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Bioactivity of *Laurus Nobilis* and *Mentha Piperita* essential oils on some phytopathogenic fungi (*in vitro* assay)

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Abstract
Extraction of essential oils from locally available plants: *Laurus nobilis and Mentha piperita* were carried out using steam-distillation method. Extracted oils were screened for their chemical composition and antifungal activity in order to find new metabolite compound which are characterized by a biological activity. Twenty-two constituents, representing 99.7% of the essential oil of *Laurus nobilis* was determined by GC-MS analysis. The main compounds identified are 1,8-Cineole (45.36%), followed by bornylene (17.25%), linalool (8.13%), and sabinene (7.48%), when Twenty-three compounds were identified, representing 99.9% for *Mentha piperita* oil. The principal components are: Carvone (51.04%), Limonene (36.37%) and β-Pinene (1.66%), which compose 89.07% of the oil. The antifungal activity of oils was tested using the direct contact method against the growth of *Fusarium moniliforme, Fusarium solani, Fusarium oxysporum* and Stemphylium*solani*. The results showed great potential of natural antimicrobial activity against strains tested. Our results clearly demonstrate that the essential oils of selected plants can well present an interesting alternative naturel may be useful as biofongicides.

Keywords: Essential oil; *Laurus nobilis*; *Mentha piperita*; GC/MS; Antifungal Activity; Biofongicides.

1. Introduction

Chemical control seems to be the most effective way to curb the fungal parasite in agriculture. However, it has many disadvantages such as environmental pollution, the poisoning of operators and consumer issues, elimination of useful entomofauna, the residue accumulation in the food chain including the appearance resistant fungi. To overcome the many constraints related to the use of chemical pesticides, other strategies have been considered, namely biological control that aims to control and kill pathogens by biopesticides.

For this purpose, the crude extracts of the plants begin to have great interest as a potential source of bioactive natural molecules. These constituents are classified into two types of primary and secondary metabolism. Secondary metabolites are produced often complex chemical structures, widely dispersed and very different in different species. There are more than 200,000 secondary metabolites classified according to their chemical affiliations [1]. We cite for example, essential oils and phenolics more particularly flavonoids.

The study of essential oils is the topic that intrigues research laboratories despite its age where new perspectives are implemented for a sophisticated design of plant biotechnology. Essential oils are used in

aromatherapy, pharmacy, perfumery and cosmetic products [2] due to their wealth of active ingredients that are loaded by a vital energy of natural origin. Also, they are used in biological control as biopesticides due to their inhibitory action on growth and toxinogenesis of several bacteria and fungi.

Laurus nobilis (Lauraceae) leaves and essential oils are used as precious spice, which are used as flavoring agent in culinary and processed foods [3]. In traditional medicine, Laurus nobilis is used for its antiseptic, anti-cancer, anti-spasmodic, anti-mutagenic, anti-cancer and as a treatment of digestive disorders [4-7].

Peppermint (*Mentha piperita*) one of those plants that is native of Middle East. It is the result of hybridization between water mint (*Mentha aquatica*) and spearmint (*Mentha spicata*) [8, 9]. In traditional medicine, it is used for its antiseptic, antiviral, antispasmodic, antibacterial and antioxidant activity. [10-14]

This work was focus on the study of the antifungal activity of essential oils from aromatic plants which two *Laurus nobilis* and *Mentha piperita* which respectively belong to the family of Lamiaceae and Lauraceae. This study was conducted to illustrate the use of these essential oils as a natural alternative to chemical (biopesticide).

2. **Experimental**

2.1.*Vegetal Material*

The leaves of *Laurus Nobilis* and aerial parts of *Mentha Piperita* were harvested in March and June (2013) respectively in the regions of Skikda (North Algeria (N 36 ° 52'18.011 'E 6 ° 53'14.786')) and Ouargla (South Algeria (N 36 ° 52'18.011 "E 5 ° 20'36.7")). These two species were identified by botanist Pr .Abdelmajid Chahma, Medicine Faculty, University of Ouargla, Algeria. A specimen was deposited at the herbarium of the University under the number GO2013-2 and GO2013-1 respectively.

2.2 *Extraction of essential oil*

The extraction of essential oil was carried by steam distillation, in a Clevenger apparatus by immersing 100g of dry leaves in a flask of 1000 ml of water for 3 hours. The obtained essential oil was dried with MgSO4 and stored in the dark at 4°C [9].

2.3 *Gas chromatography–mass spectrometry essential oil analysis*

The essential oils were analyzed in INRAP (national institute of research and physico-chemical analysis) Tunisia, using an Agilent 6890 gas chromatograph coupled to an Agilent 5975B mass selective detector with electron impact ionization (70 eV) and an Agilent Chemstation software (Agilent Technologies, Palo Alto, USA). Separation of oil constituents was performed on HP-5MS; 5% Phenyl Methyl Siloxane capillary column (30 m × 0.25 mm, film thickness 0.25 μm) in the split mode (1:50) at 250 ◦C. The oven temperature was programmed at 50◦C for 1 min, raised to 300◦C at 2◦C min^{-1} and finally held at this temperature for 10 min. Helium was used as carrier gas at a flow of 0.8 mL min^{-1}. Linear retention indices (RI) for all compounds were determined using n-alkanes as standards. Identification of individual compounds was performed by matching their patterns mass spectral fragmentation with corresponding data (NIST 05 and Wiley7 libraries), and by the laboratory database.

2.4 *Antifungal Activity*

2.4.1 *Origin of fungal strains*

The fungi used in this study were isolated from tomato leaves, peppers and wheat leaves. These are 03 species within the genus Fusarium (*Fusarium moniliforme, Fusarium solani, Fusarium oxysporum*) and a specie belonging to the genus *Stemphylium solani* they entail considerable loss of production in several varieties of plant. The species were identified by Mr. Messaoud Bensaci, Ecosystems Protection in Arid and Semi-Arid areas Laboratory. University of Ouargla, Algeria.

2.4.2. *Direct contact method*

The evaluation of the antifungal activity of essential oils is adopted by the direct contact method with four concentrations are obtained by the addition of 30, 150, 300, and 450 µl of essential oils upon 60ml warm PDA in a vial with adding drops of tween 20 (concentration of 0.5%). The technique involves adding the oil at different concentrations (0.05%, 0.25%, 0.5% and 0.75%) in the middle of still liquid culture followed by 5 minutes of stirring in order to homogenize the medium PDA with essential oil. After shaking the vials, the mixture (PDA + HE + Tween 20) is poured into petri dishes.

The inoculation is done under the hood where depositing at the center of the box a mycelial disc of 0.6 cm in diameter. The Witnesses (fungal strains + PDA + Tween 20) are made in the same conditions without essential oil and the measure is taken

after 72 hours of incubation. These boxes (control and test) are incubated at 25 ± 2 ° C for 7 days respectively [15]. All tests are repeated three times.

2.4.3.*Inhibition rate (% TI)*

The calculation of inhibition percent of growth compared to the control allows to evaluate the effect of oil concentrations on fungal growth. The technique consists of measuring the diameters of the various fungal colonies after the required incubation time [16].

$$\mathbf{TI(\%) = 100 \times (dC - dE)/dC}$$

TI (%) = Inhibition rate expressed as a percentage
dC = Diameter of settlements in the dishes "positive control"
dE = Diameter of colonies in the dishes containing the plant extract

2.4.4. *Determining the mycelial growth rate (VC):*

According Cahagnier B et al [17], the rate of mycelial growth of each concentration is determined by the formula:

$$\mathbf{VC} = [D1/Te1] + [(D2 - D1)/Te2] + [(D3 - D2)/Te3] + \cdots + [(Dn - D_{n-1})/Ten]$$

D: Diameter of the growth zone of each day and Te: Incubation time.

3. Results and discussion

3.1. *Gas chromatography–mass spectrometry analysis of essential oil*

Essential oils yields have been calculated based on the dry plant material of the aerial part of the plant. *Laurus nobilis* is provided a rate of 0.78% relatively lower than that obtained from *Mentha Piperita* (0.84%). The chromatographic analysis of essential oils have identified 22 compounds representing approximately 99.74% for *Laurus Nobilis* and 23 compounds representing 99.9% for *Mentha Piperita* (Table 1).

Laurus Nobilis essential oil of Algeria is composed mainly by 1,8-cineole (45.36%) accompanied by other constituents in contents: bornylene (17.25%), linalool (8.13%) and sabinene (7.48%) totaling approximately 78.22% (figure 01) [7].

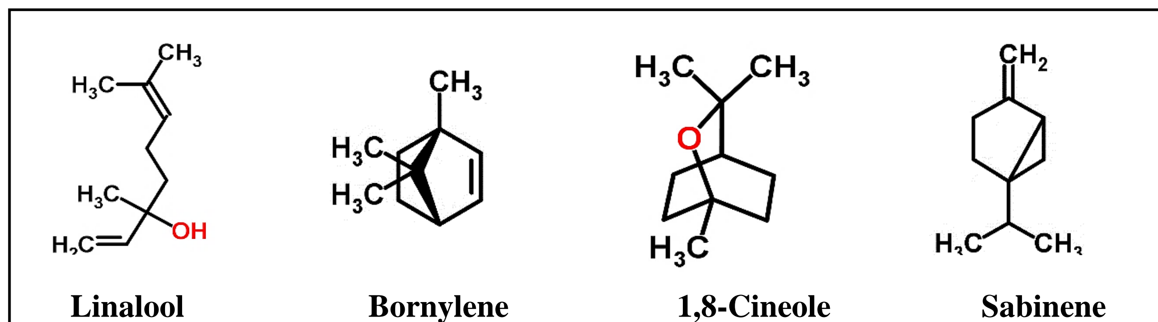

Figure 01: Majority components of the essential oil of *Laurus nobilis*.

This chemical composition is substantially similar to those published for other geographical regions: the monoterpene 1,8-cineole is reported to be the main component of the essential oil of Morocco [18, 19], Tunisia [20, 21], Egypt [22], Turkey [23, 24], Iran [25], Italy [26], Portogal [27] and Argentina [28]. The essential oil's content showed variations in the same plant of different geographical origin, and also in different parts of the tree. In comparison between the *Laurus nobilis* oil's composition collected in Tunisia which showed a high content of 1,8-cineole (56.31%), borneol (11.04%) and valencene (11.03%) [21] and oil's composition of Morocco, who gave 1,8-cineole (39.81%), 2-carene (13.03) and trans-ocimene (7.05) [19], we noticed considerable differences.

The essential oil of *Mentha Piperita* was richer in Carvone with a higher rate than 50% followed by Limonene (36.37%) and β-Pinene (1.66%)(figure 02) [13].

Table 01: Chemical composition of the essential oils of *Mentha Piperita* and *Laurus nobilis*

Compounds	RT	*M.piperita %*	*L.nobilis%*
α-Thujene	705	0.13	0.24
α-Pinene	763	1.07	3.18
Camphene	819	0.2	0.4
β-Terpinene	933	0.96	-
β-Pinene	943	**1.66**	2.32
Sabinene	945	**-**	**7.48**
β-Myrcene	1023	1.5	0.38
(+)-4-Carene	1147	-	0.27
Limonene	1241	**36.37**	-
1,8-Cineole	1253	**-**	**45.36**
γ-Terpinene	1373	**-**	0.57
Terpinolene	1535	**-**	0.2
iso-amyl-2-methyl butyrate	1621	0.12	-
Linalool	1655	-	**8.13**
cis Limonene oxide	1801	0.2	-
trans-Limonen oxide	1831	0.18	-
Borneol	2002	0.27	0.19
L-Borneol	2017	0.45	-
4-Terpineneol	2090	-	2
α-Terpineol	2186	-	2.14
trans-Dihydrocarvone	2206	1.52	-
cis-Carveol	2461	0.46	-
Carvone	2602	**51.04**	-
cis-carvone oxide	2741	0.15	-
L-bornyl acetate	2766	-	0.64
Pseudolimonene	2970	-	0.6
Piperitenone	3119	0.47	
Bornylene	3216	-	**17.25**
Piperitenone oxide	3283	0.74	-
β-bourbenene	3365	0.3	-
Caryophyllene	3571	0.37	-
Methyleugenol	3574	-	6.84
γ-Muurolene	4144	0.17	-
Elemicine	4444	-	0.29
Nerolidol	4466	-	0.15
Spathulenol	4511	-	0.5
Caryophyllene oxide	4526	0.82	0.61
α-Cadinol	4868	0.84	-
Total		99.99	99.74

RT : Retention indices

Similarly, the essence of *Mentha Piperita* from Federal District, Brazil is dominated by the Carvone but with only 30.5% [29]. However, the main constituents of *Mentha Piperita* oil from: Morocco (Menthol (46.32%), Menthfuran (13.18%) and Menthyl acetate (12.1%) Fadil, Farah [30], Turkey (+)-menthol (38.06%), Menthol (35.64%) and Neo-menthol (6.73%) Kizil, Hasimi [9] and α-terpinene (19.7%), Pipertitinone oxide (19.3%) and trans-carveol (14.5%) for Iran [31].

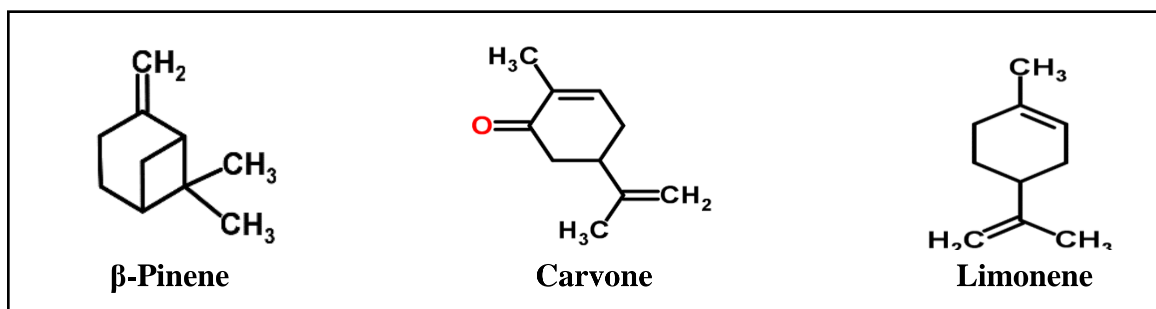

Figure 02: Majority components of the essential oil of *Mentha piperita.*

Generally, it is learned that the variation in the chemical composition of essential oils could be attributed to the geographical origin of the plant, the extraction technique, the time of harvest and climatic factors [32-34].

3.2. *Antifungal Activity*

3.2.1 *Kinetics of mycelial growth*

The mycelial growth kinetics was evaluated every 24 hours by measuring the mean of three perpendicular diameters passing through the center of the puck. This reading is always performed in comparison with control cultures that they are started on the same day under the same conditions. Any even slight growth of each fungus will be considered negative action that the essential oil in question does not have any inhibitory action against fungal growth. The Figure 03 summarizes the results of mycelial growth (cm) of the fungal strains as a function of incubation time and the concentration of essential oil of *Laurus nobilis*.

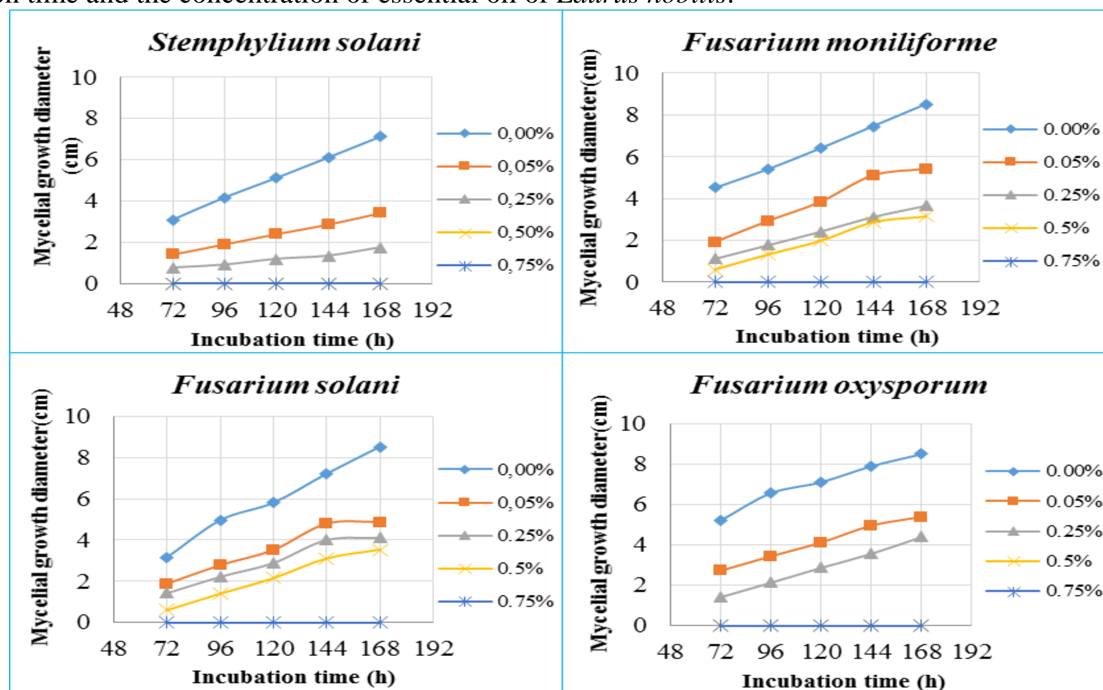

Figure 03: Kinetics of mycelial growth as a function of time and concentration of essential oils from *Laurus nobilis*.

The essential oil of *Laurus nobilis* effect (Figure 03) shows a reduction in mycelial growth with increasing concentration. *Stemphylium solani* and *Fusarium solani* revealed more sensitive when no growth recorded by a concentration of 0.5%. There was no mycelial growth of any fungal strain at a concentration of 0.75%.
For the essential oil of *Mentha piperita* and with different concentrations of extracted essential oil, we notice that the mycelial growth *Stemphylium solani* is reduced by a concentration of 0.25%, beyond this concentration no growth observed. In addition, the mycelial growth is reduced with increasing concentration for other fungal strains and for a concentration of 0.75% essential oil, no growth is reported.

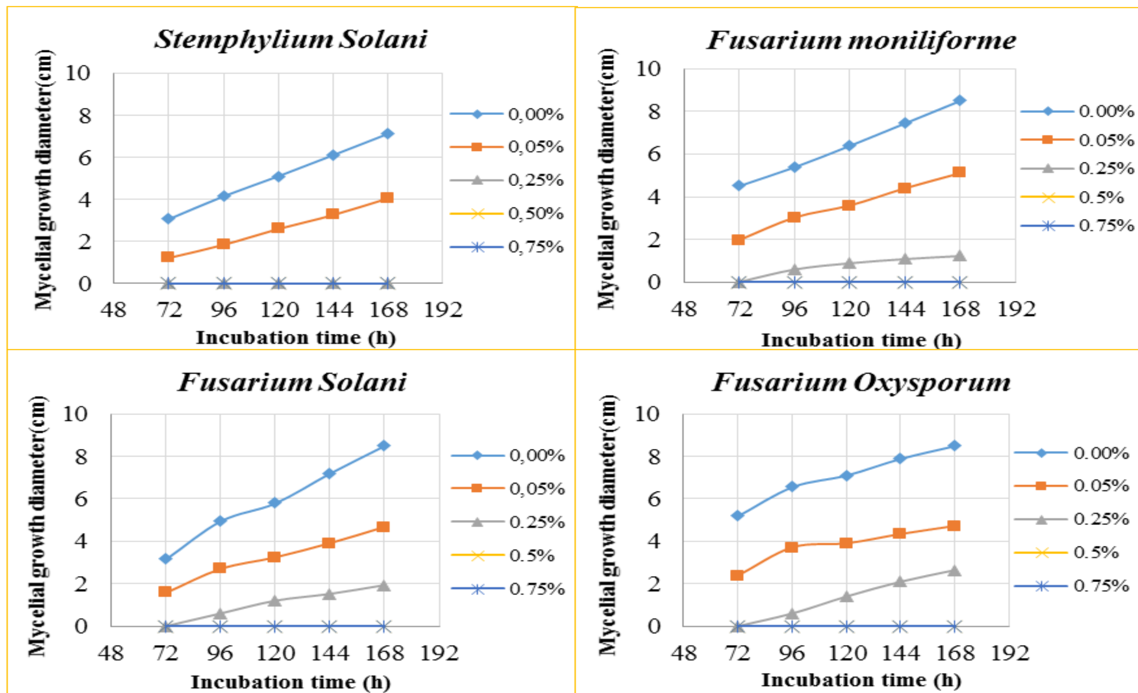

Figure 04: Kinetics of mycelial growth as a function of time and concentration of essential oils of *Mentha Piperita*

3.2.2 *Antifungal Index:*
Inhibition rates of essential oils studied are given in Figure 05

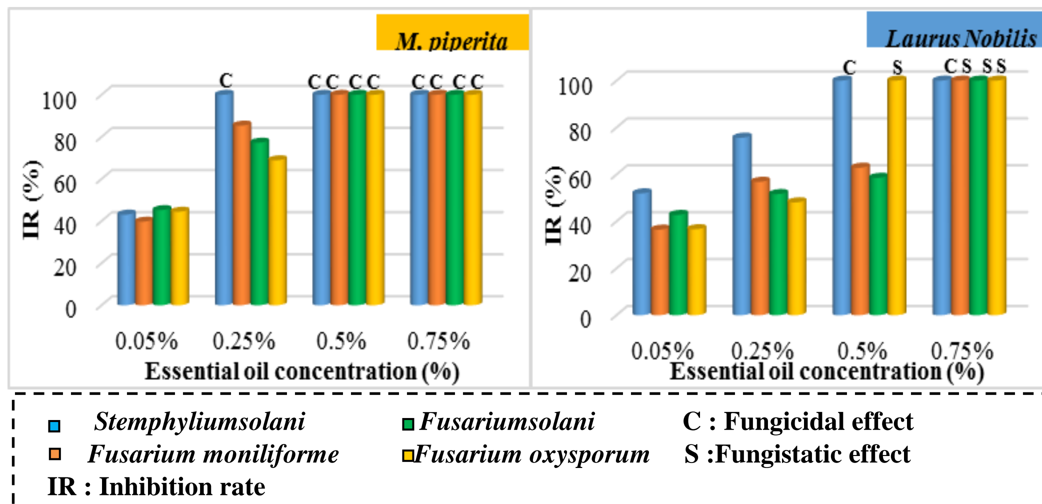

Figure 05: strains inhibition rate as a function of the concentration of essential oils from plants studied

We note that according to Figure 05 all concentrations of essential oils applied were partially inhibited the growth of fungal strains tested. Figure 05 show that the inhibition rate is increased with increase in concentration of oils, and the minimal inhibitory concentration (MIC) is in the order with a good antifungal effectiveness shown by the essential oil of each plant studied.
Indeed, for *Laurus nobilis*, the MIC is between 0,25% and 0.5% for *Stemphylium solani* strains with a fungicidal effect and *Fusarium oxysporum* with a fungistatic effect. For the *Fusarium moniliforme* and *Fusarium solani*, the MIC is located between 0,5% and 0.75%.
The essential oil of *Mentha piperita* effect notes a fungicidal effect, the MIC is among 0.05% and 0.25% for *Stemphylium solani* and (0.25-0.5)% for other strains tested

3.2.3 *Speed of mycelial growth*
The speed of the mycelial growth of the four fungal strains as a function of the concentration of essential oils is shown in Figures 06 and 07.

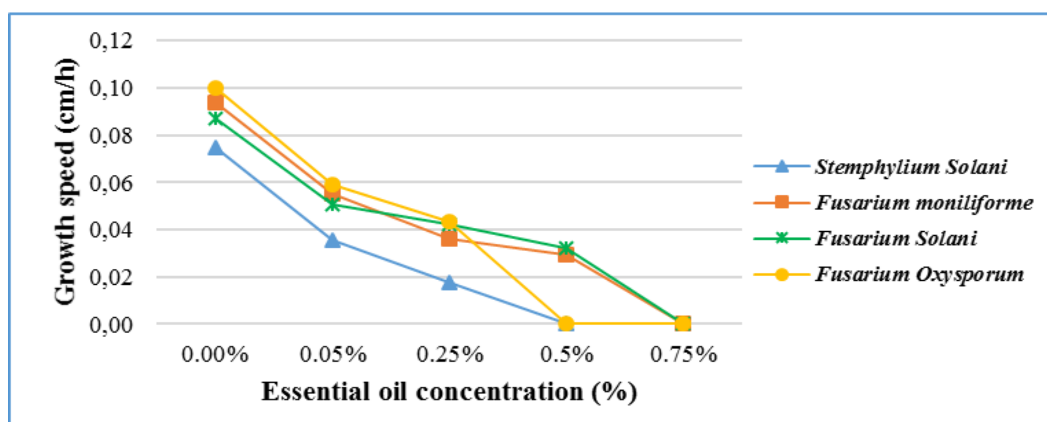

Figure 06: mycelial growth speed under the effect of increasing the concentration of essential oil of *Laurus nobilis*.

According to the results of Figure 06, there is a decreasing speed of mycelial growth by increasing the concentration of essential oil. The speed of the strains is decrease to total inhibition (0 cm/h) in 0.5% concentration of essential oil to *Fusarium oxysporum, Stemphylium solani* and 0.75% for other strains.

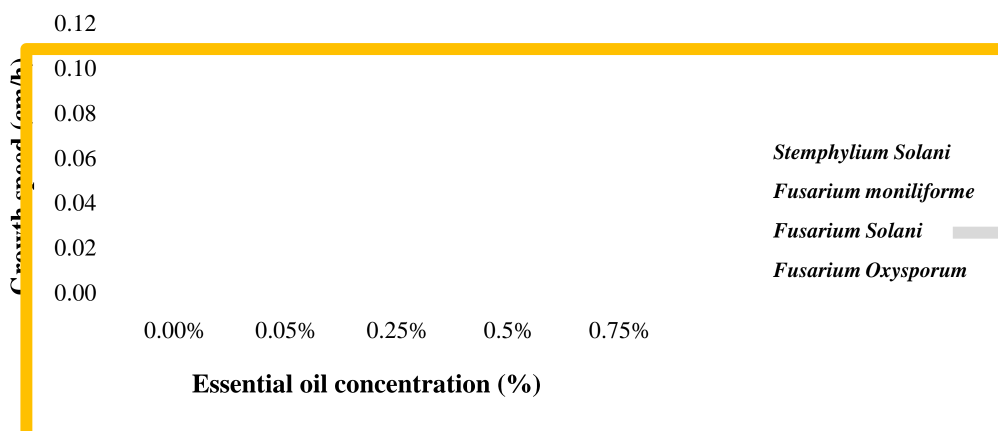

Figure 07: mycelial growth speed under the effect of increasing the concentration of essential oil of *Mentha Piperita*

Figure 07 shows that the speed of the strains decreases to total inhibition (0cm / h) in the 0.25% concentration of essential oil to *Stemphylium solani* and 0.5% for other strains. The technique of contact direct comprises contacting the essential oils and micro-organisms, then observe the growth of the latter. Plant oils exert a

significant inhibitory activity against the strains tested. The diameters, the speed and the antifungal index of the mycelium growth decreases each time it increases the concentration of each essential oil to the non germination on disc at determined MIC, this is confirmed by the work of Mehani and their collaborators. [35]
According Chami, Prasad et al , the difficulty of developing an antifungal molecule is linked to the ultrastructure of fungal cell which presents three barriers: the cell wall, chitin, membrane ergosterol and eukaryotic nucleus in firstly; and secondly, antifungal molecules themselves that can lead to resistance.[36,37]
Indeed, biological control through the use of natural alternatives gave a lot of interest in this moment. Many Researchers noted that the possibility of using the extract from plants as an effective natural alternative.
A study performed on the essential oils of three plants which *Laurus nobilis* one of them for 17 fungal species that are: *Aspergillus niger, A. ochraceus, A. versicolor, A. flavus, A. terreus, Alternaria alternata*, *Aureobasidium pullulans, Penicillium ochrochloron, P. funiculosum, Cladosporium clado-sporioides, C. fulvium, Trichoderma viride, Fusarium tricinctum, F. sporotrichoides, Phoma macdonaldii, Phomopsis helianthi et Mucor mucedo*. The results obtained showed that this plant that is rich in 1,8-Cineole has moderate power over the mushrooms tested [38].

Essential oil of peppermint presents a strong antifungal activity against strains of *Aspergillus Niger* in a study conducted by Mr. Moghtader [39]. Menthol, the main constituent of the essential oil of peppermint, was considered responsible for the oil antifungal property.
Furthermore, Ferdeş et al. confirmed that essential oils from aromatic plants such as lemon, mint, juniper and rosemary, have antifungal activity against the strains of *Aspergillus niger, Fusarium oxysporum*, *Monascus purpureus et Penicillium hirsutum* molds. Also, oils show various antifungal activities; the most effective against all strains tested was mint oil [40].
The antifungal potency of essential oils studied could be attributed by the presence of components has an antifungal activity cause severe membrane damage and loss of homeostasis in which cell death or total inhibition. The majority of our essential oils constituents: Sabinene, Bornylene, 1,8-Cineole, Linalool, Carvone and Limonene proved by several researchers that have power antifungal [41-45].
Moreover, the antifungal activity of essential oils can be explained by the synergistic effect between the different essential oil compounds. However, as the majority compounds are often responsible for the antifungal activity. More, these minor components can contribute significantly to the activity of essential oils.

Conclusions

This work has been devoted to the study of the antifungal activity of essential oils of the species *Laurus nobilis* and *Mentha Piperita*. The chemical characterization of these essential oils has been determined in order to contribute to valorization and to redefine as their better exploitation. The qualitative and quantitative analysis of the essential oil from the plant of *Laurus nobilis* identified 22 components dominated by the 1,8-cineole (45.36%). On the other hand, the analysis of *Mentha Piperita* essential oil revealed by 23 components where Carvone is majority (51.04%). Bioassays have shown considerable activity of essential oils studied. This results are absolutely a rich source of information on chemical properties and antifungal essential oil of endemic species from Algerian flora. such activity has a natural attractive alternative suggests prospects of application as biopesticides.

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