0 | 0 | 0 |
| 11 | Endo-Borneol | 0.42 | 0.39 | 0.33 | 0.41 | 0.36 | 0.42 | 0.36 |
| 12 | Neodihydrocarveol | 0 | 0 | 0.18 | 0 | 0.2 | 0 | 0.1 |
| 13 | 3-Dodecyne | 0 | 0.25 | 0.5 | 0.27 | 0.59 | 0.17 | 0.8 |
| 14 | trans-p-metha$^{-1}$(7)8, diene-2-ol | 0 | 0 | 0.38 | 0.49 | 0.36 | 0.46 | 0.27 |
| 15 | N-Methyl-N-(2-propynyl)-2-propen$^{-1}$-amine | 0 | 0 | 0 | 0 | 47.1 | 0 | 0 |
| 16 | d-Menthol | 3.44 | 0 | 0 | 0 | 0 | 0 | 0 |
| 17 | Trans-Carveol | 0.85 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | (-)-Carvone | 66.89 | 71.67 | 66.81 | 70.7 | 21.9 | 72.53 | 68.18 |
| 19 | L-Verbenone | 0.18 | 0.16 | 0.15 | 0.15 | 0.14 | 0.14 | 0.14 |
| 20 | (-)-cis-Carvyl acetate | 0 | 0 | 0 | 0 | 0.17 | 0 | 0.21 |
| 21 | Eugenol | 0.29 | 1.4 | 0 | 0 | 0 | 0 | 0 |
| 22 | 2-Isopropylidenecyclohexanone | 0 | 0 | 0.27 | 0 | 0 | 0 | 0 |
| 23 | α-Bourbonene | 0.87 | 0.83 | 0.9 | 0.89 | 0.54 | 1.09 | 0.59 |
| 24 | 2, 4-Diisopropenyl$^{-1}$-methyl$^{-1}$-vinylcyclohexane | 0 | 0 | 0.17 | 0.18 | 0.1 | 0.22 | 0.11 |
| 25 | 3-methyl-2-pent-2-enyl-cyclopent-2-enone | 0 | 0.2 | 0 | 0 | 0 | 0 | 0 |
| 26 | 2, 4-Diisopropenyl$^{-1}$-methyl$^{-1}$-vinylcyclohexane | 0.22 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27 | Isocaryophyllene | 2.47 | 2.01 | 1.79 | 2.08 | 1.25 | 2.77 | 1.29 |
| 28 | β-ylangene | 0.18 | 0 | 0 | 0.19 | 0 | 0 | 0 |
| 29 | 7, 11-Dimethyl-3-methylene$^{-1}$, 6, 10-Dodecatriene | 0.48 | 0 | 0 | 0.46 | 0.22 | 0.57 | 0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 30 | cis-α-Bisabolene | 0.29 | 0 | 0.17 | 0.23 | 0.13 | 0.28 | 0.24 |
| 31 | Germacrene-D | 2.17 | 1.66 | 1.48 | 1.8 | 1.05 | 2.5 | 0 |
| 32 | Tetracyclol nonane | 0.17 | 0 | 0 | 0 | 0 | 0 | 0 |
| 33 | α-Cadinol | 0.21 | 0 | 0 | 0 | 0 | 0.1 | 0 |
| 34 | Cis-Z-α-Bisabolene epoxide | 0.14 | 0 | 0 | 0.19 | 0 | 0 | 0 |
| 35 | β-Farnesene | 0 | 0.41 | 0.36 | 0 | 0 | 0 | 0 |
| 36 | β-Copaene | 0 | 0.1 | 0 | 0 | 0 | 0 | 0 |
| 37 | Patchoulane | 0 | 0.2 | 0.34 | 0 | 0 | 0.28 | 0 |
| 38 | Germacrene-D-4-ol | 0 | 0 | 0.12 | 0.14 | 0.12 | 0.17 | 0.1 |
| 39 | 3-Tetradecyne$^{-1}$-ol | 0 | 0 | 0 | 0.1 | 0 | 0 | 0 |
| 40 | Dihydro Carvyl acetate | 0 | 0 | 0 | 0 | 0 | 0 | 0.15 |

S1:0.05mM Mel treatment & collected after 2 days; S2: 0.05mM Mel treatment & collected after 5 days

S3: 0.1mM Mel treatment & collected after 2 days; S4: 0.1mM Mel treatment & collected after 5 days.

S5: 0.2mM Mel treatment & collected after 2 days; S6: 0.2mM Mel treatment & collected after 5 days.

After comparing the chemical profile of the EO of control with the Mel-treated plants, it was observed that the EO of control shows different GC fingerprints in terms of EO-phytoconstituents and their percentage. For example, considering the most abundant phytoconstituents, the percentage of (-) - carvone in total EO of control was 66.89%, whereas the EOs of S4 and S5 contained the lowest (21.7 %) and the highest (72.53 %) amount of (-) - carvone, respectively. Similarly, isocaryophyllene (C: 2.47 %; S4: 1.25 %; S5: 2.77 %), germacrene- D (C: 2.17 %; S6: 0 %; S5: 2.5 %), α- bourbonene (C: 0.87 %; S6: 0.59 %; S5: 1.09 %), β- ocimene (C: 0.64 %; S2: 0.61 %; S6: 0.68 %), α- pinene (C: 0.45 %; S5: 0.33 %; S6: 0.78 %), β- pinene (C: 0.45 %; S1: 0.38 %; S6: 0.63 %), endo-borneol (C: 0.42 %; S4: 0.39 %; S5: 0.42 %) are some other phytoconstituents along with their percentages in total EO, which got changed after Mel treatment (Table 4).

In addition to variations in the percentage of various phytoconstituents in total EOs, some components either disappeared from GC chromatograms of the EOs of Mel-treated plants or newly appeared (Table 4). The phytoconstituents such as d-menthol, p-menthan-3-one, trans-carveol, eugenol, β-ylangene, α-cadinol, cis-Z-α-Bisabolene epoxide are examples of disappeared components, whereas neo dihydro carveol, cis-carvyl acetate and dihydro carvyl acetate, β-farnesene, β-copaene. Patchoulane, and germacrene-D-4-ol are the newly appeared phytoconstituents.

Based on the findings of the GC fingerprint of the EOs extracted from control and Mel-treated plants, it was observed that Mel may increase the percentage and total number of various EO phytoconstituents.' However, in most cases, the EOs extracted from Mel-treated plants' chemical profiles were similar among the S1-S3-S5 and S2-S4-S6.

The chemical profile of EO of Mentha Spicata showed a significant variation in response to Mel concentration and the time points after treatment. For example, the most abundant component of EO, (-)-carvone % in total EO in control was 66.89%, whereas EO obtained from $S_4$ contained the lowest amount of (-)-carvone (21.7%), and the highest percentage was found in $S_5$ (72.53%). Similarly, isocaryophyllene (C: 2.47%; $S_4$: 1.25%; $S_5$: 2.77%), germacrene-D (C: 2.17%; S6: 0%; $S_5$: 2.5%), α-bourbonene (C: 0.87%; S6: 0.59%; $S_5$: 1.09%), β-ocimene (C: 0.64%; $S_2$: 0.61%; S6: 0.68%), α-pinene (C: 0.45%; $S_5$: 0.33%; S6: 0.78%), β-pinene (C: 0.45%; $S_1$: 0.38%; S6: 0.63%), endo-borneol (C: 0.42%; $S_4$: 0.39%; $S_5$: 0.42%) were some other phytoconstituents, which their amount got changed after Mel treatment. In addition to difference in amount of various components in total EO, some of them disappeared after Mel treatment; d-menthol, p-menthan-3-one, trans-carveol, eugenol, β-ylangene, α-cadinol, cis-Z-α-Bisabolene epoxide; or newly synthesized; neo dihydro carveol, cis-carvyl acetate anddihydro carvyl acetate, β-farnesene, β-copaene. patchoulane, germacrene-D-4-ol.

About these data, there was an increase in the percentage of different components and their total number in the composition of EO obtained from Mentha Spicata after Mel treatment. These differences in the chemical composition of EO obtained from control and Mel-treated Mentha Spicata were confirmed by the UV and FTIR spectra of each. In these spectra, some peaks were present only for control, some for Mel-treated EO samples, and some bands were observed in the same wavelength and wave numbers or within the same range of frequencies. These data are essential because the presence or absence of bands or peaks indicates the occurrence of specific molecules in a mixture.

Our results are justified by the previously published data on the effect of growth regulators, specifically Mel, on SMs. Coskun *et al*. (2019) reported that the application of Mel caused a different chemical profile of EO in rosemary plants. There were components in the EO of Mel-treated plants that were not observed in the EO of the control. They also stated that different concentrations of Mel affected EO components differently. In this study, the foliar spray of Mel caused a variation in the percentage of principal components in the composition of Mentha Spicata EO in a dose- and time-dependent manner. In Mentha Spicata, the (-)-carvone content in control was 66.89%, whereas this amount differed in Mel-treated plants' EO in the following order: 72.53% in $S_5$ > 71.67% in $S_1$ > 70.70% in $S_3$ > 66.81% in $S_2$ > 68.18% in S6 > 21.9% in $S_4$.

### *Antioxidant activity (DPPH assay)*

The antioxidant activity of the EOs extracted from control and Mel-treated Mentha Spicata was measured by DPPH, and the results are summarized below (Table 5). The EOs of control and Mel-treated plants were able to reduce the free radical DPPH to DPPHH and change its stable violet color to yellow with IC50 values of 3.27± 0.02 µg/mL for control, 1.61± 0.05 µg/mL for $S_4$, and 3.155± 0.02 µg/mL for S6. It was observed that the EO of Mel-treated leaves exhibited higher antioxidant activity than the EO of the control. However, compared

to ascorbic acid (IC50= 0.27± 0.03 µg/mL), a potent radical scavenging agent, EOs of control and Mel-treated Mentha Spicata exhibited weak antioxidant potential.

*Table 5. Antioxidant activity of the EOs extracted from control and Mel-treated* Mentha Spicata *leaves*

| No | Sample | IC50 Value (µg/mL) |
|---|---|---|
| 1 | Ascorbic acid | 0.27± 0.03 |
| 2 | Control | 3.27± 0.02 |
| 3 | S4 | 1.61± 0.05 |
| 4 | S6 | 3.155± 0.02 |

S4: 0.1mM Mel treatment & collected after 5 days. S6: 0.2mM Mel treatment & collected after 5 days.

Foliar application of Mel increased the antioxidant activity of EO. There was a difference between the anti-oxidative potential of EO obtained from control and Mel-treated plants. The results of the DPPH assay showed that the antioxidant activity of the EO of Mentha Spicata before Mel treatment was less than the EO extracted from Mel-treated plants. The EO sample from control and Mel-treated plants were able to reduce the free radical DPPH to DPPHH and change its stable violet color to yellow with IC50 values of 3.27±0.02µg/mL for control, 1.61±0.05µg/mL for S4, and 3.155±0.02 for S6. This result agrees with the experiment done by Sarrou *et al*. (2015), which concluded that the application of 15mM Mel enhanced the total phenolic and flavonoid content and their antioxidant activity of bitter orange leaves. These effects may be due to an increase in the percentage of hydrogenated compounds in the composition of EO.

## Conclusion

As per the objectives of this study, which mainly aimed to evaluate the effect of Mel, both in-vivo and in-vitro, on EO yield percentage, the phytochemical composition of EO, antioxidant potency of EO, and as a phytohormone and considering the results of all in-vivo and in-vitro experiments as well as the possible mechanisms, through which Mel can influence the production of EO in Mentha Spicata, it is concluded that Mel, a well know biogenic amine, which initially comes from the pineal gland of animals and functions as a potent free-radicle scavenging molecule, circadian rhythm regulator, a neurologic bio-amine, etc., could be considered as an efficient elicitor to induce biosynthesis of EO in plants in a dose and time-dependent manner. This endogenic indoleamine could change the EO yield percentage of Mentha Spicata, improve the phytochemical profile of its volatile compounds qualitatively, enhance the free-radical scavenging capacity of plant's EO, and regulate plant growth and development in a concentration and time-dependent mode. So, it may act as a promising molecule to increase aromatic crop yield with the desired quality. To get a much more straightforward and better result from the application of Mel as a promising phytohormone for improving SM production in medicinal plants, including Mentha Spicata,

it is recommended to use different concentrations of Mel in a broader range and at various time points.

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