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Production and Quality of Menthol Mint Essential Oil and Antifungal and Antigerminative Activity

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Abstract

Agricultural products certified as organic and free of pesticides cannot use synthetic chemicals in the production process. In this context, the search for new natural products appears as an alternative to the use of conventional pesticides, aiming to combat agricultural diseases. Menthol is a natural product obtained from plants that has importance in different branches of industry, mainly due to the feeling of freshness it provides in contact with skin and mucous. Menthol (70% - 90%) is the main compound of the menthol mint essential oil, followed by menthyl acetate (7% - 12%), which is an indicator of maturation. There are references to the period of maturation of menthol mint essential oil corresponds the period of flowering, on the other hand, are also presented evidence that the maturation of menthol mint essential oil is controlled by leaf expansion, namely with the physiological age of each leaf. Besides evaluating the essential oil production and quality extracted by hydrodistillation of young leaves (3rd to 5th node) and adult (6th to 8th node), was also proposed in this work to study the effect of menthol mint essential oil on the development of fungi of agricultural significance and commercial seeds of lettuce and tomato. After the extraction of the essential oil, was noted that adult leaves presented a higher content of essential oil, combined with the best commercial quality, showing higher levels of menthyl acetate and menthol. The menthol mint essential oil exhibited average fungitoxicity and antigerminative activity on *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii*. On the other hand, menthol and terpineol, two essential oil components, showed the maximum fungitoxicity activity under this species and no inhibitory effect on the germination of lettuce and tomato.

Keywords

***Mentha arvensis*, Antigerminative Activity, Antifungal Activity, Gas Chromatography**

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1. Introduction

The production of aromatic species to obtain essential oils has a great economic importance, mainly due to the increasing demand generated by food, cosmetics and pharmaceutical industries [1]-[3]. Among many aromatic species, menthol mint has a great market demand, a consequence of its various applications in different sectors of industry [4].

The genus *Mentha* belongs to the Lamiaceae family and comprises a large number of species, including *M. arvensis*. This specie is herbaceous; the composition of the essential oil varies a lot among the varieties, during the year and at different stages of its development, but is mainly composed of monoterpenes as menthol (70% - 90%), which is the major substance [5] [6] (see **Figure 1**).

Essential oils are complex mixtures containing different classes of substances, mainly terpenoids and phenylpropanoids, which have diverse chemical structures that are part of special metabolism [7] [8]. Moreover, essential oils perform multiple roles for the plants, but can be involved mainly in species-specific ecological interactions [9]-[11].

For example, the important role in attracting pollinators [12], the inhibitor of herbivory [13] [14], in protection of internal oxidation processes [15] and light stress [16]-[18].

On the other hand, others authors showed the action of essential oils and natural products on microorganisms of agricultural significance [19] [20] and on the seed germination [21] [22]. In this context, the search for new natural products becomes a valuable alternative to the conventional use of pesticides, used against cultivated plants diseases.

The pathogenic fungi are microorganisms that cause economic damage directly in the agricultural production system, besides indirectly cause ecological problems, due to the use of pesticides for its control [23].

The production of essential oils involves biosynthesis, transport, storage and degradation, each of these processes governed by genes and influenced by factors such as heredity, stage of growth and the environment [24] [25].

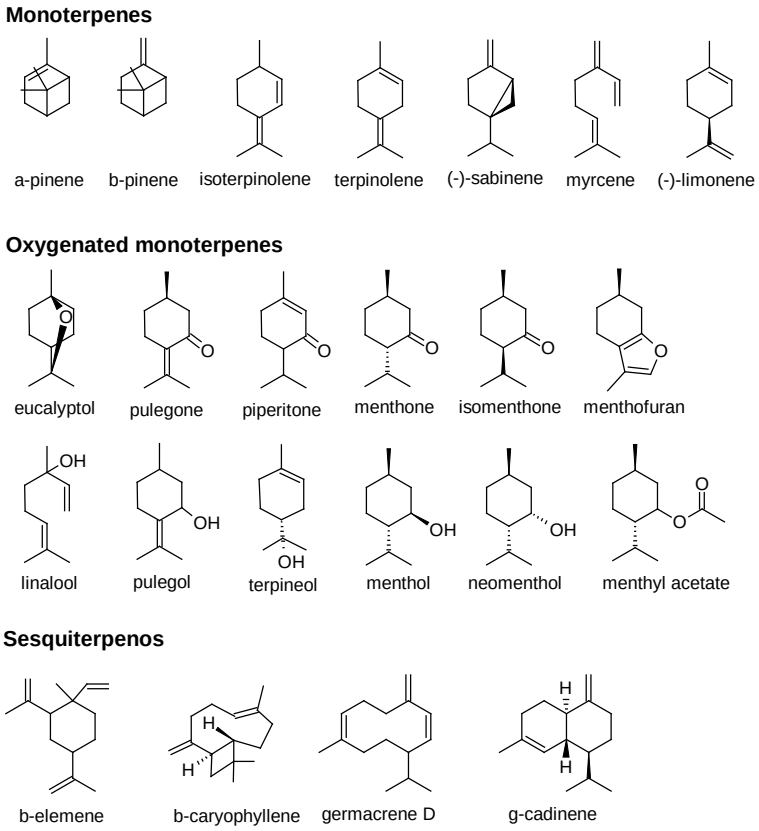

Figure 1. Substances usually found in the essential oils of the genus *Mentha*.

It is common to relate the flowering period as the best time to harvesting and extraction of the essential oil. However, the doubt persists if the flowering causes a homogeneous response leading to production and maturation of essential oil in the whole plant or if these events are controlled individually by expansion of the leaves?

According to Gershenzon [26], the leaves are organs that have different ages in architecture of a plant and therefore it may present qualitative and quantitative changes in the essential oil composition. By this point of view, the variability would be caused by the age of organs effect, which makes it more specific and localized control.

The objective of this work was to evaluate the essential oil production and quality extracted by hydrodistillation of young leaves (3rd to 5th node) and adult (6th to 8th node), as well as to evaluate the antifungal and antigerminative potential of menthol mint essential oil and some isolated substances of this oil, on the fungus *F. oxysporum*, *R. solani* and *S. rolfsii* and commercial seeds of lettuce e tomato, searching for alternatives to the use of fungicides in the treatment of commercial seeds.

2. Materials and Methods

2.1. Materials

For the experiment were obtained the following materials: alkane standard solution C_8-C_{40} (Fluka, St. Louis, USA); Analytical standards (−)-limonene, (+)-menthofuran, eucalyptol, sabinene, (−)-*α*-pinene, (−)-menthone, *α*-terpineol, (+)-neomenthol, (−)-pulegone, (−)-menthol; the solvents dichloromethane, dimethylsulfoxide (Sigma-Aldrich, SP, Brazil); commercial seeds of lettuce, White Boston variety and tomato, Santa Cruz Kada variety (Isla Seeds Ltd., RS, Brazil); seedlings of menthol mint (*Mentha arvensis* L.) IAC 701 variety provided by Linax Corporation (Votuporanga, SP) and pure fungal cultures were obtained from mycology collection of the sectors of phytopathology of the Federal University of Viçosa (MG, Brazil).

2.2. Plant Material, Essential Oil Extraction and Analyses

Seedlings of menthol mint IAC 701 variety were propagated directly in definitive beds at Chemistry Department of Universidade Federal Rural do Rio de Janeiro (UFRRJ). The plants were grown for approximately 60 days, when young leaves (3rd to 5th node) and adult (6th to 8th node) were collected for extraction of essential oil by hydrodistillation (50 g of fresh plant material), with Clevenger apparatus, during one hour.

The oil content (% w/w) was evaluated and the composition was analyzed by gas chromatography (GC) (Hewlett-Packard 5890 Series II, Palo Alto, USA) equipped with flame ionization detection and a split/splitless injector, in a split ratio of 1:20 was used to separate and detect the constituents in the essential oil. The substances were separated into the fused silica capillary column CPSil-8CB [30 m × 0.25 mm (i.d.) × 0.25 µm (film thickness)]. Helium was used as the carrier gas at a flow rate of 1 mL·minute^{-1}. The column temperature was programmed as follows: 60˚C for 2 minutes followed by heating at 5˚C·minute^{-1} to 110˚C, followed by heating at 3˚C·minute^{-1} to 150˚C and finally followed by heating at 15˚C·minute^{-1} until 290˚C and holding constant for 15 minutes. The injector temperature was 220˚C and the detector temperature was 280˚C. The percentage of components in the essential oil was calculated from the relative area of each peak analyzed by flame ionisation detector.

The gas chromatography coupled with mass spectrometry (GC-MS) was used for the essential oil analysis using a Varian Saturn 2000 (Palo Alto, CA). The flow of the helium gas carrier, the capillary column and the temperature conditions for the GC-MS analysis were the same as described for the GC. The temperature of the injector was 220˚C and the temperature of the interface was 250˚. Mass spectra were obtained with an ion-trap detector operating at 70 eV, with 40 - 400 m/z mass range and scanning rate equal to 0.5 scan·second^{-1}. The identification of the oil constituents was based on comparisons of their GC retention indexes and their mass spectra with authentic standards, NIST database (2008) and retention index (RI) [27]. The RI was obtained from the co-injection of an alkane standard solution.

2.3. Description of the Experiment and Statistical

To evaluate the antifungal and antigerminative effects of menthol mint essential oil and same chemical substances isolated (−)-limonene, (+)-menthofuran, eucalyptol, sabinene, (−)-*α*-pinene, (−)-menthone, *α*-terpineol, (+)-neomenthol, (−)-pulegone and (−)-menthol were performed the following procedures.

Antigerminative activity-seeds of tomato and lettuce were previously separated, 50 seeds per replicate, treated

with a solution of dichloromethane (DCM) with 1.0 and 5.0 mg·mL^{-1} of essential oil and their compounds isolated, respectively. The seeds were immersed in the solution until the total evaporation of the DCM, and then were transferred to Petri dishes containing filter paper and 3 ml distilled water. Finally, the dishes were sealed with plastic film and placed in the germination chamber with photoperiod of 12 hours and 25˚C (±1˚C). Two controls were prepared under the same condition, with DCM and the untreated seeds. At the end of the experiment, were considered as germinated seeds with healthy free leaflets and the radicle developed.

Antifungal activity was utilized dilution method, in which the essential oils and isolated substances were diluted on potatoes dextrose agar (PDA) medium at 1.0 and 5.0 mg·mL^{-1}, respectively. To the dilution of the essential oils and chemical substances was used the dimethylsulfoxide (DMSO, 0.5% v/v). Approximately 5 ml of PDA medium containing the active substances were transferred to Petri dishes and, after solidification, were placed in the center of these dishes disks with 5 mm diameter containing propagative structures of *F. oxysporum*, *R. solani* and *S. rolfsii*. Two controls were prepared in the same conditions, with DMSO (0.5%) and only the culture medium. To avoid the growth of bacteria was used in the culture medium gentamicin (200 µg·mL^{-1}), an antibiotic of broad spectrum. The dishes were placed in a thermostatic chamber at constant temperature of 25˚C (±1˚C) and were monitored daily the average diameter of the colonies in the two orthogonal directions until that controls reach ±80% of the total diameter of the plates.

Plants of menthol mint were randomly collected in the center of three replicate blocks, until completing 50 g of young and adult leaves. Each treatment of antigerminative and antifungal activity test were composed of five replicates. The mean and confidence interval (CI) were calculated in Sigma Stat 2.03 (Chicago, USA). The graphics were created in Graph Pad Prism, version 5 (Graph Pad Software Inc., San Diego, USA).

3. Results and Discussion

3.1. Essential Oil Content and Chemical Analysis

Table 1 shows a significant difference (0.15%) between the content of essential oil obtained from young and mature leaves, with a significant increase in oil content of adult leaves (about 20%). Similar results with the genus *Mentha* showed evidences that the maturity of the leaves is the physiological parameter responsible for the increase in the content of essential oil [26] [28].

Table 1. Essential oil content and chemical profile of menthol mint extracted by hydrodistillation of young and adult leaves.

EO[1]	RT[2]	Substance	KI[3]	RI[4]	Content (%)		ID[6]
					Position leaves		
					Young	Adult	
1	10.06	*β*-pinene	980	985	0.09 ± 0.00[5]	0.18 ± 0.01	AS. MS. KI
2	11.68	limonene	1031	1036	1.42 ± 0.05	0.48 ± 0.02	AS. MS. KI
3	15.60	isopulegol	1146	1158	0.49 ± 0.00	0.39 ± 0.01	MS. KI
4	15.86	menthona	1154	1166	13.34 ± 0.22	3.83 ± 0.02	AS. MS. KI
5	16.13	isomenthona	1164	1175	2.55 ± 0.07	2.64 ± 0.05	AS. MS. KI
6	16.26	neomenthol	1165	1179	1.43 ± 0.02	1.68 ± 0.06	AS. MS. KI
7	16.60	menthol	1173	1190	67.27 ± 1.65	73.35 ± 0.18	AS. MS. KI
8	18.41	pulegone	1237	1250	0.31 ± 0.03	0.05 ± 0.00	AS. MS. KI
9	18.90	piperitone	1252	1266	2.49 ± 0.03	2.28 ± 0.06	MS. KI
10	19.80	menthyl acetate	1294	1297	2.33 ± 0.13	10.03 ± 0.55	MS. KI
11	23.20	*β*-elemene	1391	1417	0.12 ± 0.00	0.43 ± 0.03	MS. KI
12	25.31	germacrene D	1480	1497	1.75 ± 0.06	1.14 ± 0.02	MS. KI
		Ketone monoterpenes			18.69 ± 0.26	8.79 ± 0.14	
		Alcohols and esters monoterpenes			71.52 ± 1.55	85.46 ± 0.33	
		Total of terpenes			93.59 ± 1.39	96.48 ± 0.16	
		Essential oil content			0.74 ± 0.02	0.89 ± 0.03	

[1]EO, elution order; [2]RT, retention time in minutes; [3]KI, Kovats index [29]; [4]RI, Retention Index calculated as described in materials and methods; [5]Values are means ± confidence interval (95%) of the samples (n = 3); [6]ID, sample identification by comparison with authentic analytical standards (AS); mass spectrum (MS) and KI.

In the chemical profiles of the essential oils obtained by hydrodistillation of the fresh leaves, the results were compatible with those found in the literature [6]. However, between the young and adult leaves, it was observed increased concentrations of menthol (9%) and menthyl acetate (330%) in mature leaves and a decrease of menthone (71%) in young leaves (**Table 1**). Similar results were showed by others authors [26] [28] [30].

The results showed that the young leaves are associated with higher content of limonene and ketone monoterpenes, intermediates in the biosynthesis of menthol, on the other hand, the adult leaves presented higher contents of alcohols and esters monoterpene (Note in **Figure 2** the numbers marked with asterisk) [30].

3.2. Antifungal Activity

Table 2 shows that essential oil affected partially the development of the fungus *F. oxysporum*, *R. solani* and *S. rolsii*. Under these conditions the antifungal effect is characterized as fungistatic and may be caused by the synergism of substances of the essential oil.

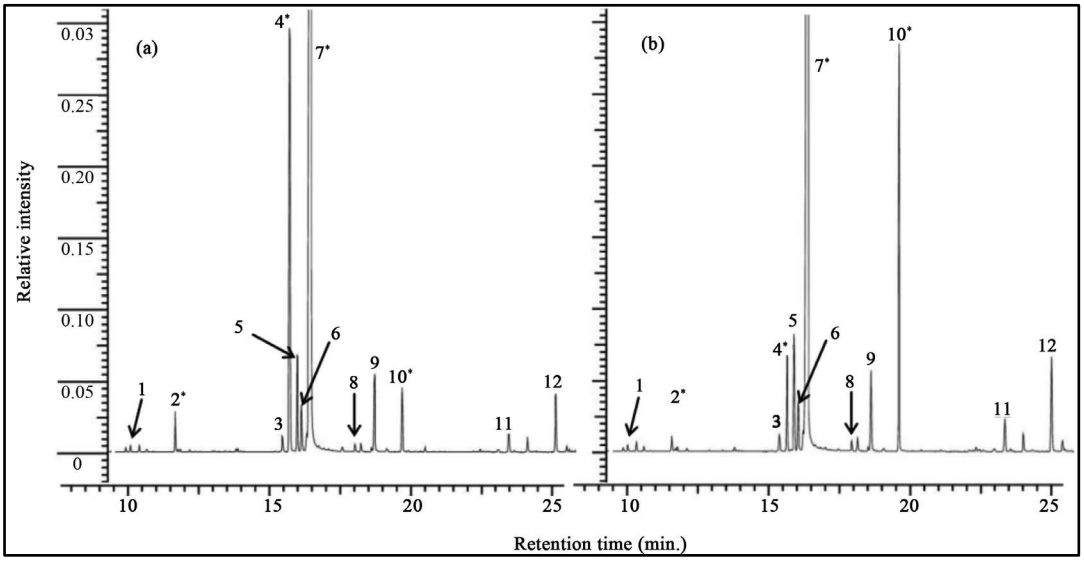

Figure 2. Total ion chromatogram of menthol mint essential oil obtained by hydrodistillation of young (a) and adult (b) leaves. Compounds in order of elution (see **Table 1**).

Table 2. Percentage of antigerminative and antifungal activity of menthol mint essential oil and compounds usually found in essential oil of plants of genus *Mentha*.

Substances	Concentration (mg·mL^{-1})[2]	Inhibition (%)				
		Mycelial growth[3]			Germination[4]	
		F. oxysporum	*R. solani*	*S. rolfsii*	Tomato	Lettuce
Essential oil[1]	1.0	55 ± 2.95	49 ± 4.5	47 ± 3.8	28 ± 2.1	32 ± 3.1
Limonene	5.0	9 ± 0.5	17 ± 2.1	0 ± 0.0	100 ± 4.5	100 ± 9.8
Menthofuran	5.0	0 ± 0.0	52 ± 3.9	4 ± 0.5	94 ± 3.9	23 ± 1.1
Eucaliptol	5.0	5 ± 0.4	20 ± 1.3	0 ± 0.0	90 ± 4.2	100 ± 6.1
Sabinene	5.0	83 ± 3.2	81 ± 6.2	55 ± 4.6	80 ± 3.1	98 ± 4.1
Pinene	5.0	10 ± 1.1	100 ± 7.2	0 ± 0.0	61 ± 2.9	73 ± 2.1
Menthona	5.0	7 ± 0.9	34 ± 1.5	64 ± 4.1	25 ± 3.0	100 ± 5.9
Terpineol	5.0	100 ± 9.5	100 ± 7.8	100 ± 4.3	0 ± 0.0	0 ± 0.0
Neomentol	5.0	23 ± 1.9	100 ± 8.6	100 ± 7.8	0 ± 0.0	65 ± 1.9
Pulegone	5.0	17 ± 1.1	100 ± 8.2	100 ± 5.9	0 ± 0.0	65 ± 2.3
Menthol	5.0	100 ± 9.8	100 ± 9.7	100 ± 8.1	0 ± 0.0	0 ± 0.0

[1]Essential oil of menthol mint obtained by hydrodistillation of all leaves of plant; [2]concentration of substances in mg·mL^{-1} were dissolved in [3]DMSO or [4]DCM; [5]values are means ± confidence interval (95%) of the samples (n = 5).

The fungitoxic activity analysis showed that the isolated compounds terpineol, neomenthol, pulegone and menthol inhibited 100% of *S. rolfsii* and *R. solani* growth, menthol and terpineol inhibited 100% of the development of *F. oxysporum* and pinene inhibited 100% of the development of *R. solani*. Menthofuran showed no fungitoxicity to *F. oxysporum*, as well as limonene pinene and eucalyptol against *S. rolfsii*.

Natural compounds act on the internal mechanisms of the fungus leading to malformation of important structures, cytoplasmic granulation, disorganization of cell contents, disruption of the plasma membrane and inhibition of fungal enzymes, consequently inhibiting germination, germ tube elongation and reduction or inhibition of the mycelial growth [31].

3.3. Antigerminative Activity

The essential oil showed partial antigerminative action on the seeds of lettuce and tomato, but limonene inhibited 100% of the germination of tomato seed and limonene, eucalyptol and menthone inhibited 100% of the lettuce seed development. The germination of the tomato seeds was not inhibited by terpineol, neomenthol, pulegone and menthol. Also was not inhibited the germination of lettuce seeds by terpineol and menthol.

These results indicate the potential of natural products as inhibitors of germination, in the same way that other researchers showed antigerminatives effects on commercial seeds of cultivated species and weeds [32]-[36].

On the other hand, if the purpose involves protection of seeds against phytopathogenic fungi, the ideal search involves the use of substances which have antifungal effect with no antigerminative action. In this context, some substances are shown to be adequate for the treatment of tomato seeds (terpineol, neomenthol, pulegone and menthol) and lettuce (terpineol and menthol).

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