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# Antimicrobial Potential and Chemical Profiling of Leaves Essential Oil of *Mentha* Species Growing under North-West Himalaya Conditions

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## Abstract

***Mentha* essential oil is one of the most utilized essential oil in the food and pharmaceutical industries. The present study reports the chemical composition and antibacterial properties of leaf essential oils of *Mentha* species. Further, the effect of the harvesting period on essential oil yield was also investigated. Firstly, the cultivated *Mentha* piperita and wild *Mentha longifolia,* revealed significant differences in their chemical profile. *M. longifolia* essential oil was characterized with endo-borneol (1.12-6.2%), caryophyllene (2.72-7.03%), isopipertenone (0.07-0.36%), germacrene D (0.98-3.22%), 3-cyclopentene-1-one,2-hydroxy-3-(3-methyl-2-butenyl)- (21.91-56.72%) and piperitone oxide (8.96-39.31%), whereas, M. piperita leaves essential oil was found rich in isomenthone (5.97-6.75%), 1-menthone (7.32-18.32%) and menthol (18.03-58.53%), etc. The essential oils of both *Mentha* species exhibited strong antimicrobial activity as evaluated using poisoned food technique, dry weight method, and disc diffusion method against *Candida albicans, Fusarium oxysporum, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae* and *Escherichia coli.* Secondly, the maximum essential oil yield was observed in July month, 0.63±0.01 and 0.56±0.01%, respectively for *M. piperita* and *M. longifolia.***

**Keywords:** Disc diffusion method, Poisoned food technique, Harvesting period, Gas chromatography-mass spectrometry

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## INTRODUCTION

*Mentha* is one of the most important genera of the Lamiaceae family, and its medicinal and aromatherapeutic effects have been known since ancient times.[1] It is made up of about 25-30 species that are found in Asia, Africa, Australia, Europe, and North America.[2] *M. piperita* L. and *M. longifolia* L. are two of the most important medicinal plant species in terms of ethnobotanical significance among these species.

*M. longifolia,* often known as wild mint, is a plant native to Africa, Europe, and Asia that has long been traditionally used to cure bronchitis, nausea, anorexia, flatulence, ulcerative colitis, and liver problems.[3,4] The antioxidant, anti-inflammatory, antimicrobial, analgesic, stimulant, carminative, diaphoretic, antiemetic, antispasmodic, anticatarrhal, emmenagogue, and anticarcinogenic properties have also been documented for the species.[2,3] *M. piperita* L., commonly known as peppermint, is a popular tonic herb and flavoring ingredient. It is a sterile hybrid from a cross between spearmint (*M. spicata*) and water mint (*M. aquatica*).[5] In the past, headache, migraine, vomiting, bronchitis, menstrual cramps, and digestive diseases like irritable bowel syndrome and constipation have all been treated with this species.[6] Additionally, this species also exhibit astringent, antiseptic, antipyretic, analgesic, anti-carcinogenic, antioxidant, and antispasmodic property.[7]

All these medicinal properties in *Mentha* species are due to the presence of essential oil, a complex mixture of low molecular weight volatile compounds. It is a product of secondary metabolism.[8] In folk medicine, essential oils have been used for treating various ailments such as joint swelling, skin diseases, cuts, and wounds.[9,10] They demonstrated antibacterial, antiviral, antioxidant, insecticidal, and other biological effects.[10] Interestingly, these essential oils are also utilized in the treatment of cancer.[11] Further, they have a wide range of therapeutic applications, including food preservation, pharmaceuticals, and alternative treatments.[11,12] *M. longifolia* essential oil was found rich in pulegone, carvone, piperitone, menthone, menthol, Isomenthone, 1,8-Cineole, while menthol, menthone, menthyl acetate, menthofuran, piperitinone oxide, $\alpha$-Terpinene, carveol, etc. constitute the bioactive composition of *M. piperita*.[13]

Secondary metabolites (alkaloids, phenols, flavonoids, and so on) have therapeutic characteristics; their production and accumulation are influenced by the genotype as well as the plant's interaction with its environment. The photoperiod, temperature, relative humidity, and other climatic conditions influence the chemical profile because some chemicals accumulate over time in response to environmental changes.[14] The plant secondary metabolite products are also affected by harvesting time, plant age, and crop density.[15] As a result, it is critical to understand the appropriate time and age for plants to have a rich chemical profile with robust biological activities, so they may be used for essential oil extraction and medicine development against deadly infections in the long run.

The researchers in India,[16] Saudi Arabia,[17] Turkey,[18] Serbia,[19] and others have previously identified seasonal change in the phytochemical content and biological properties of essential oils in both *Mentha* species. The motive of this study was to analyze the chemical profiles of essential oils from *M. piperita* and *M. longifolia* leaves harvested in the northwestern Himalaya, as well as to look into their antibacterial efficacy against pathogenic bacterial strains (*Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae, and Staphylococcus aureus*) and fungi (*Fusarium oxysporum* and *Candida albicans*)

## MATERIAL AND METHODS

### Plant collection

In April-July 2018, 100 g leaves of cultivated *M. piperita* and wild *M. longifolia* were harvested from Y.S. Parmar University, Nauni, Solan nursery (30°85'70" N, 77°16'74" E) and Giripul (Distt. Sirmour; 30°52'53" N, 77°12'37" E), Himachal Pradesh, India, respectively, for the extraction of essential oil. The leaves were randomly sampled from the growing plot/area using a stratified random sampling method in the morning hours before noon for each month (April, May, June, and July).

### Identification of plants

The *Mentha* species in the field were identified based on morphological characters

(*M. logifolia* is characterized by ovate-lanceolate, greyish-green leaves with white hairs from the above, and purplish-white flowers, whereas in *M. piperita* leaves are ovate serrate, green-colored with long petiole, and purple flowers).[20,21] Collected plants were further authenticated in the herbarium of Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, H.P., India. (UHF-Herbarium No. 13571). The voucher specimens have also been deposited in the herbarium of the School of Biological and Environmental Sciences, Shoolini University, Solan, H.P., India.

**Isolation of essential oil**

The leaves were harvested from plants (90 plants/3 quadrat) of *M. piperita* and *M. longifolia* and utilized for essential oil extraction using the hydrodistillation method.[22] Briefly, fresh leaves (100 g) were chopped into bits and combined with distilled water (1 litre) in a flask. In continuation, Clevenger-type equipment was used to distil the mixture for 3 hours at 60°C. The essential oil was extracted and stored at 4°C for further use.

**Essential oil yield**

Using the formula below, the essential oil yield in the percentage of each sample was computed:

$$\text{Oil yield} = \frac{w1}{w2} \text{ X } 100$$

Where, w1 = net weight of oil (g); w2 = total weight of fresh leaves (g)

**Gas chromatography-mass spectrometry analysis of leaves essential oil**

GC-MS analysis of *M. piperita* and *M. longifolia* leaves essential oil was performed as per the standard method of Okut et al. in the SERF laboratory of Punjab University, Chandigarh, India.[23] The analysis was done using a GC-MS instrument (Thermo Trace 1300 GC coupled with Thermo TSQ 8000 Triple Quadrupole MS) fitted with a TG 5MS capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) for qualitative determination. The essential oil was diluted 1/10 in n-hexane (v/v) before analysis. The Autosampler was built for the injection of essential oils. The injector temperature was 250°C. The temperature of the column was programmed from 60°C to 250°C, held for 60°C for 2 minutes, moderately raised to 200°C, and kept for 5 minutes with an increase of 15°C /minute to 220°C. Helium was employed as a carrier gas with 1.5 mL/minutes flow rate, and mass spectra were taken in scan mode. The ionization voltage and split ratio were 70 eV and 1:20, respectively. The ion source and interface were both at 230°C and 250°C, respectively. The solvent cut time was 3 minutes. A total of 1.0 µl of the sample was used in the analysis. The percentage of constituents was also determined using the response factor, and retention indexes (RIs) were calculated using the retention duration of n-alkanes (C10-C20) injected at the same temperature and under the same conditions. The phytoconstituents were identified by comparing their RI to those described in the literature, and by comparing their mass spectra to those recorded in the NIST 2.0 electronic library and the Wiley 275 libraries, with Match Factor (SI) and Reverse Match Factor (RSI) greater than 900.

Antimicrobial activity

**Microbial strains and growth conditions**

To evaluate the antimicrobial potential, four bacterial [Gram positive bacteria (*Bacillus subtilis* (MTCC 5521) and *Staphylococcus aureus* (MTCC73) and Gram-negative bacteria (*Escherichia coli* (MTCC739) and *Klebsiella pneumoniae* (MTCC109))] and two fungal strains [*Fusarium oxysporum* (SR266-9), *Candida albicans* (ATCC90028) were selected. All microbial strains were procured from the Shoolini University's Yeast Biology and Plant Pathology laboratory. The bacteria were maintained on Nutrient Agar (NA) at 37°C for 18 to 24 h. The fungal strain such as *F. oxysporum* was maintained on Potato Dextrose Agar (PDA) medium, while *C. albicans* was maintained on Yeast Peptone Dextrose Agar (YPD) media at 25°C for 48 to 72 h.

Agar disc diffusion method

NA and YPD agar were spread with bacterial strains and yeast (0.5 McFarland standard), respectively.[24,25] The 6 mm diameter discs of sterile filter paper soaked with various concentrations (1.5 - 3.0 mg/mL) of diluted essential oil (1:10 in DMSO, v/v) were kept on the inoculated plates. For positive control Ampicillin and Fluconazole (50 µg/mL) were used against tested bacterial and fungal strains, respectively. After the incubation period of 18-24 h at 37°C (in case of bacteria) and 48-72 h at 25°C (for fungi), inhibition zones were observed in the

plates around the paper discs. An inhibition zone diameter was measured using HiMedia Zone scale. The experiment was carried out in triplicates.

**Poisoned food technique**

The leaves essential oils of both *Mentha* species (1:10 diluted in DMSO, v/v) at varying concentrations (1.5- 3.0 mg/mL) were transferred to conical flasks containing 25 mL of sterilized PDA and then poured into Petri plates.

On the other hand, in the center of the Petri dish, 6-7 days old culture of *F. oxysporum* was placed and incubated at 25°C. After seven days, the radial development of fungal mycelium was checked. The findings were compared with negative control (plates with DMSO only).[26]

The inhibition percentage of fungi in treatments was estimated using the formula below:

$$\text{Percent inhibition} = \frac{C - T}{C} \text{ X100}$$

Where C represents the colony radius in the control plate, and T represents the pathogen radial growth in the presence of essential oil.

**Dry weight method**

Briefly, five test tubes containing sterile potato dextrose broth (5 mL) were inoculated with actively growing *F. oxysporum* mycelium (20 µL containing 1x10$^6$ spores/mL) obtained from a 6-7 days old culture. In four test tubes, 20 µL (1.5-3.0 mg/mL) of essential oil (1:10 in DMSO, v/v) was added, while one test tube was left without essential oil as a negative control, and all tubes were incubated at 25°C for 7 days. In the tubes, growth of visible mycelia represents the degree of effectivity of the essential oil. The fungal mycelia were further separated by filtration using Whatman filter paper No. 1. To achieve constant weight, the filter paper was dried at 60°C.

The growth inhibition of fungi was calculated by observing the control and sample mycelial dry weight.[27] The percentage growth inhibition was calculated using the following formula:

$$\% \text{ Growth inhibition} = \frac{\text{Control - Test}}{\text{Control}} \text{ X 100}$$

**Minimum inhibitory concentration (MIC)**

The broth dilution method described by the Clinical and Laboratory Standards Institute was used for evaluating MIC. Briefly, essential oil was diluted geometrically (5-0.0095 %) in a 96-well microtiter plate, having growth control (NB/PDB/YPD broth containing DMSO) and positive control well (NB/PDB/YPD broth inoculated with each microbial culture and containing 10 mg/mL of Ampicillin, Hygromycin B and Fluconazole separately). Further, an equal number of cells (2 × 10$^6$ CFU mL-1, 0.5 McFarland) were inoculated into each well. At 37°C the microtiter plates were incubated for 24 h in case of bacteria and 25°C for 48 h in case of fungal strains.[26,28] The 15 µl of the resazurin dye was added to each well and plate was further incubated for 2-4 hours at 37°C. Any color changes after incubation period were observed. The blue color indicated bacterial growth whereas pink color indicated bacterial growth. Following the incubation period, the MIC values were reported as the lowest concentration of oil that suppresses observable microorganism growth. NB was used for antibacterial activity, while PDB and YPD Broth were used for antifungal activity.

**Data analysis**

Wherever necessary, analysis of variance (ANOVA), average and standard deviation were evaluated using GraphPad Prism 5.0. A p value <0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

**Essential oil yield**

In all harvesting months, there was a significant variation in the yield of peppermint and mint leaves essential oil with respect to the plant's harvesting age (Table 1). The results showed that the least essential oil yield was achieved in April (0.26±0.02% and 0.63±0.01%, respectively) and the maximum was achieved in July month

**Table 1.** The average essential oil yield (%) of *M. longifolia* and *M. piperita* from April to July month in year 2018

| Months | Essential oil content (%±SD) | |
|---|---|---|
| | *M. longifolia* | *M. piperita* |
| April | 0.24±0.01$^a$ | 0.26±0.02$^a$ |
| May | 0.40±0.02$^b$ | 0.45±0.01$^b$ |
| June | 0.49±0.02$^c$ | 0.58±0.01$^c$ |
| July | 0.56±0.01$^d$ | 0.63±0.01$^d$ |

[SD: Standard deviation; Different letters (a, b, c, d) were used when mean values were statistically different (p<0.05)]

**Table 2.** Phytocompounds observed in the essential oil from the leaves of *M. piperita* and *M. longifolia* (harvested during April-July, 2018)

| No. | Compounds | RI (Obs) | RI (Lit) | RT | Concentration (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | April | May | June | July |
| | | | | *Mentha piperita* | | | | |
| 1 | 1-Menthone | 1155 | 1150 | 5.57 | 7.32 | 12.69 | 18.32 | 13.24 |
| 2 | Isomenthone | 1141 | 1148 | 5.69 | - | 6.53 | 5.97 | 6.75 |
| 3 | Menthol | 1168 | 1165 | 5.89 | 58.42 | 51.05 | 18.72 | 18.03 |
| 4 | Menthyl acetate | 1282 | 1291 | 6.98 | 4.78 | 6.35 | 6.89 | 0.72 |
| 5 | Caryophyllene | 1408 | 1405 | 8.15 | 1.54 | 0.85 | 0.21 | 0.05 |
| 6 | Germacrene-D | 1475 | 1485 | 8.67 | 1.89 | 0.63 | 0.88 | 0.72 |
| | | | | *Mentha longifolia* | | | | |
| 1 | Endo-Borneol | 1154 | 1153 | 5.71 | 6.02 | 2.95 | 1.12 | 4.59 |
| 2 | α- Terpineol | 1178 | 1175 | 5.96 | - | 0.28 | 0.12 | 0.15 |
| 3 | Piperitone oxide | 1218 | 1230 | 6.65 | 39.31 | 15.22 | 17.08 | 8.96 |
| 4 | Carvone oxide | 1254 | 1260 | 6.69 | - | - | - | 0.01 |
| 5 | Isopiperitenone | 1264 | 1266 | 6.79 | 0.18 | 0.07 | 0.20 | 0.36 |
| 6 | Carvacrol | 1269 | 1278 | 7.08 | - | 1.06 | 0.64 | 0.32 |
| 7 | 3-Cyclopentene-1-one,2-hydroxy-3-(3-methyl-2-butenyl)- | 1341 | 1346 | 7.76 | 21.91 | 30.54 | 56.72 | 55.08 |
| 8 | Cinerolon | 1645 | 1641 | 8.03 | 0.17 | 0.08 | 0.25 | 0.19 |
| 9 | Caryophyllene | 1418 | 1418 | 8.15 | 7.03 | 5.57 | 3.85 | 2.72 |
| 10 | cis-a-Farnescene | 1465 | 1480 | 8.41 | 1.97 | 1.52 | 1.08 | 1.03 |
| 11 | Germacrene D | 1472 | 1475 | 8.67 | 3.22 | 2.18 | 1.95 | 0.98 |
| 12 | Caryophyllene oxide | 1570 | 1575 | 9.51 | 0.12 | 0.79 | 0.51 | 0.19 |

(- Absence of compound; RT- Retention time; RI (Obs)- Retention index observed; RI (Lit)- Retention index Listed)
*M. piperita*: Monoterpenoids (1, 2, 3, 4); Sesquiterpene (5, 6)
*M. longifolia*: Monoterpenoids (1, 2, 5, 6, 8, 10); Sesquiterpene (9, 11, 12); Monocyclic terpene ketone (3); Monoterpene ketone (4); Cyclic ketone (7).

(0.63±0.01 % and 0.56±0.01 %, respectively) in both *M. piperita* and *M. longifolia* leaves. The *M. piperita* essential oil yield was higher as compared to *M. longifolia* essential oil.

The monthly variation in essential oil yield could be related to the fact that as the plant ages, the number of leaves and their surface area grows, resulting in the creation of more oil glands and hence a rise in essential oil content. The plant density and harvesting time, according to Mansoori, have a major impact on dry biomass and oil yield production because higher density improves plant vegetative growth and the number of leaves per plant, which enhances light interception and so increases plant essential oil yield.[29]

**Essential oil chemical composition**

The volatile and semi-volatile components in the essential oil collected in each month, i.e., from April to May, were investigated using the GC-MS technique that revealed a unique chemical profile of essential oil of both *M. piperita* and *M. longifolia*. A total of six prominent phytochemical compounds (1-menthone, isomenthone, menthol, menthyl acetate, cryophyllene, and germacrene-D) were observed in the essential oil of *M. piperita* from April to July month (Table 2; Fig. 1).

Whereas, in *M. longifolia* leaves essential oil endo-borneol, α- terpineol, isopiperitenone, piperitone oxide, carvone oxide, 3-cyclopentene-1-one, 2-hydroxy-3-(3-methyl-2-butenyl)-, carvacrol, caryophyllene, germacrene D, and cis-a-farnescene were observed (Table 2; Fig. 2).

Most of the phytocompounds found in *M. piperita* essential oil, such as menthol, menthyl acetate, and menthone, have been reported previously in the literature, with notable differences in essential oil composition and quantity.[17,19,30] Other phytocompounds found in *M. longifolia* essential oil have also been described in previous papers, except 3-cyclopentene-1-one,2-

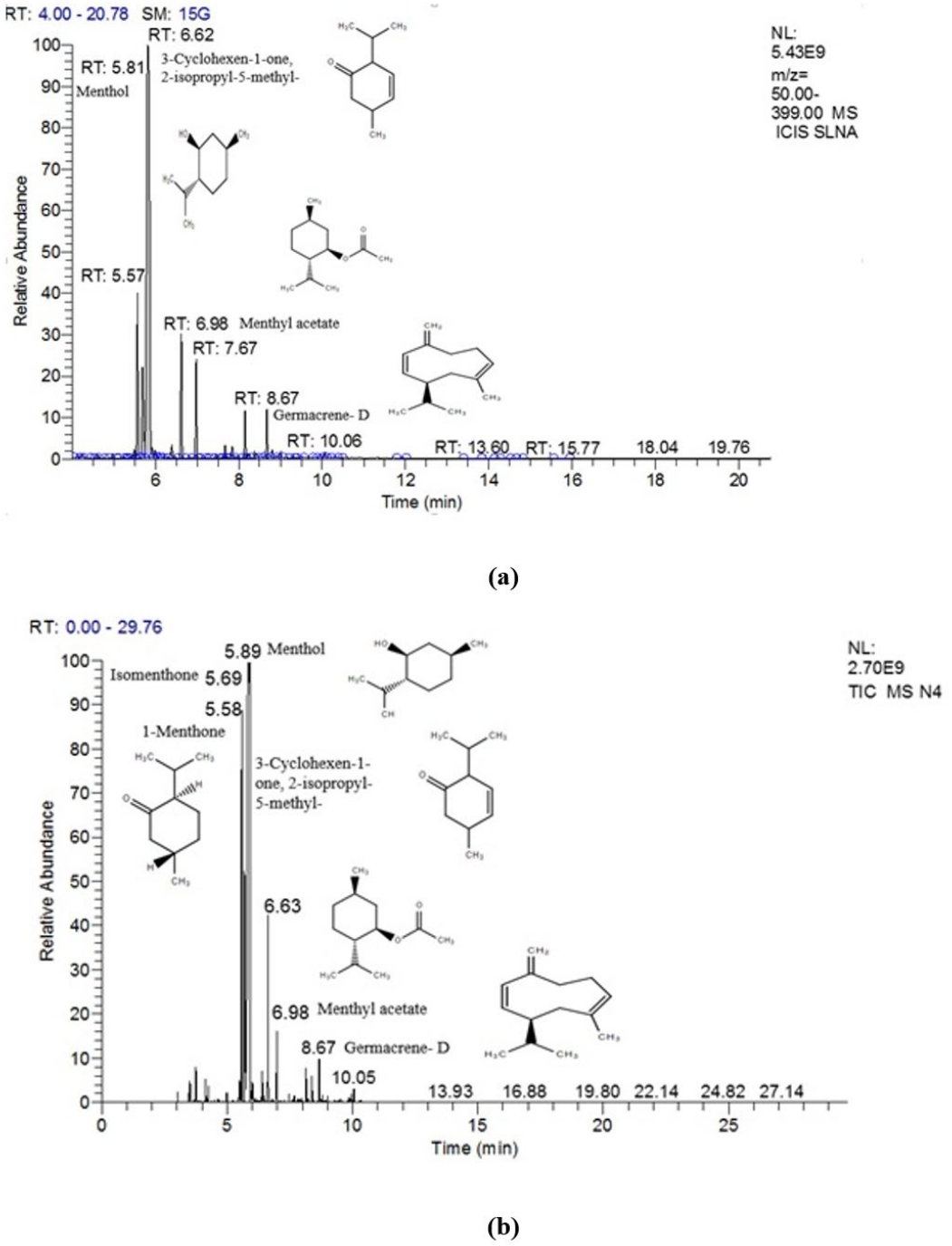



**Fig. 1.** GC-MS chromatograms of essential oil of *M. piperita* obtained from leaves in the month of April (a) and July (b).

hydroxy-3-(3-methyl-2-butenyl)-, which is a new compound with unknown action.[31-33] For instance, Verma et al. observed piperitenone oxide, germacrene D, (E)-caryophyllene, α-humulene, 2- hydroxy piperitone, thymol, and α-longipinene in the *M. longifolia* essential oil collected from the Garhwal region of Western Himalaya.[34]

In the literature, the effect of seasonal change in the essential oil chemical composition from a few *Mentha* species has

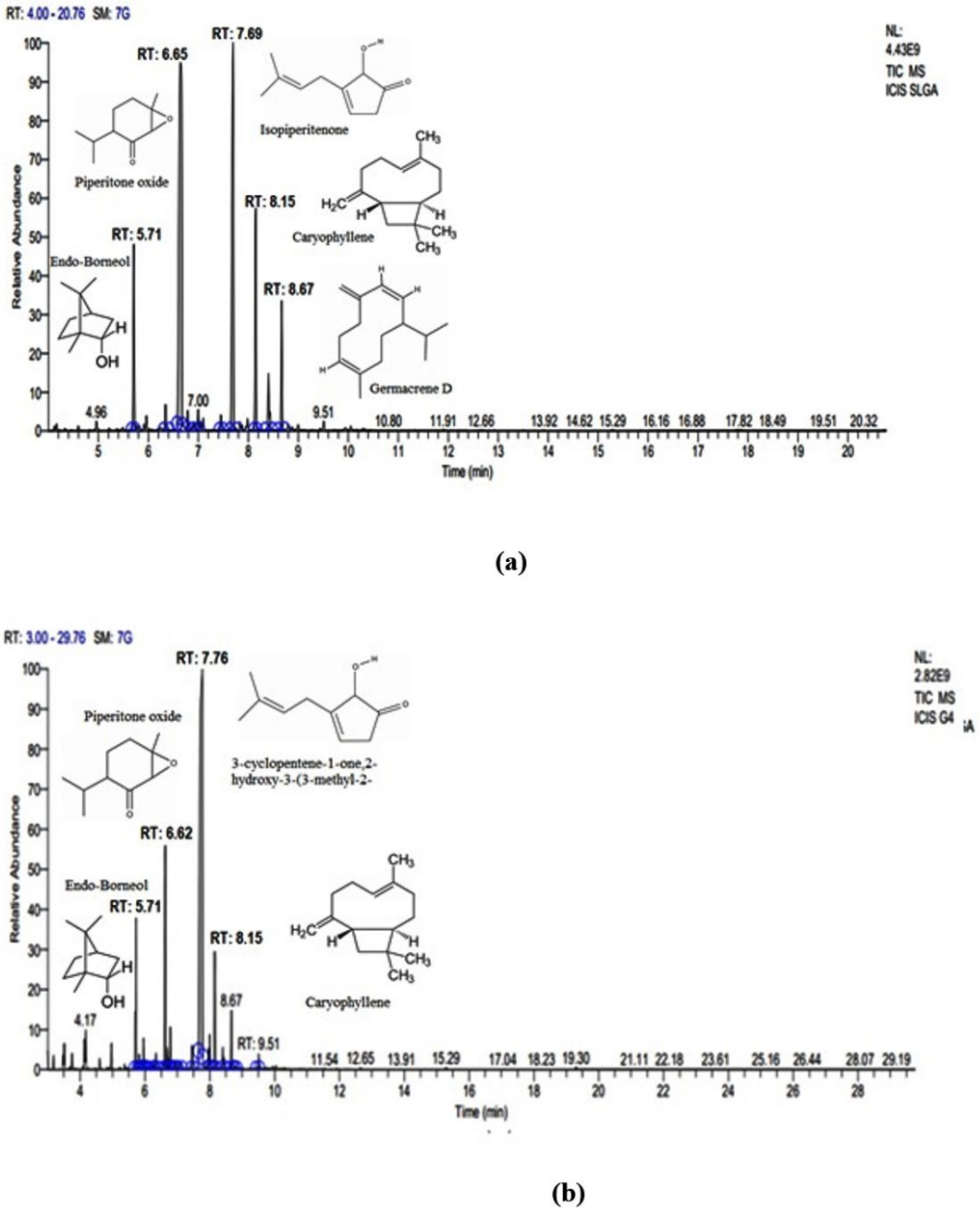
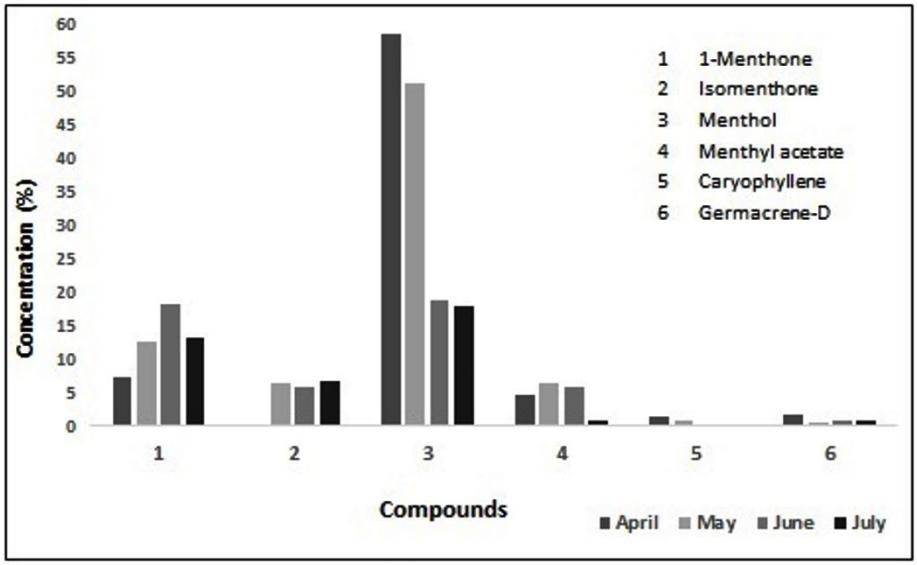


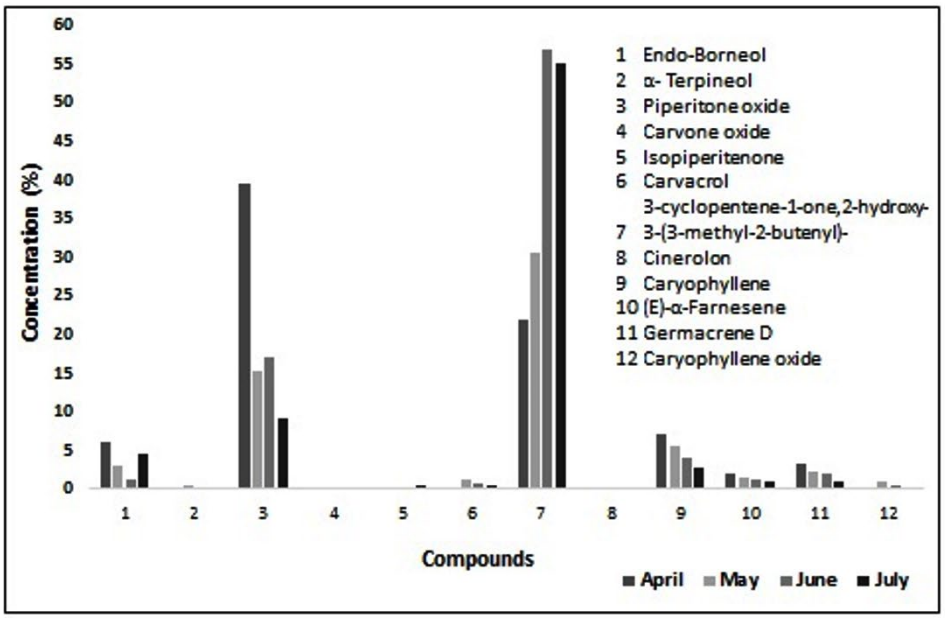

**Fig. 2.** GC-MS chromatograms of essential oil of *M. longifolia* obtained from leaves in the month of April (a) and July (b).

been documented.[23,31-33] The current study also revealed variation in the concentration (%) of phytocompounds detected in the essential oil of both *Mentha* species in each studied month samples (Table 2 and Fig. 3).

For example, in *M. piperita* essential oil, the amount of 1-menthone was increased from April month to June month (7.32% - 18.32%) and then decreased in the July month (13.24%). Menthol was also reduced from April to July




a




b

**Fig. 3.** Monthly variation in the composition of essential oil of the leaves of *M. piperita* (a) and *M. longifolia* (b).

**Table 3.** Antibacterial activity of essential oil of leaves of *M. piperita* and *M. longifolia*

| Bacterium | Months | Inhibitory zones (in mm) measured by the Hi media scale (mean + SD) | | | | |
|---|---|---|---|---|---|---|
| | | Ampicillin | Essential oil | | | |
| | | 50 µg/ml | 1.5 mg/ml | 2.0 mg/ml | 2.5 mg/ml | 3.0 mg/ml |
| | | | | *Mentha piperita* | | |
| *B. subtilis* | May | 20±0.6 | 14±0.6$^{a}$ | 16±0.6$^{b}$ | 17±0.6$^{c}$ | 19±0.6$^{d}$ |
| | June | 19±0.6 | 11±0.6$^{a}$ | 12±1.0$^{a}$ | 16±1.0$^{b}$ | 18±1.0$^{c}$ |
| | July | 19±0.6 | 13±1.0$^{a}$ | 14±0.6$^{a}$ | 17±0.6$^{b}$ | 18±0.6$^{c}$ |
| *K. pneumoniae* | May | 18±1.5 | 11±0.6$^{a}$ | 13±1.0$^{b}$ | 14±1.2$^{c}$ | 16±1.5$^{d}$ |
| | June | 19±0.6 | 12±0.6$^{a}$ | 14±0.6$^{b}$ | 15±0.6$^{c}$ | 17±0.6$^{d}$ |
| | July | 18±0.6 | 11±0.6$^{a}$ | 13±0.6$^{b}$ | 14±0.0$^{c}$ | 15±0.6$^{d}$ |
| *S. aureus* | May | 19±0.6 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 14±0.6$^{b}$ | 15±0.6$^{c}$ |
| | June | 18±0.6 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 14±1.2$^{b}$ | 16±0.6$^{c}$ |
| | July | 18±1.0 | 13±0.6$^{a}$ | 15±0.6$^{b}$ | 16±0.6$^{c}$ | 17±0.6$^{d}$ |
| *E. coli* | May | 15±0.6 | 12±0.6$^{a}$ | 13±1.0$^{a}$ | 14±0.6$^{b}$ | 15±0.6$^{c}$ |
| | June | 15±0.6 | 10±0.6$^{a}$ | 11±0.6$^{a}$ | 12±0.6$^{b}$ | 14±0.6$^{c}$ |
| | July | 15±0.6 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 14±0.6$^{b}$ | 15±0.6$^{c}$ |
| | | | | *Mentha longifolia* | | |
| *B. subtilis* | May | 20±0.6 | 10±0.6$^{a}$ | 12±0.6$^{b}$ | 15±0.6$^{c}$ | 17±0.6$^{d}$ |
| | June | 19±0.6 | 10±0.0$^{a}$ | 11±0.6$^{b}$ | 14±0.6$^{c}$ | 15±0.6$^{d}$ |
| | July | 20±1.0 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 15±0.6$^{b}$ | 16±1.0$^{c}$ |
| *K. pneumoniae* | May | 16±1.0 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 13±0.6$^{a}$ | 15±1.0$^{a}$ |
| | June | 16±0.6 | 10±0.0$^{a}$ | 12±0.0$^{a}$ | 12±0.6$^{a}$ | 14±0.6$^{a}$ |
| | July | 17±0.6 | 11±0.6$^{a}$ | 13±0.6$^{a}$ | 14±0.6$^{a}$ | 17±1.0$^{a}$ |
| *S. aureus* | May | 18±0.6 | 11±0.6$^{a}$ | 12±0.6$^{a}$ | 14±0.6$^{b}$ | 16±0.6$^{c}$ |
| | June | 17±0.6 | 10±0.6$^{a}$ | 11±0.6$^{a}$ | 12±0.6$^{b}$ | 14±0.6$^{c}$ |
| | July | 17±0.6 | 13±0.6$^{a}$ | 14±0.6$^{a}$ | 17±1.0$^{b}$ | 19±0.6$^{c}$ |
| *E. coli* | May | 12±0.6 | 11±0.6$^{a}$ | 13±0.6$^{b}$ | 15±0.6$^{c}$ | 16±0.6$^{d}$ |
| | June | 13±0.6 | 10±0.0$^{a}$ | 11±0.6$^{b}$ | 13±0.6$^{c}$ | 14±0.6$^{d}$ |
| | July | 13±0.6 | 10±0.6$^{a}$ | 12±0.6$^{b}$ | 13±0.6$^{c}$ | 16±1.0$^{d}$ |

[SD- Standard deviation; Different letters (a, b, c, d) were used when mean values were statistically different ($p<0.05$)].

(58.43% - 18.03%), whereas menthyl acetate was elevated from April to May (4.78% - 6.35%) and subsequently declined from June to July (58.43 percent-18.03 percent) (5.89% - 0.72%). In May and June, there was a higher concentration of menthyl acetate. Similarly, in *M. longifolia* essential oil, endo-borneol concentration was decreased from April (6.02%) to June (1.12%) and suddenly increased in July (4.59%). The increasing trend of the compound 3-cyclopentene-1-one, 2-hydroxy-3-(3-methyl-2-butenyl) was observed from April (21.91%) to July (55.08%). In continuation, from April (7.03 %) to May (5.57%), the caryophyllene level increased, then declined from June (3.85%) through July (2.72%). On the other hand, piperitone oxide (39.31-8.96 %) decreased from April (39.31%) to May (15.22 %), insignificantly increased in June (17.08 %) and then decreased in July (8.96 %) month. The remaining compounds like germacrene D (3.22-0.98 %) and cis-a-farnescene (1.97-1.-3 %) showed a decreasing trend from April to July month. Fig. 3 shows the increasing and decreasing order of concentration of detected phytocompounds.

In comparison to previous findings, the principal compounds observed in *M. longifolia* oil were menthone, pulegone, limonene, and terpinolenone, but the present investigation revealed that several of these compounds were absent in the *M. longifolia* essential oil. A chemical, 3-cyclopentene-1-one,2-hydroxy-3-(3-methyl-2-butenyl)-, was also observed in the GC-MS profile of *M. longifolia* essential oil, which is a new compound with unknown action. Similarly, most researchers identified menthofuran, limonene, and 1,8-cineole as important components in *M.*

**Table 4.** Minimum inhibitory concentration (MIC) of *M. piperita* and *M. longifolia* leaves essential oil against *B. subtilis, K. pneumoniae, S. aureus* and *E. coli*

| Months | MIC of Bacterial strains (mg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *B. subtilis* | | *K. pneumoniae* | | *S. aureus* | | *E. coli* | |
| | Oil | Ampicillin | Oil | Ampicillin | Oil | Ampicillin | Oil | Ampicillin |
| | | | | *Mentha piperita* | | | | |
| May | 0.62 | 0.07 | 0.62 | 0.07 | 1.25 | 0.03 | 1.25 | 0.07 |
| June | 1.25 | 0.07 | 1.25 | 0.07 | 1.25 | 0.03 | 2.50 | 0.07 |
| July | 0.62 | 0.07 | 0.62 | 0.07 | 0.62 | 0.03 | 1.25 | 0.07 |
| | | | | *Mentha longifolia* | | | | |
| May | 0.31 | 0.07 | 0.62 | 0.07 | 0.62 | 0.03 | 0.62 | 0.07 |
| June | 0.62 | 0.07 | 1.25 | 0.07 | 0.62 | 0.03 | 2.50 | 0.07 |
| July | 0.31 | 0.07 | 0.31 | 0.07 | 0.31 | 0.03 | 1.25 | 0.07 |

*piperita* essential oil, however, our investigation found none of these chemicals.[35,36] This difference in the essential oil chemical composition from plant species can be attributed to variation in climatic, edaphic conditions, harvesting period and drying technique, etc.[37] Additionally, chemical profile is influenced by all such factors that can boost the biosynthesis of some molecules while inhibiting the biosynthesis of others.[38]

**Antibacterial activity**

The study revealed that as the concentration of essential oil (*M. piperita* and *M. longifolia*) increased from 1.5 - 3.0 mg/mL the inhibition zone diameter was also increased from May to July month, and fluctuation in the essential oil activity of both *Mentha* species in each month was extremely minute (Table 3).

In addition, *M. piperita* essential oil showed the lowest MIC (0.62 mg/mL) against *B. subtilis, K. pneumoniae* (MIC - 0.62 mg/mL), and *E. coli* (MIC - 1.25 mg/mL) in May and July month harvest, and against S. aureus, (MIC - 0.62 mg/mL)

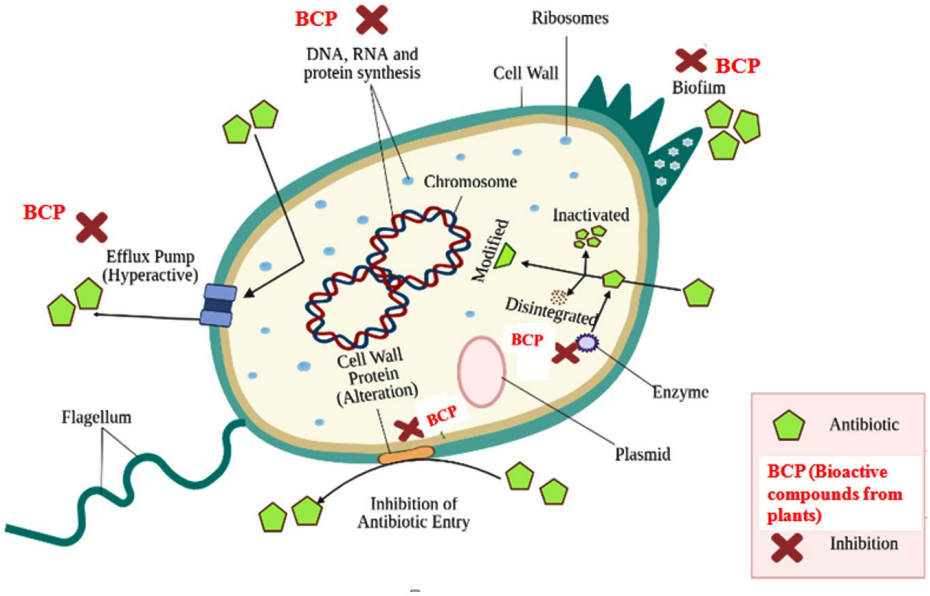



**Fig. 4.** Antibiotic resistance mechanisms and mode of action of plant bioactive compounds against bacterial strains. Reproduced from[48] under the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

**Table 5.** Antifungal activity of essential oil of leaves of *M. piperita and M. longifolia* against *C. albicans*

| Fungal strains | Months | Inhibitory zones (in mm) measured by the Hi media scale (mean+SD) | | | | |
|---|---|---|---|---|---|---|
| | | Fluconazole | Essential oil | | | |
| | | 50 µg/ml | 1.5 mg/ml | 2.0 mg/ml | 2.5 mg/ml | 3.0 mg/ml |
| | | | | *Mentha piperita* | | |
| *C. albicans* | May | 12±0.6 | 10±0.6$^{a}$ | 10±0.6$^{a}$ | 12±0.6$^{b}$ | 13±0.6$^{c}$ |
| | June | 12±0.6 | 11±0.6$^{a}$ | 12±0.0$^{a}$ | 13±1.0$^{b}$ | 15±0.6$^{c}$ |
| | July | 13±0.6 | 12±0.6$^{a}$ | 12±0.6$^{a}$ | 14±1.0$^{b}$ | 16±0.6$^{c}$ |
| | | | | *Mentha longifolia* | | |
| *C. albicans* | May | 12±0.6 | 10±0.6$^{a}$ | 11±1.0$^{a}$ | 12±0.6$^{b}$ | 13±1.0$^{c}$ |
| | June | 12±0.0 | 14±1.0$^{a}$ | 16±1.2$^{b}$ | 18±1.2$^{c}$ | 18±0.6$^{d}$ |
| | July | 13±1.2 | 12±0.6$^{a}$ | 18±0.0$^{b}$ | 18±0.6$^{c}$ | 19±1.0$^{d}$ |

[SD- Standard deviation; Different letters were used when mean values were statistically different (p<0.05)].

**Table 6.** Antifungal activity of essential oil of leaves of *M. piperita* and *M. longifolia* against *F. oxysporum*

| Method | Months | Essential oil concentration | | | |
|---|---|---|---|---|---|
| | | 1.5 mg/ml | 2.0 mg/ml | 2.5 mg/ml | 3.0 mg/ml |
| | | % Inhibition (mean+SD) | | | |
| | | | *Mentha piperita* | | |
| Poison food technique | May | 39.1±4.06$^{a}$ | 48.50±2.34$^{b}$ | 53.92±2.34$^{c}$ | 60.69±2.34$^{d}$ |
| | June | 24.61±2.03$^{a}$ | 32.85±3.53$^{b}$ | 39.92±3.53$^{c}$ | 56.41±2.03$^{d}$ |
| | July | 15.36±2.25$^{a}$ | 20.56±2.25$^{b}$ | 30.98±2.25$^{c}$ | 44.00±2.25$^{d}$ |
| Dry weight technique | May | 43.22±0.44$^{a}$ | 50.69±0.91$^{a}$ | 64.95±1.34$^{b}$ | 79.61±0.40$^{c}$ |
| | June | 38.36±1.03$^{a}$ | 47.56±1.57$^{b}$ | 62.63±0.57$^{c}$ | 74.22±0.94$^{d}$ |
| | July | 28.67±0.90$^{a}$ | 40.09±0.87$^{b}$ | 60.92±0.34$^{c}$ | 73.22±0.79$^{d}$ |
| | | | *Mentha longifolia* | | |
| Poisoned food technique | May | 41.72±2.34$^{a}$ | 48.50±2.34$^{b}$ | 52.56±2.34$^{c}$ | 60.70±4.69$^{d}$ |
| | June | 28.14±2.03$^{a}$ | 38.74±2.03$^{b}$ | 49.35±2.04$^{c}$ | 58.77±2.04$^{d}$ |
| | July | 18.48±0.90$^{a}$ | 24.47±2.25$^{b}$ | 36.19±2.25$^{c}$ | 47.91±2.25$^{d}$ |
| Dry weight method | May | 50.63±0.60$^{a}$ | 66.61±0.85$^{b}$ | 78.17±0.41$^{c}$ | 90.90±0.79$^{d}$ |
| | June | 33.04±0.77$^{a}$ | 47.92±0.75$^{b}$ | 77.57±1.20$^{c}$ | 87.90±0.76$^{d}$ |
| | July | 31.55±1.07$^{a}$ | 43.89±0.49$^{b}$ | 71.56±0.87$^{c}$ | 85.89±0.95$^{d}$ |

[SD-Standard deviation; Different letters were used when mean values were statistically different (p<0.05)].

in July month. Subsequently, May and July month harvest was found to be most effective against *E. coli* with MIC-1.25 mg/mL (Table 4). On the other hand, *M. longifolia* essential oil (May and July month harvest) showed the lowest MIC (0.31 mg/mL) against *B. subtilis.* Further, a similar MIC was observed against *S. aureus* and *K. pneumoniae* with the July month harvest (Table 4).

The *M. piperita* and *M. longifolia* essential oils were observed to be extra effective against *B. subtilis, K. pneumoniae,* and *S. aureus* than *E. coli*. The present findings are consistent with previous research.[39-45]

Although, the effect of season on the antibacterial property of *M. longifolia* and *M. piperita* is known, there is no report on the ideal harvesting time for *M. longifolia* and *M. piperita* in terms of antibacterial potential of essential oil within one growing season.[32,37,46] In contrary to previous reports, in this study almost similar activity was reported against Gram positive as well as Gram negative strains. As per existing

**Table 7.** MIC values of *M. piperita* and *M. longifolia* leaves essential oil against *C. albicans* and *F. oxysporum*

| Months | MIC of fungal strains (mg/mL) | | | |
|---|---|---|---|---|
| | *C. albicans* | | *F. oxysporum* | |
| | Essential oil | Ampicillin | Essential oil | Hygromycin B |
| | *Mentha piperita* | | | |
| May | 1.25 | 0.15 | 0.62 | 0.03 |
| June | 0.62 | 0.15 | 0.62 | 0.03 |
| July | 0.31 | 0.15 | 1.25 | 0.03 |
| | *Mentha longifolia* | | | |
| May | 0.62 | 0.15 | 0.31 | 0.03 |
| June | 0.31 | 0.15 | 0.62 | 0.03 |
| July | 0.15 | 0.15 | 0.62 | 0.03 |

literature, Gram positive bacterial strains are more susceptible to antibacterial agents as compared to Gram negative ones as the latter has a lipid bilayer that protects them against antimicrobials.[47] The bioactive content of *M. longifolia* and *M. piperita* essential oils is considered to be responsible for their antibacterial properties.

Balkrishna et al.[48] described antibiotic resistance mechanisms such as molecular target transformation, efflux pump hyperactivity, biofilm growth, enzyme induced destruction, and drug transformation. They demonstrated that, antibacterial activity of plants is linked to their bioactive composition, and can be mediated by inhibiting efflux pump expression level, protein and DNA synthesis, biofilm, and others as shown in Fig. 4.

The essential oils of *Mentha* spp. might work through the mechanisms described above due to diverse bioactive components.

Fungicidal activity

The analysis revealed that as the concentration of the essential oil (0.5 mg/mL-3.0 mg/mL) increased, inhibition of fungal growth also increased (Tables 5 and 6). The lowest MIC values were observed with essential oil of *M. piperita* against *C. albicans* in July and May months (MIC-0.31 mg/mL).

Whereas, the essential oil extracted in June and May months showed the same MIC value of 0.62 mg/mL against *F. oxysporum* (Table 7). On the other hand, the *M. longifolia* essential oilMIC values revealed the maximum fungicidal activity against *C. albicans* in July month (MIC-1.5 mg/mL), whereas, essential oil of May month harvest showed maximum antifungal activity (MIC-1.5 mg/mL) against *F. oxysporum* (Table 7). These findings proved that both *Mentha* species essential oil were more efficient against *Candida albicans* than *F. oxysporum*. Additionally, *M. longifolia* essential oil has stronger fungicidal action than *M. piperita* essential oil, similar to the antibacterial results. Hussain et al. observed the maximum antifungal activity of the *M. longifolia* oil in the summer season and *M. piperita* oil in the winter season against *F. oxysporum*.[33] Kizil et al. also observed the significant antibacterial effect of *M. piperita* essential oil against C. albicans, and *E. coli*.[49]

In the light of existing literature, it was found that, the antifungal and antibacterial potential of essential oil is attributed to the presence of some phytoconstituents with various mechanisms including hydrophobicity.[50] In addition, the mode of action of plant bioactive content is still unknown; it is thought that they interfere with cell membrane organization, resulting in a drop in membrane potential and a lower amount of ATP synthesis.[48]

The antibacterial effect of *M. piperita* oil is due to menthol, menthone, piperitenone oxide, and carvone, according to the available literature.[34] Subsequently, according to Zouari-Bouassida et al. monoterpenoids are responsible for the antimicrobial potential of *M. piperita* essential oil.[45] Similarly, in *M. longifolia* essential oil the antimicrobial activity is due to the existence of menthol, menthone, piperitone oxide, carvone, etc.[33] However, some of these components were missing from *M. longifolia* essential oil obtained in the northwestern Himalaya. The compounds observed during the present study were piperitone oxide, 3-cyclopentene-1-one,2-hydroxy-3-(3-methyl-2-butenyl)-, borneol, caryophyllene, cis-a-farnesene, germacrene D, etc. and most of these compounds have been reported in the literature with various biological activities.[19,31,39,41,51-54] Hence, the variation in antimicrobial potential of the essential oils harvested in the examined months could be related to variations in the quantity of these phytoconstituents in each month from April to July.

## CONCLUSION

In conclusion, *M. piperita* essential oil harvested in July month was more effective against *Klebsiella pneumoniae, Staphylococcus aureus* and *Candida albicans,* whereas May month harvest showed good activity against *Bacillus subtilis*, *Escherichia coli,* and *Fusarium oxysporum.* Similarly, *M. longifolia* essential oil from the July month harvest was more potent against *K. pneumoniae, E. coli,* and *Candida albicans,* as compared to May month harvest (moderately effective against *B. subtilis,* and *F. oxysporum*). This may be due to the cumulative effect of biologically active phytocompounds such as menthol, isomenthone, piperitone oxide, carvone oxide, and others, which accumulated in July, and germacrene D, caryophyllene oxide, caryophyllene, a-terpineol, and others in May. As a result, the harvesting time was found to be a key player for the antimicrobial potential of *Mentha* essential oils, like *M. piperita*, *M. longifolia* essential oil can be utilized in the pharmaceutical, perfumery, and food industries.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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None.

## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript

## ETHICS STATEMENT

Not applicable.

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