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Variation in essential oil composition, antioxidant and mosquito larvicidal activity during three cuts dates of five *Mentha* species

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Abstract

This study aimed to evaluate the variation in essential oil production, constituents, antioxidant and mosquito larvacidal activities of *Mentha suaveolens*, *Mentha pulegium*, *Mentha longifolia*, *Mentha spicata* and *Mentha viridis* among three cuts dates. The highest essential oil yields of all species were obtained at the third cut on 1st August. While *M. pulegium* showed the maximum values at the second cut on 12th June. Monoterpenes represented the majority in the essential oil of all cuts and the oxygenated compounds were dominated (75.78 - 93.93 %) for all species. The second cut on 12th June produced the highest value of linalyl acetate in *M. suaveolens*, pulegone in *M. pulegium* and *M. longifolia* and carvone in *M. spicata* and *M. viridis*. While the delay to the third cut on 1st August increased the biosynthesis of L-linalool in *M. suaveolens* and D-Limonene in *M. viridis*. The first cut on 4th May in *M. suaveolens* showed the highest antioxidant activity followed by *M. viridis* then *M. longifolia* and *M. pulegium*. Concerning the larvicidal activities against *Culex pipiens*, the lowest LC50 and LC90 values were 0.019 and 0.0457 µl with *M. pulegium* in the second cut and 0.07 and 0.28 µl with *M. longifolia* in first cut. The second cut on 12th June resulted in 100% mortality of mosquito larvae with the lowest concentrations of oils 0.125, 0.25 and 0.5 µl for *M. pulegium*, *M. longifolia* and *M. suaveolens*, respectively. For *M. spicata* and *M. viridis* oils the third cut on 1st August recorded 100% mortalities at concentration of 2 microliters. It could be concluded that the date of cut affects rate of accumulation of the major constituents of mint species essential oil and the activity for mortality of mosquito larvae. The essential oil of mint species can be used as natural larvicidal agents and have the potential to provide efficient and safer insecticide for humans and the environment.

Keywords: *Mentha* spices, *Culex pipiens*, Essential oil, Antioxidant activity and Natural insecticide

1. Introduction

Mentha species, one of the oldest and popular plants, are widely used in cooking, cosmetics and as therapy especially in the treatment of gastrointestinal disorders. The essential oils and extracts of mint species showed numerous biological and pharmaceutical activities. Mint species vary in their essential oil content and composition depending upon plant species, geographical location, harvesting time, drying, extraction methods (1), plant age, crop density (2), physiological and environmental conditions (3), genetic pathways (4), temperature, photoperiod, nutrition (5) and the abiotic environmental stress factors as salinity (6) and drought (7).

M. suaveolens Ehrh is a common wild species and is known as apple mint. The major constituents of apple mint oil are linalool, ρ-menth-1-en-8-ol and geranyl acetate (7, 8). *M. suaveolens* has antioxidant, anti-inflammatory, analgesic, cytotoxic, antihypertensive, hepatoprotective, candidacidal, virucidal and antifungal activities (9, 10, 11, 12, 13).

M. pulegium L. is commonly known as pennyroyal and its essential oil varies among geographic-climatic zones. Pulegon was the major component of the essential oil of pennyroyal plants grown in Portugal (14), Uruguay (15), Bulgaria (16), Massachusetts in U.S.A (6), Italy (17) and Egypt under desert agro-system (5,7). The essential oil is consumed mainly for

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its antioxidant, antiseptic, carminative, antispasmodic, diaphoretic, anti-inflammatory, abortifacient, anticancer, antifungal, antimicrobial and insect repelling properties (18-22).

M. longifolia L. essential oil contains pulegone, 1,8 cineole, L-menthone, βpinene, α-pinene, cisiso-pulegone and piperitenone (23). *M. longifolia* is used widely in traditional medicine as antimicrobial, anti-inflammatory, carminative, antispasmodic and stimulant (23, 24).

M. spicata L. (spearmint) can be characterized as a carvone chemotype and the most abundant constituents of its essential oil are oxygenated monoterpenes. The major constituents are carvone, limonene and dihydrocarveol. The essential oil is a valuable source of natural phenolic antioxidants, Alzheimer's enzymes inhibitors, cholinesterase inhibitors and pancreatic lipase inhibitors as well as antiproliferative, antidermatophytic and antifungal agents (25-27).

M. viridis essential oil is mainly consists of carvone, 1.8-Cineole and Terpinen-4-olare as the major constituents (28). Due to the biological activity of these components, the oil is used for insecticidal, acaricidal (**29**), antimicrobial, antherpetic, antioxidant, antidiabetic, dermato-protective and antidermatophyte properties. Moreover, *M. viridis* is considered as a folkloric remedy for treating colds and flu, respiratory tract problems, gastralgia, hemorrhoids and stomachache (30), beside its use in the industry of flavors and products for oral hygiene (28, 31).

Mosquitoes (Diptera: Culicidae) transmit many diseases to human or animals such as malaria, filariasis, yellow fever, Japanese encephalitis, dengue fever and Zika virus. *Culex pipiens* is the most common mosquito in Egypt and is widely distributed in North Africa (32, 33). Vector control programs now employ many synthetic insecticides, which include carbamates, organophosphates and pyrethroids for effective control of mosquito (34). However, the continuous overuse of a limited number of chemical pesticides results in fast development of multiple resistances in mosquitoes (35). Besides, synthetic chemical insecticides also cause side effects to humans, non-target species and environment (36). The need for effective environmentally friendly alternatives is critical because chemical mosquito control has been associated with several negative effects, such as resistance development (33). Several methods for the management of mosquitoes' insecticide resistance have been explored. Among these methods using plant-essential oils were evaluated and revealed effective and applicable in use for control in a wide range (37).

The purpose of this study was to evaluate the variation in essential oil, production, constituents, and antioxidant activities of *Mentha suaveolens*, *M. pulegium*, *M.* longifolia, *M. spicata* and *M. viridis* during three cuts and their insecticidal efficacy against 4th instar larvae of *C. pipines*.

2. Material and methods

Cultivation of *Mentha* species and essential oil study:

This study was carried out at the Agricultural Experimental Station of the National Research Centre at Nubaria district, Behira Governorate, west of the Nile Delta of Egypt (its location is latitude 30º 30' 1.4" N, and longitude 30º 19' 10.9" E, Egypt), using a drip irrigation system during the two successive seasons of 2019 and 2020.

The data of physical and chemical analyses of the experimental soil following (38) are shown in Table (1). The soil was sandy in texture.

Compost (20.2 ton ha^{-1}) and calcium superphosphate (15.5% P_2O_5) at the rate of 1.2 ton ha^{-1} were added during soil preparation, whereas 715 kg ha^{-1} of ammonium sulphate (20.5% N) and 360 kg ha^{-1} of potassium sulphate (48.5% K_2O) fertilizers were added in three doses during plant growth. Irrigation of the plants was done using drip system (4 L hour^{-1} and 10 L day^{-1}) as the need of plants.

Mentha suaveolens and *M. pulegium* seedlings were established from cuttings originally secured from the Medicinal Plant Program in the Department of Plant Soil and Insect Sciences at the University of Massachusetts, Amherst, USA. Sufficient numbers of plants were kept in the nursery of the National Research Centre as a source of the cuttings used in this experiment. *M. longifolia, M. spicata and M. viridis* were obtained from Horticulture Research Institute, Agricultural Research Center, Egypt. All mint seedlings were transplanted on 5thand 3rd of March 2019 and 2020, respectively into the experimental field with 30 cm adjusted to dripper lines, which were 75 cm apart. The metrological data of the experimental farm region during the growing period are presented in Table (2).

The layout of the experiment was in completely randomized design of three replicates. Three cuts were taken during the growing seasons of the five mint species. The dates of the three cuts were1stcut was on 4thMay, the 2nd cut was on 12th June and the 3rd cut was on 1stAugust.

Essential oil percentages of fresh herb were determined by hydro-distillation using Clevenger-type apparatus according to (8, 39) and the essential oil yields (Lha^{-1}) were calculated. The resulted essential oil was separately dried over anhydrous sodium sulfate and was kept in the refrigerator till used for chemical and biological analyses. To identify the main constituents and to determine their relative percentages, the essential oils were separately subjected for GC-MS analysis using gas chromatography-mass spectrometry instrument stands at the Department of Medicinal and Aromatic Plants Research, National Research Centre following the conditions mentioned by (40).

Table 1. The physical and chemical properties of the experimental soil during 2019 and 2020 seasons

Physical properties							
	Very coarse sand (2-1 mm)	Coarse sand (1-0.5 mm)	Medium sand (0.5-0.25 mm)	Fine sand (0.25-0.1 mm)	Very fine sand (0.1-0.05 mm)	Silt + Clay (0.5> mm)	Texture
2019	13.83	53.91	2.35	18.08	9.75	2.08	**Sandy**
2020	11.92	57.15	1.97	19.12	8.03	1.81	**Sandy**

Chemical properties										
	pH	E.C. (dSm^{-1})	Cations (meq l^{-1})				Anions (meq l^{-1})			
	(2.5:1)	(1:1)	Ca^{++}	Mg^{++}	Na^{+}	K^{+}	CO_3^{--}	HCO_3^{-}	Cl^{-}	So_4^{--}
2019	8.04	0.96	3.5	0.9	4.62	0.6	--	1.9	6.1	1.62
2020	8.07	1.23	4.5	0.75	6.38	0.75	--	3.0	7.4	1.98

Table 2. Monthly average of some metrological data of the experimental area during 2019 and 2020 seasons

2019 season					2020 season				
Month	Air temperature ºC			R.H.	Month	Air temperature ºC			R.H.
	Max.	Min.	Aver.	%		Max.	Min.	Aver.	%
Mar. 2019	**24**	**7**	**15**	**75**	**Mar. 2020**	**29**	**7**	**16**	**71**
Apr. 2019	**36**	**9**	**18**	**67**	**Apr. 2020**	**33**	**9**	**18**	**69**
May 2019	**45**	**12**	**23**	**64**	**May 2020**	**41**	**12**	**22**	**63**
Jun. 2019	**35**	**18**	**26**	**70**	**Jun. 2020**	**41**	**15**	**24**	**66**
Jul. 2019	**39**	**20**	**27**	**69**	**Jul. 2020**	**33**	**21**	**26**	**71**
Aug. 2019	**34**	**20**	**27**	**73**	**Aug. 2020**	**34**	**20**	**27**	**72**

R.H.: Relative humidity

Antioxidant activity of essential oil:

The ability of the essential oil to scavenge DPPH free radicals was assessed using the method described by (41).

Statistical analysis:

All previously recorded data were analyzed as completely randomized design by analysis of variance (ANOVA) using the General Linear Models procedure of CoStat (42). Least significant difference (LSD) test was applied at 0.05 probability level to compare the means of the treatments.

Larvicidal activity of five essential oils of *Mentha* species against fourth instar of *Cx. pipiens* larvae:

Fourth instar of *Cx. pipiens* larvae (Diptera: *Culicidae*) were selected as the target insect for *in vitro* assay of five essential oils of *Mentha* species (*M. suaveolens*, *M. pulegium*, *M. longifolia*, *M. spicata* and *M. viridis*) during three cuts. A local strain was obtained from Department of Mosquito Research, Medical Insect Research Institute, Al-Agouzah, Giza Governorate, Egypt. The larvae were placed into plastic containers (30×20×10 cm) with 1500 ml distilled water at room temperature. Larvae were fed on equal parts of dried yeast, biscuits and dried milk powder, according to the method of (43).

Larvicidal bioassays

In accordance with pilot study, the fold of different concentrations (4, 3, 2, 1.9, 1.7, 1.5, 1, 0.5, 0.25, 0.125, 0.0625 and 0.0321 µl/ml) were adjusted for each essential oil on 4thlarval stages of *Cx. pipiens*. In the pilot study with *M. suaveolens*, *M. pulegium* and *M. longifolia* a full larval mortality rate (100%) was achieved at concentration of 0.5 µl/ml. Therefore, a four-fold increasing concentration (0.0312, 0.0625, 0.125, and 0.25 µl/ml) was applied for *M. spicata* and *M. viridis*.

Control replicates were treated with distilled water only. Larval mortality was recorded after 24h post-treatment, and five replicates were performed (n = 5) in each experiment as recommended by (44). Briefly, 5 larvae were placed in each well of a sterile standard 12-wells tissue culture test plate (Nunclone Delta Surface, Thermo-Fischer Scientific, Denmark) with 2 ml d H_2O mixed with the assigned concentration of oils or distilled H_2O for the negative control larval group. Each bioassay was run for 24h, during which larvae were fed to avoid mortality caused by starvation. A lack of larvae reaction to gentle prodding with a glass pipette was considered as mortality according to (45). After 24h, the percentage of larval mortality was calculated for each concentration using Abbott's formula (46). Bioassays of each concentration were replicated five times for every experimental tested.

Histopathology of 4th instar *Cx. pipiens* larvae by light microscopy

After 24 hours, the exposed larvae to the 1st cut of the higher concentration of different essential oils (2 µl/ml *M. suaveolens*, 4 µl/ml *M. viridis*, 3 µl/ml *M. spicata*, 2 µl/ml *M. pulegium* and 1 µl/ml *M. longifolia*), were fixed in 10% formalin buffer solution for 24 hrs. The specimens were dehydrated, cleared and embedded into paraffin blocks. Paraffin sections 5 µm thick were prepared, stained with hema-toxylin and eosin, and examined microscopically.

Statistical analysis*:*

Cx. pipiens larval mortality data were subjected to analysis of variance (ANOVA) of arcsine square root transformed mortality percentages. The lethal concentrations required to kill 50, and 90 (LC_{50}, and LC_{90}) of larvae 24 h post-treatment were calculated by probit analysis with a reliability interval of 95% using the SPSS 16 software.

3. Results and discussion

Essential oil production:

The results illustrated in Table (3) revealed that the highest values of oil percentage and yield of *M. suaveolens, M. longifolia, M. spicata and M. viridis* were obtained at the 3rd cut on 1st August in both seasons. While in *M. pulegium*, the maximum values of oil percentage and yield were obtained at the 2nd cut on 12th June.

These results are agreed with (47) on four *Mentha* species, (8) on *M. pulegium* and *M. suaveolens*. The variations in oil percentage and yield throughout the cuts may be attributed to some factors strongly affect the essential oil such as harvest time, temperature (5), light intensity, duration of sunshine, relative humidity and plant age (2, 48).

Table 3. Essential oil percentage (%) and yield (L hectar^{-1}) of five *Mentha* species during 2019 and 2020 season

Cuts	Oil Percentage		Oil yield (L hectar^{-1})	
	1st season	2nd season	1st season	2nd season
***Mentha suaveolens* Ehrh.**				
1st Cut	0.16	0.17	9.5	9.5
2nd Cut	0.25	0.23	15.0	13.6
3rd Cut	0.28	0.28	16.3	18.1
LSD at 5%	**0.038**	**0.049**	**2.24**	**2.95**
***Mentha pulegium* L.**				
1st Cut	0.39	0.33	12.9	9.3
2nd Cut	0.50	0.48	20.1	21.7
3rd Cut	-	-	-	-
LSD at 5%	**0.072**	**0.042**	**2.54**	**1.61**
Mentha longifolia L.				
1st Cut	0.80	0.79	32.0	22.6
2nd Cut	1.02	1.01	64.9	86.7
3rd Cut	1.67	1.61	103.9	119.5
LSD at 5%	**0.245**	**0.247**	**14.81**	**18.19**
Mentha spicata				
1st Cut	0.40	0.38	16.1	16.3
2nd Cut	0.51	0.40	36.4	34.5
3rd Cut	0.72	0.62	52.2	49.7
LSD at 5%	**0.067**	**0.079**	**4.37**	**6.20**
Mentha viridis				
1st Cut	0.35	0.33	10.4	8.8
2nd Cut	0.44	0.44	22.4	30.6
3rd Cut	0.71	0.67	46.5	40.1
LSD at 5%	**0.121**	**0.063**	**7.76**	**3.88**

Essential oil constituents:

The identified components of five essential oils of mint species are shown in Tables (4-8). The relative percentages of the main constituents varied in the essential oils the three cuts Monoterpenes represented the majority (82.03 - 99.02%) in all three cuts of mint species, and the oxygenated compounds were dominated (92.48, 84.29, 93.93, 84.38, 77.54 and 75.78%) comparing to the nonoxygenated compounds for *M. suaveolens, M. pulegium, M. longifolia, M. spicata and M. viridis*, respectively. In *M suaveolens* (Table, 4), the major constituent was Linalool followed by linalyl acetate then eucalyptol. The highest relative percentages of l-linalool (53.63%) and eucalyptol (7.42%) were detected in the 3rd cut on 1st August. While the 2nd cut on 12th June recorded the highest relative percentage of linalyl acetate (22.5 %). The inverse correlation between linalool and linalyl acetate could be attributed to the fact that linalyl acetate is the acetate ester form of linalool.

Table 4. Essential oil constituents of *Mentha suaveolens* during 2020 season

RT.	Compounds	KI	Area %		
			1st cut	2nd cut	3rd cut
3.73	α-Pinene	921	--	0.67	0.46
4.61	Sabinene	955	0.72	0.92	0.54
4.74	β-Pinene	960	1.20	1.61	1.60
5.04	β- Myrcene	970	1.49	1.26	0.27
5.39	3-Octanol	981	0.72	0.48	--
6.23	Eucalyptol	1006	3.93	4.99	7.42
6.34	trans-β-Ocimene	1009	0.75	--	--
6.60	β –Ocimene	1017	0.82	1.07	--
8.27	Linalool	1064	40.69	41.78	53.63
8.39	1-Octen-3-yl-acetate	1067	4.03	3.61	--
8.71	3-Octanol, acetate	1075	4.58	6.23	4.38
11.37	L-α-Terpineol	1139	--	--	0.16
11.69	Terpinen-4-ol	1147	--	--	0.12
12.37	α –Terpineol	1162	4.70	4.60	3.35
13.24	cis-Geraniol	1181	0.60	--	--
14.30	Linalyl acetate	1203	21.42	22.58	19.86
14.64	Carvone	1211	--	--	3.25
14.79	Geraniol	1115	1.38	0.48	--
15.80	4-Hexen-1-ol, 5-methyl-2-(1-methylethenyl)-, acetate	1239	--	0.63	--
18.85	Nerol acetate	1308	1.25	0.92	--
19.58	Geranyl acetate	1319	2.41	1.21	--
19.75	(-)-β-Bourbonene	1321	--	--	0.28
19.96	Pentadecanal-	1330	--	--	--
21.06	Caryophyllene	1355	--	--	0.23
22.87	(+)-epi-Bicyclosesquiphellandrene	1393	--	0.49	--
23.64	Germacrene D	1411	5.06	5.77	3.80
23.74	Ledol	1907	--	--	--
24.21	Elixene	1425	--	--	0.15
24.71	β-Elemene	1437	--	--	0.09
25.29	Dihydro-β-agarofurane	1451	0.69	0.69	0.31
28.36	Viridflorol	1522	2.18	--	--
30.41	Guaiol	1571	1.38	--	--
Monoterpines			82.03	93.04	95.04
Sesquiterpenes			7.92	6.95	4.86
Total of non-oxygenated compounds			9.21	11.79	7.42
Total of oxygenated compounds			80.74	88.2	92.48
Total of identified compounds			100	99.99	99.90

Pulegone was found to be the main constituent of *M. pulegium* oil (Table, 5) and its highest value was obtained from the 2nd cut on 12th June. In *M. longifolia* (Table, 6), the major constituents were pulegone, eucalyptol and I-menthone in order. The highest relative concentration of pulegone (59.07%) was obtained from the 2nd cut on 12th June. The 1st cut on 4th May produced the highest relative concentration of the I-menthone (10.24%) D-Limonene (3.08%), while the highest relative percentages of eucalyptol (12.05%) and β-Pinene (6.54%) were recorded from the 3rd cut on 1st August. These results agreed with (8) on *M. pulegium* as well as (24) on *M. longifolia*. (49) stated that there is a negative correlation between limonene and β-pinene. Moreover, (50) concluded that limonene is readily incorporated into menthone, pulegone and other oxygenated derivatives.

Carvone (59.93%) was the major component of the oxygenated compounds in *M. spicata* oil (Table, 7), followed by D-limonene (13.34%) and eucalyptol (6.49%). The highest relative concentration of carvone and eucalyptol were produced from the 1st cut and 2nd cut on 4th May and 12thJune. While the 3rd cut on 1st August recorded the highest concentration of D-Limonene. It was observed that, carvone was negatively correlated with limonene. Kjonaas and Croteau (1983) found that d,l-[9-3H]limonene in *Mentha spicata* is definitely incorporated into carvone confirming its role as the precursor of oxygenated p-menthane monoterpenes.

The main component in the essential oil of *Mentha viridis* were carvone, D-limonene and 1,8-cineole (Table, 8). The 2nd cut on 12th June recorded the highest relative percentage of carvone (59.51%), while the 1st cut on 4thMay produced the highest concentration of 1,8-Cineole (9.82%). D-Limonene (15.90%) reached the greatest value in the 3rd cut on 1st August. The results indicated reverse correlation between carvone and D-limonene. These results agreed with (51, 52) who found that carvone increased gradually from winter to spring where it reached 59.09%; the percentage reached its maximum level of 76.82 and 75.18% in summer and fall months, respectively and accompanied by an increase in concentration of limonene, dihydrocarveol, pulegone, and cis-carveol.

Table 5. Essential oil constituents of *Mentha pulegium* during 2020 season

R.T.	Compounds	KI	Area %	
			1st cut	2nd cut
3.73	α-Pinene	**921**	1.86	2.15
4.61	Sabinene	**955**	0.34	0.41
4.74	β-Pinene	**960**	1.47	1.69
5.04	β- Myrcene	**970**	0.18	0.19
5.39	3-Octanol	**981**	1.55	1.60
6.13	D-Limonene	**1002**	3.09	0.89
6.23	Eucalyptol	**1006**	--	0.24
7.09	Butane-1,1-dicarbonitrile,1-cyclohexyl-3-methyl	**1032**	0.04	--
8.27	Linalool	**1064**	0.23	--
8.71	3-Octanol, acetate	**1075**	--	--
10.40	d-Camphor	**1115**	0.03	--
10.71	I-Menthone	**1123**	1.73	4.12
11.07	L-Menthone	**1132**	--	--
11.34	endo-Borneol	**1139**	0.04	0.06
11.51	Isopulegone	**1143**	2.03	1.98
12.37	α –Terpineol	**1162**	0.35	0.43
14.16	Pulegone	**1199**	83.87	84.29
16.48	Bicyclo[3.2.0]heptan-2-one,5-formylmethyl-6-hydroxy-3,3-dimethyl-6-vinyl	**1254**	--	0.21
21.06	Caryophyllene	**1355**	1.14	0.54
22.56	Humulene	**1387**	1.98	1.01
Monoterpines			96.81	98.26
Sesquiterpenes			3.12	1.55
Total of non-oxygenated compounds			10.1	6.88
Total of oxygenated compounds			89.83	92.93
Total of identified compounds			99.93	99.81

Table 6. Essential oil constituents of *Mentha longifolia* during 2020 season

RT.	Compounds	KI	Area %		
			1st cut	2nd cut	3rd cut
3.73	α-Pinene	**921**	3.12	3.07	3.85
4.61	Sabinene	**955**	2.89	3.08	4.35
4.74	β-Pinene	**960**	5.12	5.22	6.54
5.04	β- Myrcene	**970**	0.43	0.67	1.12
6.13	D-Limonene	**1002**	3.08	2.32	1.63
6.23	Eucalyptol	**1006**	11.19	10.35	12.05
10.07	β-Sabinyl acetate	**1106**	0.22	0.36	0.46
10.28	Verbenol	**1112**	0.11	0.18	0.18
10.71	I-Menthone	**1123**	10.24	9.40	6.68
11.07	L-Menthone	**1132**	--	--	5.67
11.34	endo-Borneol	**1139**	--	--	1.60
11.37	L-α –Terpineol	**1139**	0.76	1.13	--
11.51	Isopulegone	**1143**	1.12	1.19	1.14
12.37	α –Terpineol	**1162**	0.75	1.34	2.21
14.16	Pulegone	**1199**	59.18	59.07	50.86
16.48	Bicyclo[3.2.0]heptan-2-one, 5-formylmethyl-6-hydroxy-3,3-dimethyl-6-vinyl	**1254**	0.17	--	--
18.67	Verbenone	**1299**	0.64	0.94	0.56
21.06	Caryophyllene	**1355**	0.63	0.83	0.62
22.56	Humulene	**1387**	0.14	0.20	0.14
23.64	Germacrene D	**1411**	--	0.40	0.21
Monoterpines			**99.02**	**98.32**	**98.9**
Sesquiterpenes			**0.77**	**1.43**	**0.97**
Total of non-oxygenated compounds			**15.41**	**15.79**	**18.46**
Total of oxygenated compounds			**84.38**	**83.96**	**81.41**
Total of identified compounds			**99.79**	**99.75**	**99.87**

Table 7. Essential oil constituents of *Mentha spicata* during 2020 season

RT.	Compounds	KI	Area %		
			1st cut	2nd cut	3rd cut
3.73	α-Pinene	**921**	2.53	2.05	3.37
4.61	Sabinene	**955**	2.34	2.20	2.83
4.74	β-Pinene	**960**	3.57	3.31	4.33
5.04	β- Myrcene	**970**	0.64	0.84	1.29
6.13	D-Limonene	**1002**	11.20	7.19	13.34
6.23	Eucalyptol	**1006**	6.49	6.20	5.54
7.52	trans-sabinene hydrate	**1044**	0.90	1.44	0.72
8.27	Linalool	**1064**	0.42	--	--
10.71	I-Menthone	**1123**	--	0.39	--
11.34	endo-Borneol	**1139**	1.36	1.64	1.27
11.69	Terpinen-4-ol	**1147**	--	1.12	0.77
12.37	α –Terpineol	**1162**	0.57	1.86	1.02
12.54	trans-Dihydrocarvone	**1166**	--	1.24	0.50
13.40	trans-Carveol	**1184**	0.60	0.45	0.66
14.16	Pulegone	**1199**	0.51	3.27	0.84
14.50	Carvone	**1208**	59.62	59.93	58.68
19.58	(-)-β-Bourbonene	**1321**	2.07	1.84	1.62
21.06	Caryophyllene	**1355**	1.27	1.31	0.96
22.87	(+)-epi-Bicyclosesquiphellandrene	**1393**	1.35	--	0.41
23.64	Germacrene D	**1411**	2.07	2.16	1.00
24.21	Elixene	**1425**	1.20	1.01	0.51
24.71	β-Elemene	**1437**	0.53	0.55	--
25.36	trans-calamenene	**1452**	0.78	--	0.35
Monoterpines			**90.75**	**93.13**	**95.16**
Sesquiterpenes			**9.27**	**6.87**	**4.85**
Total of non-oxygenated compounds			**29.55**	**22.46**	**30.01**
Total of oxygenated compounds			**70.47**	**77.54**	**70.00**
Total of identified compounds			**100.02**	**100**	**100.01**

Table 8. Essential oil constituents of *Mentha viridis* during 2020 season

R.T.	Compounds	KI	Area %		
			1st cut	2nd cut	3rd cut
3.73	α-Pinene	**921**	2.71	2.17	3.41
4.61	Sabinene	**955**	2.53	2.36	3.15
4.74	β-Pinene	**960**	4.46	3.44	4.56
5.04	β-Myrcene	**970**	0.58	1.02	1.41
6.13	D-Limonene	**1002**	10.50	8.59	15.90
6.23	1,8-Cineole	**1006**	9.82	6.88	5.82
7.06	γ-Terpinene	**1031**	--	0.44	0.40
7.52	trans-sabinene hydrate	**1044**	1.47	1.89	1.03
11.34	endo-Borneol	**1139**	1.53	1.53	1.11
11.69	Terpinen-4-ol	**1147**	0.53	1.34	1.12
12.37	α –Terpineol	**1162**	1.38	1.91	1.46
12.42	Dihydrocarveol	**1164**	--	--	0.38
12.54	trans-Dihydrocarvone	**1166**	1.23	0.89	0.89
13.40	trans-Carveol	**1184**	0.19	0.66	0.94
14.16	Pulegone	**1199**	--	1.17	0.36
14.50	Carvone	**1208**	53.98	59.51	55.13
19.58	(-)-β-Bourbonene	**1321**	2.04	1.73	1.01
21.06	Caryophyllene	**1355**	1.69	1.18	0.76
22.56	Humulene	**1387**	0.29	--	--
23.64	Germacrene D	**1411**	2.61	1.95	0.81
24.21	Elixene	**1425**	1.44	0.87	0.37
Monoterpines			**90.91**	**93.8**	**97.07**
Sesquiterpenes			**8.07**	**5.73**	**2.95**
Total of non-oxygenated compounds			**28.85**	**23.75**	**31.78**
Total of oxygenated compounds			**70.13**	**75.78**	**68.24**
Total of identified compounds			**98.98**	**99.53**	**100**

It might be concluded that the 2nd cut on 12th June was found to be suitable for producing the highest value of linalyl acetate in *M. suaveolens*, pulegone in *M. pulegium*,*M. longifolia* and increasing carvone in *M. spicata*. While, the delay to the 3rd cut on 1st August increased the biosynthesis of Linalool and eucalyptol *in M. suaveolens* as well as D-limonene in *M. spicata* and *M. viridis*. The cuts dates affect the rate of accumulation of the major constituents of mint species. These results agreed with (53) on thyme, (54) on *Cymbopogon martini*, (55) on *Cinnamomum osmophloeum* and (56) on *Mentha viridis* who stated that harvesting time and climate conditions affect the essential oil constituents.

The chemical variability in phytochemical profiles of *Mentha* essential oils could be due to the impact of harvesting time at different stages, phenological status, physiological and environmental conditions which affect the regulation of the biosynthesis of essential oil (1, 57, 3, 47). Moreover, (58) found that plants collected at different seasons may produce different novel compounds with other activities.

Antioxidant activity of essential oils:

Data presented in Table (9) showed that the antioxidant activity of the five *Mentha* species was significantly affected by the three cuts date. The higher antioxidant activity were found in the 1st cut on 4th May in *M. suaveolens* followed by *M. viridis* then *M. longifolia* and *M. pulegium*, while the delay to the 3rd cut on 1st August decreased the antioxidant activities of oils but increased it in *M. spicata* oil. The variation in essential oil constituents of five *Mentha* species Tables (4-8) during three cuts dates can affect the antioxidant activities of oils. The higher antioxidant activity could be explained by the high amount of monoterpenes represented the majority (82.03 - 99.02%) in all three cuts dates of mint species Tables (4-8). As well as the oxygenated compounds were dominated (77.54 - 93.93%) in which producing the highest relative concentrations of linalool, linalyl acetate, pulegone, carvone, D-limonene which increased the antioxidant activities of *Mentha* species. This result agreed with literature data in which terpenes are described as good antioxidant compounds (59, 60). The antioxidant activity of the mint essential oils could be attributed to the major monoterpenoids including menthol, menthone, carvone, 1,8-cineole (61-63) and also to the high percentage of oxygenated compounds (64, 65) on *Argemone ochroleuca*, terpenes such as terpinyl acetate and eucalyptol which were described as good antioxidant compounds (59, 60, 66).

Table 9. Mean values of antioxidant activity (%) of the essential oil of five *Mentha* species during 2020 season

	M. suaveolens	***M. pulegium***	***M. longifolia***	***M. spicata***	***M. viridis***
1st Cut	**83.5**	**69.4**	**70.3**	**58.1**	**78.3**
2nd Cut	**75.5**	**62.8**	**65.2**	**73.2**	**71.7**
3rd Cut	**59.3**	**-**	**51.1**	**78.3**	**63.4**
LSD at 5%	**3.51**	**1.97**	**4.91**	**4.75**	**8.13**

Essential oils against 4th instar *Cx. pipiens* larvae:
Essential oils of *Mentha* species during 3 cuts dates had a significant effect on the 4th larval stage as compared with the control. The means of the mortality percentages are given in Table (10).

The mean mortality of larvae increased with increased concentrations. The mortality (%) of five essential oils of *Mentha* species on the 4th larval stage varied with concentration as well as cuts date. The strongest effect of *M. suaveolens* oil (100% mortality) at 1st, 2nd and 3rd cuts were observed at concentrations of 2, 0.5 and 2 µl/ml, respectively. However, *M. pulegium* oil resulted in 100% mortality rate at the higher concentration (2.0 µl/ml) and the lower concentration (0.125 µl/ml) at the 1st and 2nd cuts. While the most effective concentrations (1, 0.25 and 1 µl/ml) were observed with *M. longifolia* oil at 1st, 2nd and 3rd cuts, respectively. Also, *M. spicata* and *M. viridis* oils, larval stage showed 100% mortalities rate at 1st, 2nd and 3rd cuts at concentrations of 3, 2 and 2 µl/ml and 4, 4 and 2 µl/ml , respectively.

In addition, the LC_{50} and LC_{90} were calculated at 24 h and were illustrated in Table (10) for each essential oil. The highest LC_{50} and LC_{90} (lethal concentration) values were (2.113, 3.242 µl/ml) with *M. viridis at* 1st cut, (1.85, 2.235 µl/ml) with *M. suaveolens* at 1st cut and (1.067, 1.925 µl/ml) with *M. spicata at* 1st cut. However, the lowest LC_{50} and LC_{90} values were (0.019, 0.0457 µl/ml) with *M. pulegium* in the 2nd cut and (0.07, 0.28 µl/ml), (0.152, 0.249 µl/ml) and (0.367, 0.632 µl/ml) with *M. longifolia*in the 1st, 2nd and 3rd cut, respectively.

Generally, the 2nd cut on 12th June was the most economic cut which resulted in 100% mortality with the lowest concentrations of oils 0.125, 0.25 and 0.5 µl/ml for *M. pulegium*, *M. longifolia* and *M. suaveolens*, respectively. While in *M. spicata* and *M. viridis* oils, the delay to the 3rd cut on 1st August recorded 100% mortalities at concentration 2 µl/ml.

The activity of these oils against mosquito larvae is generally associated with the presence of one or more of mono-terpenoid constituents. The oxygenated compounds were dominated which have been well-documented as active fumigants and insecticides. On the other hand, oxygenated terpenoids are more toxic than the non-oxygenated ones. It might be concluded that the 2nd cut on 12th June was found to be suitable for producing the highest relative concentration of pulegone, carvone, eucalyptol in *M. pulegium*, *M. longifolia*, *M. spicata*, respectively. While the delay to the 3rd cut on 1stAugust increased the accumulation of l-linalool and linalyl acetate in *M. suaveolens* and *D-Limonene in M. viridis.* Generally, the monoterpenoids and sesquiterpenes are associated with insecticidal properties of many essential oils (67).

Several researches on essential oils of *Mentha* species stated that most of the oils are rich in pulegone, carvone, limonene, menthon, menthol, 1,8-cineole and caryophyllene (68). Carvone, the main constituents of *M. spicata* and *M. viridis*, have now been successfully commercialized in the industry as antiinsecticidal agents. Menthone, alpha pinene, beta pinene, linalool and limonene have insecticidal activities against mosquito (69). The essential oil also has larvicidal effects on mosquitoes, its LC_{50} being 83.8 ppm against *An. stephensi* (70). The essential oils from the species of Lamiaceae contain linalool, linoleic acid, *p*-cymene, eucalyptol, eugenol, citral, thujone, camphor, methyl chavicol and many other terpenes, all of which are effective against mosquito. *Ocimum basilicum* essential oil has larvicidal properties, producing 100% mortality of *C. pipiens* fatigans at concentration of 0.12% (71).

Most of the arthropod-repellent constituents mentioned previously are oxygenated, having linked hydroxyl group to a primary, secondary or aromatic carbon. It should be noted that for many metabolites with linked hydroxyl group to a tertiary carbon (limonene, a-terpineol and linalool), such activity inhibited *A. gambiae*, which suggest the possibility that the type of carbon where the hydroxyl substitution is present modulates repellency

Table 10. Mortality percentages (mean ± SE) of 4th *Culex pipiens* L. (Diptera:Culicidae) exposed to different concentrations of five mint species oils after 24 h.

Cuts	Conc. (µl/ml)												LC_{50}	LC_{90}
	0.0312	0.0625	0.125	0.25	0.5	1	1.5	1.7	1.9	2	3	4		
Mentha suaveolens Ehrh.														
1st cut	-	-	-	-	-	0a	16±7.4	20±8.9	24±7.4	100c	-	-	1.85	2.235
2nd cut	0a	4±4a	8±4.89a	68±4.8c	100e	-	-	-	-	-	-	-	0.199	0.39
3rd cut	-	-	0a	4±4ab	32±8c	52±4.8b	52±4.8b	52±4.8b	76±9.7b	100c	-	-	0.76	1.72
Mentha pulegium L.														
1st cut	-	-	0a	20±8.9	28±12c	84±9.7c	84±9.7c	84±9.7c	84±9.7c	100c	-	-	0.55	1.285
2nd cut	76±9.7b	96±4c	100d	-	-	-	-	-	-	-	-	-	0.019	0.0457
Mentha longifolia L.														
1st cut	0a	44±7.4b	72±4.8c	88±8d	96±4de	100c	-	-	-	-	-	-	0.07	0.28
2ndcut	0a	4±4a	20±10.9b	100d	-	-	-	-	-	-	-	-	0.152	0.249
3rd cut	-	0a	4±4a	8±4.8ab	80±8.9d	100c	-	-	-	-	-	-	0.367	0.632
Mentha spicata L.														
1st cut	-	-	-	0a	4±4ab	48±4.8b	48±4.8b	48±4.8b	88±4.8bc	88±4.8bc	100	-	1.067	1.925
2ndcut	-	-	0a	4±4ab	24±7.4bc	88±8c	88±8c	88±8c	88±8c	100c	-	-	0.626	1.094
3rd cut	-	-	0a	8±4.8ab	12±4.8abc	40±10.9b	40±10.9b	40±10.9b	76±9.7b	100c	-	-	0.9	2.04
Mentha viridis L.														
1st cut	-	-	-	-	0a	8±4.8	8±4.8	16±4a	16±4a	16±4a	96±4	100	2.113	3.242
2ndcut	-	-	0a	8±4.89ab	16±4ab	56±7.2	56±7.2	56±7.2	56±7.2	76±11.6b	76±11.6b	100	1.002	3.19
3rd cut	-	-	0a	16±4ab	20±6.4abc	52±4.8	52±4.8	52±4.8	52±4.8	100c	-	-	0.75	1.99
F	60.16	81.7	80.7	52.3	32.2	18.75	-	-	-	39.4	0.57	-		
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	-	-	<0.001	Ns	-		

No mortality was recorded in the control. SE: Standard error of mean values. Values with different letters (a, b, c,..) are significantly different (P <0.05) within oil for each concentration (based on the non over lapping confidence limits). LC 50, 90: lethal concentrations that kill 50 and 90 % of the larvae. Ns: non-significant. R = 5, N = 5, ΣN = 25. xocuticle, en: endocuticl, cu: cuticle, m: muscles, f: fat and g: gut

Effect of different essential oils on histology *of Cx. pipiens* 4th instars larvae:

The normal cuticle of *Cx. pipiens* 4th instars larvae, as typical for the other insects studied by Wiggle Worth (1947) consist of thin layer of exocuticle, epicuticle followed by endocuticle and the muscles layer are composed of striated fibers **(**Fig.1a). However, as shown in (Fig. 2 a-e) larva exhibited many abnormalities in their body after exposure to the different essential oils. A corrugated and thinning cuticular surface and a separation of the inner cellular layer of the epidermal cells in some regions of the procuticle, while the musculature region showed a disorganized appearance. Light microscopic observations could be used to determine the ability of tested oils to penetrate through the larval cuticle (72, 73). In the current study, the histopathological effects of oils observed on the cuticle of *Cx. pipiens* larvae appeared to be in line with the results obtained by Hamouda et al. (74) who reported that the *Cx. Pipiens* (L) exposed to *Anagallis arvensis* oil extract exhibited the break-up of the cell wall and the disruption of the peritrophic membrane. Besides, Assar and El-Sobky (75) observed that the water extract of *Eichhornia crassipes* showed a dramatic effect on larval midgut as degeneration of some epithelial cells and the brush border had been occurred apically; after 48 h and 72 h most epithelial cells had been vacuolated and fully degenerated.

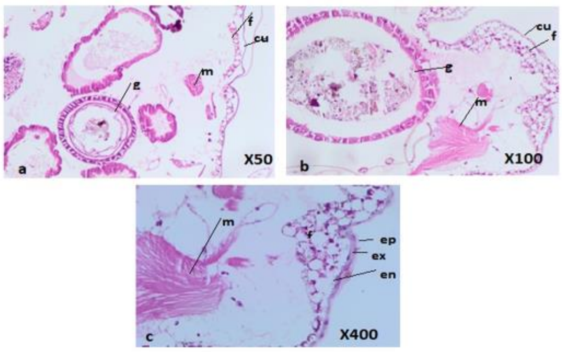

Figure 1 (a-c). Transverse H and E-stained sections of a) the cuticle and muscles of normal larvae of *Cx.pipenes*. b and c) the body wall of normal larvae has three layers; an outer electron-dense layer of epicuticle, followed by the procuticle, which is composed of the exocuticle and endocuticle, and then the inner layers of epidermal cells. ep: epicuticle, ex: exocuticle, en: endocuticle, cu: cuticle, m: muscles, f: fat and g: gut.

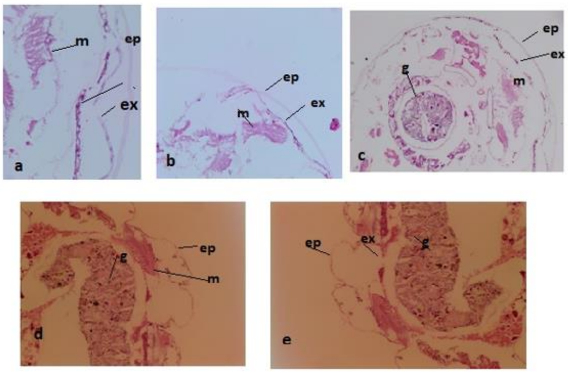

Figure 2 (a-e). Transverse H and E-stained sections of Cuticle, muscles 24 h after being exposed to (a) 100 µl *M suaveolens,* (b) 4 µl *M viridis,* (c) 3 µl *M spicata,* (d) 2 µl *M pulegium* and (e) 1 µl *M longifolia*. (10×). ep: epicuticle, ex: exocuticle, en: endocuticl, cu: cuticle, m: muscles, f: fat and g: gut

4. Conclusion

It could be concluded that the date of cut affects rate of accumulation of the major constituents of mint species, the antioxidant activity and the activity for mortality of mosquito larvae. *M. suaveolens* at the 1st cut showed the highest antioxidant activity. The essential oil of mint species can be used as natural larvicidal agents against mosquitoes and have the potential to provide efficient and safer insecticide for humans and the environment.

5. Conflict of interest

All authors declare that they have no conflict of interest

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