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CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM AERIAL PARTS OF *MENTHA PIPERITA* AND *MENTHA ARVENSIS*

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Keywords:

Essential oil, *Mentha piperita*, *Mentha arvensis*, GC-MS, FT-IR, Antifungal activity

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ABSTRACT: The present study deals with the chemistry and antifungal activity of essential oil (EO) of two species of *Mentha viz*. *Mentha piperita* and *Mentha arvensis* which was extracted using Clevenger-type apparatus. The percent yield of EO of *M. arvensis* (2.8%) was found higher than *M. piperita* (2.1%). Compositional analysis of *M. piperita* and *M. arvensis* EO oil by GC-MS revealed the presence of 51 and 35 compounds, respectively. Vibration spectroscopy performed in the 500-4000 cm^{-1} wave number region depicted variations in the presence of functional groups among the two EOs. The *M. piperita* EO exhibited better antifungal potential against *Penicillium digitatumin* comparison to *M. arvensis* EO. The results were found at par with the standard Carbendazim 50 WP at 250 µg/mL. Optical research microscopy study depicted profound morphological changes in hyphae, and spore-bearing organs of EO treated fungus, thereby supporting the better antifungal activity of *M. piperita* EO.

INTRODUCTION: Postharvest damages in fruits have been predicted to range between 10 to 40% [1]. Kinnow has very diminutive storage life at ambient conditions and economically stored at low temperature by sellers for marketing in the off period. Among various pathogens, attack by fungal pathogens is the major cause for the postharvest losses. The main fungal pathogens of kinnow during storage period are *P. digitatum*, *Penicillium italicum*, and *Aspergillus niger*. Out of these, *P. digitatum*, the green mold, causes the significant loss and is the most dominant mesophilic fungus of division Ascomycota. It is found in soils of citrus producing areas and is responsible for prevalent post-harvest diseases in citrus fruit causing huge losses due to decay caused by *P. digitatum* [2].

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Essential oils are emerging as possible alternatives for preservatives in the food industry due to their diverse antimicrobial properties. Essential oils are natural compounds produced as secondary metabolites in the plant system. These are aromatic oily volatile colorless liquids characterized by distinct odor and usually are less denser than water [3]. These are rich in secondary compounds such as flavonoids, polyphenols, and terpenoids and are generally synthesized in a specific cell or tissue types in leaves and plant stems. The main volatile components of EOs include monoterpenes, sesquiterpenes and their oxygenated derivatives such as aldehyde, ketones, alcohols, esters, and acids [4].

Many EOs and their primary compounds exhibit diverse biological activities such as antibacterial, antifungal, antioxidant, antimutagenic, and anti-microbial activities. Being natural and rich in antioxidants, these are considered safe for human consumption [5, 6]. There are 17,500 medicinal plant species among higher plants, and approximately

3,000 EOs exist among which 300 are commercially important having applications in perfume, pharmaceuticals and cosmetics industries [7, 8]. Among various plants, the genus *Mentha* (mint), an imperative member of the Lamiaceae family, is very diverse [9]. It is signified by approximately 19 species and 13 natural hybrids, mainly persistent herbs growing enthusiastically in damp or wet places. Mints can grow under an extensive range of agro-climatic conditions and are invasive and fast growing [10].

Many species and hybrids of *Mentha* also have considerable economic importance such as *Mentha spicata*, *Mentha aquatica*, *Mentha arvensis*, *Mentha canadensis*, *Mentha pulegium* and *Mentha piperita* [11]. Different species of *Mentha* are utilized across the globe for their medicinal and culinary properties. Mints are extensively cultivated for their oils and terpenoid components such as menthol, carvone, linalyl acetate, and linalool, for use in cosmetic, food, pharmaceutical, flavor, beverage and allied industries [12]. *Mentha species* essential oils have high antibacterial and antifungal activity and hence can be used as natural fungicides [13]. Commercially, the most sustainable species is peppermint (*M. piperita*), which is a hybrid of corn-mint (*M. canadensis*) and spearmint (*M. spicata*) [5]. Major bioactive compounds of *Mentha* are menthol, menthone and pulegone [14].

A lot of studies related to antimicrobial activities of EOs have been performed but the anti-mycotic potential of *Mentha* EOs against the postharvest pathogen, *P. digitatum* has not been explored as yet. The present investigation has been carried out on the compositional analysis of *M. arvensis*, and *M. piperita* leaves EOs focusing on the morphological variations of *P. digitatum* caused by the essential oils.

MATERIALS AND METHODS:

Plant Material: At the full flowering stage during May 2017, the mature fresh leaves of two *Mentha species* namely *M. arvensis* and *M. piperita* were collected from Herbal Garden, Department of Agronomy, Punjab Agricultural University, Ludhiana, India. The species were identified and authenticated by a plant agronomist, Dr. Amanpreet Singh from Department of Agronomy, Punjab Agricultural University, Ludhiana.

Extraction of Essential Oil: The fresh leaves were washed twice with double distilled water to remove dust, at room temperature. The fresh leaves of *M. arvensis* and *M. piperita* were coarsely grounded prior to the operation and after that subjected to hydro-distillation in Clevenger-type apparatus for 5h at 60 °C (until no more oil was obtained). The extracted essential oil was refluxed with diethyl ether and dried over anhydrous sodium sulfate, filtered and stored at 4 °C in dark amber colored vials till further analysis. The yield of essential oils was determined by using the formula:

$$\text{Yield (\%)} = \frac{\text{The volume of essential oil extracted (ml)}}{\text{Weight of sample taken (g)}} \times 100$$

Chemical Characterization of Essential Oils: The essential oils were analyzed using GC-MS (QP2010 Plus, Shimadzu, Japan), equipped with Rtx-5 MS capillary column (30.0 m × 0.25 mm i.d., 0.25 μm film thickness) for the separation of the components of essential oil. The injector was maintained at 260 °C and operated in split injection mode with split ratio 120.0 the split valve closed for 1 min. Helium gas was used as the carrier gas at a constant pressure of 69 kPa. The column oven was initially maintained at 50 °C for 2 min, raised to 210 °C at 3°C per min for 2 min and then to 250 °C at 6 °C per min for 6 min. The interface temperature was 270 °C and the sample was run in EI mode. Data acquisition was started 4 min after injection. The MS operating parameters used were; ionization voltage (EI) 70 eV, peak width 2 s, scan speed 1250 m/z; scan range 40-650 m/z and detector voltage 1.5 V.

Essential Oils Identification: The compounds of essential oils were identified based on the comparison of their relative retention times and mass spectra with literature data including Flavors and Fragrances of Natural and Synthetic Compounds 2 (FFNSC2), WILEY 8 and National Institute of Standards and Technology 14 (NIST14) libraries.

FT-IR Analysis: The vibrational spectroscopy of both *Mentha species* essential oil was performed on a Thermo Nicolet model 6700 FT-IR spectrometer at room temperature using ATR assembly at a resolution of 3.86 cm^{-1} in the 500-4000 cm^{-1} domains.

Determination of Antifungal Activity:

Procurement of Test Fungus: The culture of the test fungus *P. digitatum* isolated from kinnow fruit surface was procured from IARI (Indian Agriculture Research Institute), New Delhi with ITCC No. 7190.

Poisoned Food Technique: The antifungal activity of the *Mentha* essential oils against *P. digitatum* culture was evaluated by poisoned food technique [15]. All tests were performed in triplicates with different working concentrations (µg/mL) of the oil, *i.e.* 1000, 500, 400, 300, 200 and 100 µg/mL of *M. piperita* and *M. arvensis* essential oils along with standard Carbendazim 50 WP (250 µg/mL). For this purpose, each essential oil was serially diluted using 0.5% tween 20 to prepare test solutions of 1000, 500, 400, 300, 200, and 100 µg/mL. One ml of each concentration was incorporated into 15 ml molten potato dextrose agar medium and mixed well in Petri dishes. After solidification of the medium, 100 µl of prepared spore suspension containing 10^4 colony forming units (CFU ml^{-1}) of fungal spores were spread over medium. The Petri dishes were tightly sealed with parafilm and incubated at 25 °C in the incubator. The colony count was recorded until the 7^{th} day after inoculation (DAI). The percentage inhibition was calculated from the mean value of colony numbers of treated and untreated fungus.

Optical Research Microscopy of Fungal Growth: The morphological changes in fungus after treatment were studied by optical microscopy. The fungal samples were stained with lactophenol cotton-blue dye and observed under Optical research microscope (make Leica DM 5000B) at 200 and 400 X magnifications to examine any morphological changes. Untreated samples were also stained and examined. Optical micrographs were taken with the help of CCD camera attachment (Leica DFC 420C camera).

Statistical Analysis: The experimental results were expressed as the mean of three replicates. The data were analyzed using the SAS software 9.0. Differences between means were tested using the least significant difference, and treatment means were compared with Duncan's multiple range test ($P<0.05$) and t-grouping and P-value of $P<0.05$ was considered as significant.

RESULTS:

Yield and Chemical Composition of Essential Oils: Essential oils isolated from fresh leaves of both *Mentha species* were pale yellow. The percentage yield of *M. arvensis* essential oil (2.8%) was found to be higher than *M. piperita* essential oil (2.1%). The major compounds identified by GC-MS analysis in *M. piperita* and *M. arvensis* essential oils are shown in **Table 1**. A total of 51 and 30 components were identified in *M. piperita* and *M. arvensis* essential oil, respectively.

TABLE 1: MAJOR CONSTITUENTS (AREA %) IN *MENTHA PIPERITA* AND *MENTHA ARVENSIS*

Name	Area (%)	
	M. piperita	***M. arvensis***
Menthol	58.80	84.63
Pulegone	6.62	-
Isomenthne	6.42	-
L-menthol	-	4.58
Menthyl acetate	3.94	1.11
Menthofuran	3.11	-
Cyclohexanone	-	2.97
Neomenthone	2.64	-
(E)-Caryophyllene	1.95	-
Limonene	1.62	1.54
Neoisomenthol	-	1.46
Germacrene D	1.43	-
2-Cyclohexen-1-one	1.41	-
2-Isopropyl-5-methyl cyclohexanol	1.35	-
Eucalyptol	0.90	0.07
α-Pinene	0.71	0.10
(E)- β-Famesene	0.58	-
2-Isopropenyl-5-methylcyclohexanol	0.57	-
Viridiflorol	0.55	-

Table 2 showed the comparative existence of some compounds in *M. piperita* and *M. arvensis*. Menthol, the major component of both essential oils was found in higher concentration in *M. arvensis* (84.63%) than in *M. piperita* (58.80%). However, other compounds such as pulegone (6.62%), isomenthone (6.42%), menthofuran (3.11%), neomenthone (2.64%), caryophyllene (1.94%), germacrene (1.43%), 2-cyclohexen-1-one (1.41%), 2 -isopropyl -5 -methyl cyclohexanol (1.35*%) etc*. were found to be absent in essential oil of *M. arvensis*.

FT-IR Analysis: The FT-IR spectra of essential oil of both *Mentha species* revealed distinct variation for occurrence of several peaks indicating variation in compounds possessing different functional groups. The FT-IR spectrum of *M. piperita* showed a broad band at 3367.1 cm^{-1} due to bonded O-H

stretching, a band at 1706 cm^{-1} due to C=O stretching and a sharp band at 2888.7 cm^{-1} due to C−H stretching whereas in *M. arvensis* the similar stretching bands were observed at 3346 cm^{-1}, 1656 $cm^{-1,}$ and 2884.8 cm^{-1} respectively **Fig. 1A,** and **Fig. 1B**. However, a sharp bonded O−H stretch band and small overtone due to C=O band was observed in the FT-IR spectrum of *M. arvensis* in comparison to *M. piperita* essential oil.

Antifungal Activity Assay: The antifungal activities of *Mentha species* essential oils were assessed against *P. digitatum* w.r.t. absolute control and standard Carbendazim 50 WP. Results of the antifungal activity on the seventh day after inoculation (DAI) showed 100% inhibition at 1000 µg/mL in essential oils obtained from both species whereas at 100-500 µg/mL *M. piperita* essential oil showed better percent inhibition compared to *M. arvensis* essential oil **Fig. 2**. The maximum inhibition was observed at 500 µg/mL for both essential oils, *i.e.* 71% and 55% *M. piperita* and *M. arvensis* essential oils, respectively. Standard Carbendazim 50 WP showed 99.7% inhibition, which was found higher than both the essential oils **Fig. 3B**, **3C,** and **3D**. All the interactions, *i.e.* between different concentrations of both essential oils concerning control (*i.e.*, without treatment) **Fig. 3A** was significant with a coefficient of variance value of 0.765.

TABLE 2: COMPARISON OF MINOR CONSTITUENTS OF *MENTHA PIPERITA* AND *MENTHA ARVENSIS*

S. no.	Name	*M. piperita*	*M. arvensis*
1	Ethyl hexanol	+	-
2	Bicyclogermacrene	+	-
3	Caryophyllene oxide	+	-
4	Phytol acetate	+	-
5	Lavandulyl acetate	+	-
6	Neomenthol	+	-
7	α-Cadinol	+	-
8	α-Bourbonene	+	-
9	Neomenthyl acetate	+	-
10	δ-Cadinene	+	-
11	Bicyclo[3.1.0]hexane-6-methanol	+	-
12	1,6-Octadien-3-ol	+	-
13	Spathulenol	+	-
14	Trans-Nerolidol	+	-
15	2,5-diethyl-Tetrahydrofuran	+	-
16	Isomenthyl acetate	+	-
17	Eugenol	+	-
18	α-Gurjunene	+	-
19	(+)-4-Carene	+	-
20	3-Cyclohexene-1-methanol	+	-
21	BHT	+	-
22	1-Octen-3-ol	+	-
23	4,8-β-epoxy-Caryophyllane	+	-
24	P-Menthane-1,2,3-triol	+	-
25	Ispulegyl acetate	+	-

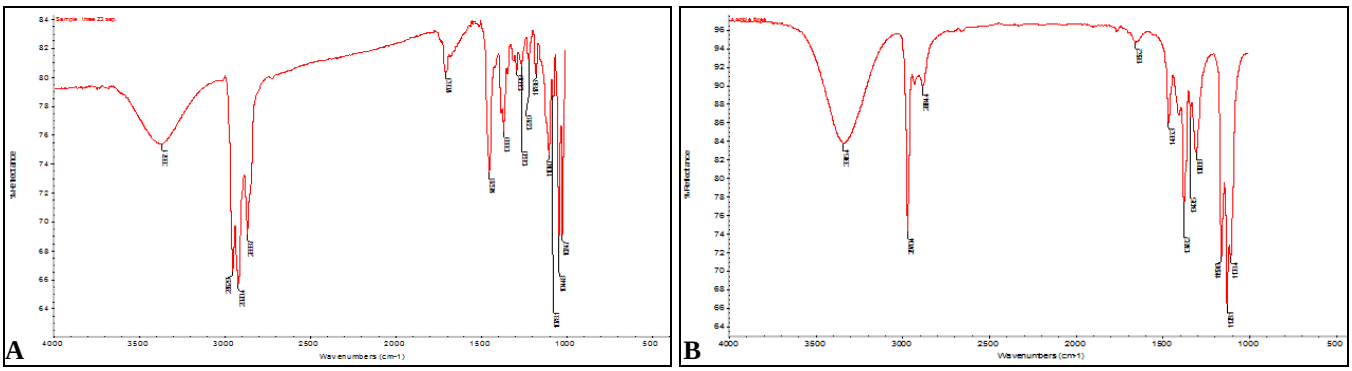

FIG. 1: FT-IR SPECTRA OF ESSENTIAL OILS EXTRACTED FROM *MENTHA PIPERATA* (1A) AND *MENTHA ARVENSIS* (1B) USING ATTENUATED TOTAL REFLECTANCE MODE

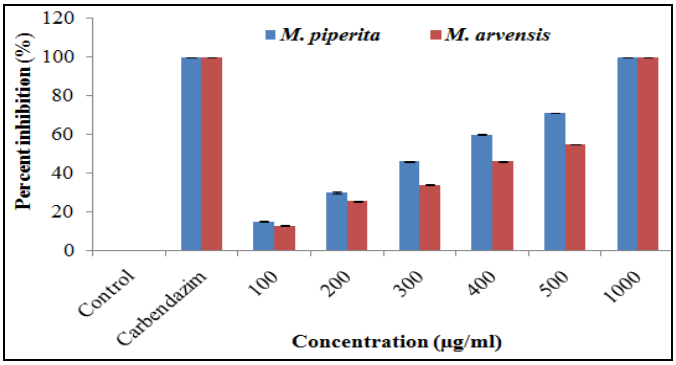

FIG. 2: ANTIFUNGAL ACTIVITY OF THE ESSENTIAL OIL OF *M. PIPERITA* AND *M. ARVENSIS* AGAINST *P. DIGITATUM*

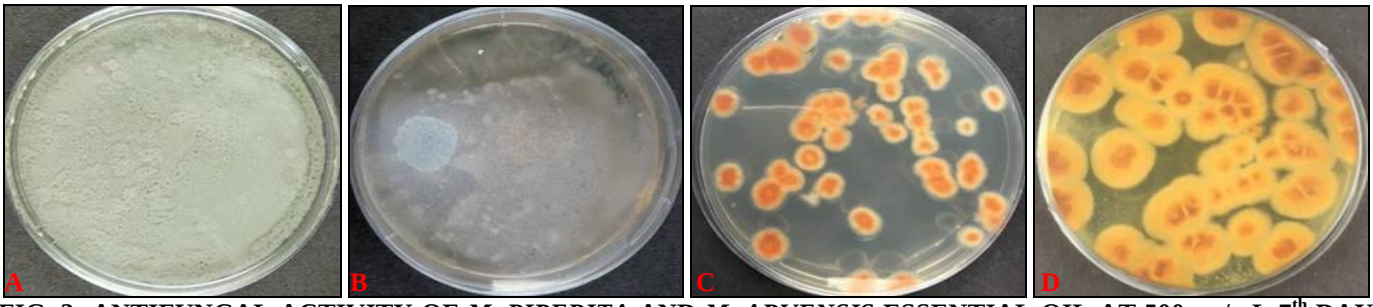

FIG. 3: ANTIFUNGAL ACTIVITY OF *M. PIPERITA* AND *M. ARVENSIS* ESSENTIAL OIL AT 500 µg/mL 7th DAY AFTER INOCULATION (3A) CONTROL (3B) CARBENDAZIM 50 WP (3C) *M. PIPERITA* AND (3D) *M. ARVENSIS*

Optical Research Microscopy of Essential Oil - *P. Digitatum* Studies: Optical research micrographs of control *P. digitatum* showed the presence of spores in groups and normal morphology of hyphae possessing dense cytoplasm **Fig. 4A** and **4B**. Whereas, on treatment with *M. piperita* **Fig. 4C**, **4D** and *M. arvensis* **4E** and **4F** essential oil, scattered spores in the form of smaller groups and as short spore chains respectively.

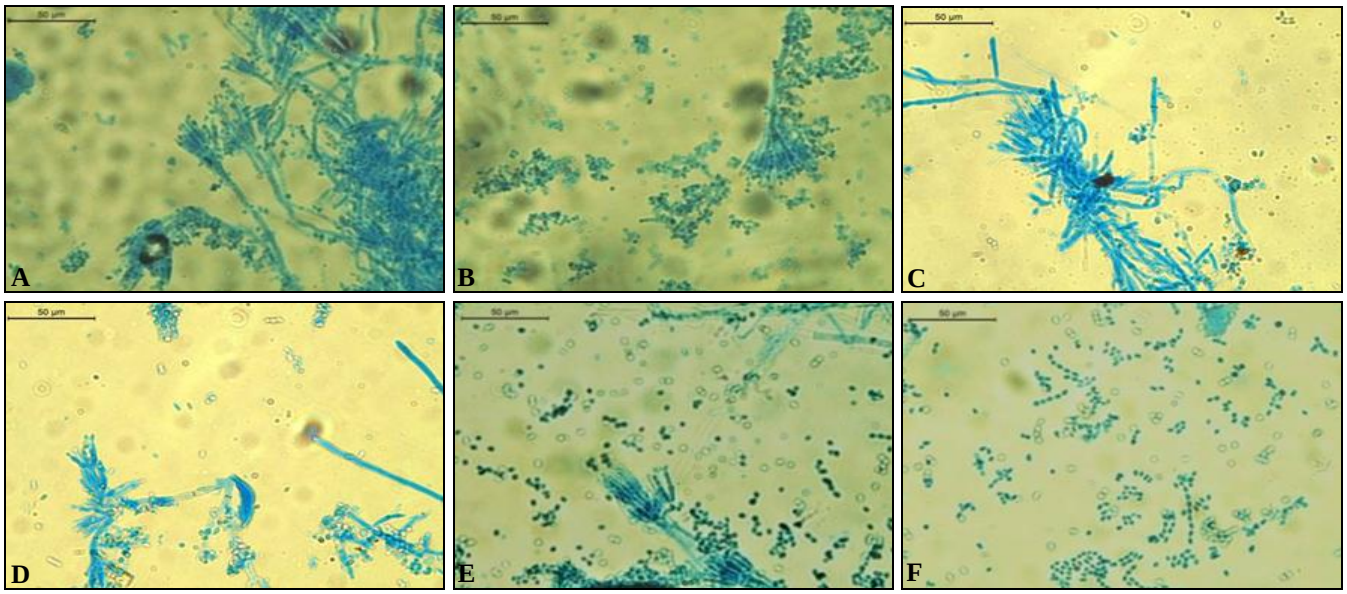

FIG. 4: OPTICAL RESEARCH MICROSCOPY SHOWING HYPHAE AND SPORE MORPHOLOGY OF CONTROL 4(A) & 4(B) FUNGUS TREATED WITH 500 µg/mL *M. ARVENSIS* EO 4(C) & 4(D) FUNGUS TREATED WITH *M. PIPERITA* EO 4(E) & 4(F)

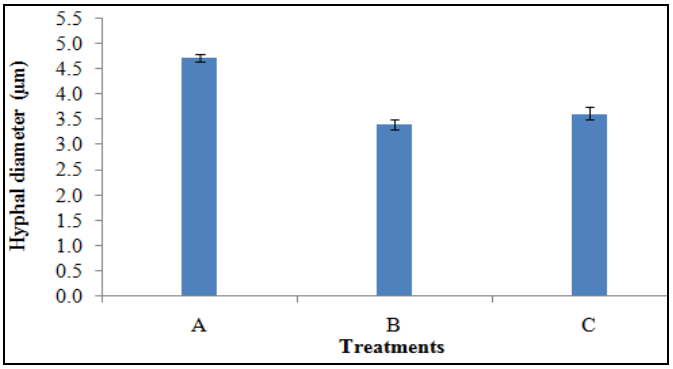

FIG. 5: HYPHAL DIAMETER (µM) OF CONTROL (A) AND FUNGAL MYCELIA TREATED WITH *M. PIPERITA* (B) AND *M. ARVENSIS*(C)

The optical micrographs also exhibited modified hyphal morphology indicated by a decrease in diameter and number of hyphal branches than control. Moreover, the cytoplasm was less dense exhibiting shrinkage which led to the fragmentation of fungal hyphae, loss of linearity including irregular hyphae and formation of warty surfaces (cell wall pitting phenomena) in fungus treated with *M. piperita* essential oil. The hyphal diameter was observed to be greater in untreated fungus followed by fungus treated with *M. arvensis* and *M. piperita* essential oil **Fig. 5**.

DISCUSSION: Percent yield of essential oils of both the species are well in correlation with the available literature with minute variations [13, 16]. The GC-MS of essential oil of both the *Mentha species* showed a broad spectrum of compounds with distinct variations. These variations are well

explained due to different climatic conditions in which the crop was reared. Further, time, the season of sample collection and soil composition of the sampling area also contributed to the composition of essential oil.

Almost all chemical compounds reported in essential oil from fresh leaves of *M. piperita* exhibited one or the other biological activity [17, 18]. Differences in the compositional analysis by GC-MS correlate very well with the FT-IR spectra of both species essential oils since more predominant bands were observed for *M. piperita* essential oil than *M. arvensis* essential oil. The FT-IR spectra of *M. piperita* depicted more interactions between various compounds may be because of greater number of compounds in it as shown by its GC-MS. The position of the absorption band due to valence vibration of O-H bonds can be used to quantify hydrogen bonding. When hydrogen bonding is stronger, the O-H bond length increases and bond force constant decreases, so the valence vibration is identified at lower frequency values compared to the values determined in the absence of association with hydrogen bonds. The association through hydrogen bonds led to wide band at 3367.1 cm^{-1} in *M. piperita* essential oil and sharp band at 3346 cm^{-1} in *M. arvensis* essential oil and a red shift occurred due to O-H stretching by 11.1 cm^{-1} of *M. arvensis* essential oil in comparison to *M. piperita* essential oil.

Samfira *et al*., [19] reported O-H stretch at 2950 cm^{-1} and C=O peak at 1700 cm^{-1} for *M. piperita* essential oil extracted from leaves collected from the western part of Romania. Prakash and Yunus [20] found O-H stretch at 3355.6 cm^{-1} and C=O peak at 1702.5 cm^{-1} for *M. arvensis* essential oil extracted from leaves collected from Lucknow city. The shift in frequency bands of functional groups was observed due to climatic changes. The results from poisoned food technique followed by percentage inhibition calculation showed more percent inhibition with less number of colonies in *M. piperita* at all tested concentrations than in *M. arvensis* at similar concentrations. At the concentration of 1000µg/mL, both species essential oils showed antifungal activity at par with standard Carbendazim 50 WP. Results observed in our studies confirmed enhanced efficacy of *M. piperita* essential oil (71%) compared to *M. arvensis* essential oil (55%) at 500µg/ml in controlling the dreaded post-harvest fungus *P. digitatum*. The essential oil of *M. piperita* leaves possess significant antifungal activity may be due to the presence of pulegone, isomenthone, menthofuran, and neomenthone, which were identified absent in *M. arvensis* essential oil. Optical research microscopy of fungus treated with essential oils revealed fragmentation of fungal hyphae, loss of linearity including irregular hyphae and formation of warty surfaces. In fungus treated with *M. piperita* essential oil malformed conidiophore structures were recorded which culminated to the formation of impaired conidia.

All these findings indicate that the antifungal mode of action of essential oil on *P. digitatum* is a result of damage to the cell plasma membrane integrity. Owing to our results, the similar phenomenon of the lipophilicity of essential oils causing membrane expansion, augmented membrane permeability and fluidity, inhibition of respiration and variation in ion transport processes of fungi which results into induced the outflow of other cellular components was observed in earlier studies [21, 22, 23].

CONCLUSION: The percent yields of *M. piperita* and *M. arvensis* essential oil obtained from leaves was calculated. The comparison showed that *M. arvensis* leaf essential oil yield (2.8%) was higher as compared to *Mentha piperita* (2.1%). Gas chromatography-Mass Spectrometry (GC-MS) of *M. piperita* and *M. arvensis* essential oil revealed the presence of 51 and 30 compounds respectively, menthol being the major compound in both essential oils. The FT-IR spectra of essential oil of both *Mentha* species showed prominent variation in compounds indicated by different shape and position of IR bands. Screening of antifungal activity revealed that *M. piperita* essential oil (71%) was found to be more effective than *M. arvensis* (55%) against *P. digitatum*. Optical research microscopy further revealed that the essential oils showed their efficacy by disrupting the cell plasma membrane, thereby causing the fragmentation of fungal hyphae.

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CONFLICT OF INTEREST: The author declares no conflict of interest.

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