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Antifungal activity of essential oils of two plants containing 1,8-cineole as major component : *Myrtus communis* and *Rosmarinus officinalis*

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Abstract
Myrtus communis and *Rosmarinus officinalis* are aromatic plants very used in traditional medicine. In the present work we studied chemical composition and antifungal activity of essential oils of these two plants. Chromatographic analysis showed that 1,8-cineole is the major component of myrtle (37.0%) and rosemary (43.16%). Antifungal activity of essential oils has been studied against three apple rot fungi by the micro-atmosphere method. The essential oils of *M. communis* was more active, it completely inhibited the growth of *Botrytis cinerea*, *Penicillium expansum* and *Alternaria alternata* at concentrations of 400, 600, 1800 µl/l respectively. While that of *R. officinalis* has not achieved total inhibition only at concentrations above 800 µl/l for *A. alternata* and 1200 µl/l for *B. cinerea*, in the case of *P. expansum*, an inhibition percentage of 89% was obtained at a concentration of 1800 µl/l.

Key words: Myrtus communis, *Rosmarinus officinalis*, antifungal activity, essential oil, *Alternaria alternata*, *Botrytis cinerea*, *Penicillium expansum*.

1. Introduction
Essential oils are secondary metabolites of aromatic and medicinal plants characterized by a very diverse chemical composition. They contain several biochemical families including acids, alcohols, aldehydes, ketones, esters, phenols, terpenes and sesquiterpenes [1]. This diversity is at the origin of various antimicrobial, antiviral, antioxidant activities of the essential oils [2 – 7].
These compounds, with varying antimicrobial and antiparasitic properties, constitute a natural reservoir of molecules that could be used as alternatives to chemical treatments that have harmful side effects on the environment and human health. Many chemical pesticides also show inefficiency due to their excessive use, and several studies have demonstrated resistance of some microorganisms to chemical treatments [8 – 10]. Examples of resistant phytopathogens are some isolates of *Alternaria alternata*, *Botrytis cinerea* and *Penicillium expansum*, which are causal agent of apple rot during the storage period [11 - 14].
Myrtle (*Myrtus communis*) and rosemary (*Rosmarinus officinalis*) are aromatic plants of Mediterranean origin and are very popular in Morocco. These two species are used for medicinal purposes. Myrtle is commonly used as an antiseptic and bactericide especially against pulmonary and urinary infections [1; 15]. While Rosemary has a stimulating effect on the central nervous system; it is an excellent tonic for the heart, liver and gallbladder; it contributes to lower the level of cholesterol in blood; it is also widely used for respiratory problems [1; 16]. Several studies reported the antimicrobial and antioxidant power of these two plants [17 - 19]; however, the activity of the essential oils of myrtle and rosemary against apple rot fungi has not yet studied.
In this context and in order to search for biological control agents based on natural molecules devoid of harmful side effects, we studied the effect of two essential oils, distributed as vapors, on the growth of three molds responsible for apple rot in storage.

2. Materials and methods

2.1. Plant material

The myrtle (*Myrtus communis* L., Myrtaceae) samples were collected in July 2009 in the region of Ouazzane in northern Morocco. The extraction of essential oils was carried out by hydrodistillation of the aerial parts in a Clevenger-type apparatus [20]. The resulting oil was dried over anhydrous sodium sulfate and stored at 4 °C in the dark. The essential oils of rosemary (*Rosmarinus officinalis* L., Lamiaceae) was delivered by the company Naturex Morocco.

2.2. Fungal material

Three fungi, *Alternaria alternata* (Fr.) Keissler 1912, *Botrytis cinerea* Pers. 1794 and *Penicillium expansum* Link ex Gray 1821, were chosen for their high frequencies to contaminate apples in storage.

These strains belong to the fungal collection of the Botanical Laboratory of the Faculty of Sciences of Rabat. They were isolated from damaged apples (**G**olden delicious) and were identified based on their morphological and microscopic features [21 – 22]. Stock cultures of molds were maintained on solid malt extract medium (1.5%) at 4°C.

2.3. Chromatographic analysis

Gas chromatographic (GC) analysis were performed with a Hewlett Packard 6890 Series equipped with a HP-Chemstation data processor, fitted with a capillary column HP-5 (30 m x 0.25 mm, 0.25 µm film thickness), with a FID detector set at 250 °C and fed with a gas mixture of H_2 / air. The mode of injection was split, the carrier gas used was nitrogen with a flow rate of 1.7 ml/min. The column temperature was programmed at a rate of 4°C/min from 50 to 200 °C.

The GC/MS analysis were carried out on a HP chromatograph (HP 6890) coupled to mass spectrometer (HP 5973 series). Fragmentation is carried out by 70 eV electron impact. A capillary column HP-5SM (30 m x 0.25 mm, 0.25 µm) was used. The column temperature was programmed from 50 to 200 °C with an isotherm at 200 °C for 5 min. The carrier gas was helium with a flow rate : 1.7 ml/min. The injection mode was split. The identification of the compounds was performed based on their Kovàts indices (KI) and the GC/MS.

2.4. In vitro test of the volatile fraction

Determination of the antifungal activity of the volatile fraction of the essential oils was performed by the micro-atmosphere method [23 – 25]. In this method, a sterile small fragment of filter paper was placed in the lid of a Petri dish containing MEA (1.5%, Biokar) [26, 27] inoculated with one of the test fungi.

The following volumes of pure essential oils were added to the filter paper: 6.5; 10; 19.5, 26; 39; 52; 65, 78 µl, which correspond to the following concentrations 100, 156, 300, 400, 600, 800, 1000 and 1200 µl/l.

The fungal cultures were sealed hermetically with Parafilm to avoid the exchange of gases and incubated cover down during 7 days in the dark at 25 °C. A control culture was prepared under the same conditions, except that, the filter paper was soaked with sterile distilled water instead of essential oils.

2.5. Percentage inhibition

The inhibition of fungal growth by the volatile fraction of the essential oils was measured by calculating the percentage inhibition [28, 29, 30]:

$$I\ (\%) = \frac{X - Xi}{X} \times 100$$

Where, X = colony diameter (mm) in the absence of essential oils (control).

Xi = colony diameter (mm) in the presence of essential oils.

2.6. Statistical analysis

All data were expressed as the mean by measuring three independent replicates. Analysis of variance using one-way ANOVA with Dunnett's post test was performed to test the significance of differences between means obtained and treatments using an GraphPad Prism version 6.07 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

3. Results and discussion

3.1. Chemical Composition

Compositions of essential oils extracted from *M. communis* and *R. officinalis* are reported in Table 1. A total of 28 and 21 constituents for *M. communis* and *R. officinalis* were identified, representing 99.80% and 99.96% of the essential oils, respectively.

1,8-cineole is the major component of the two essential oils with a concentration of 37% and 43.16% respectively for *M. communis* and *R. officinalis*. This monoterpene is accompanied, in the case of *M. communis* with α-pinene (17.9%), myrtenyl acetate (13.0%), limonene (9.3%), camphene (4.9%) and α-terpineol (4.6%). Others components have values between 0.1 and 3%. While essential oils of *R. officinalis* are constituted, in

addition to 1,8-cineole, of camphor (14.39%), α-thujene (13.46%), caryophyllene (4.89%), α-pinene (4.08%), β-pinene (3.98%) and borneol (3.57%).

Table 1: Chemical composition of essential oils of *M. communis* and *R. officinalis*.

KI	***Constituents***	***Rosemary*** (% total area)	***Myrtle*** (% total area)
910	Tricyclene	-	0,1
931	α-thujene	-	0,1
932	α-pinene	4.08	17.9
953	Camphene	-	4.9
976	Sabinene	-	0.3
986	β-pinene	3.98	-
991	Myrcene	1.34	0.2
1005	α-phellandrene	0.20	-
1016	1,4-cineole	0.54	-
1018	α-terpinene	1.47	0.2
1031	Limonene	2.35	9.3
1033	1,8-cineole	43.16	37.0
1062	ϒ-terpinene	0.63	-
1088	Terpinolene	0.32	3.0
1098	Linalool	1.05	0.2
1143	Camphor	14.39	-
1165	Borneol	3.57	-
1177	Terpinen-4-ol	0.70	0.3
1189	α-terpineol	2.37	4.6
1194	Myrtenol	-	0.9
1234	Tetrahydro linalool acetate	-	0.4
1257	Linalool acetate	-	1.0
1280	α-terpin-7-al	0.48	-
1295	bornyl acetate	-	0.2
1335	Myrtenyl acetate	-	13.8
1340	terpinen-4-ol acetate	-	0.2
1344	α-terpenyl acetate	-	0.3
1373	Longicyclene	0.12	2.7
1402	Longifolene	-	1.4
1418	Caryophyllene	4.89	0.2
1454	α-humulene	0.63	0.1
1460	Allo-aromadendrene	0.23	0.1
1524	Eugenyl acetate	0.23	0.3
1556	Germacrene B	-	0.1
1581	Caryophyllene oxide	-	0.1
Total		99.96%	99.8%

These results are somewhat similar to some previous works. Aidi Wannes and coworkers [31] reported that essential oils of myrtle is characterized by the dominance of 1,8-cineole (40.99%). Celikel and Kavas [32] and Moghrani and Maachi [33] showed that the essential oils of *M. communis*, from Turkey and Algeria

respectively, are also dominated by 1,8-cineole but at a lower concentrations than our oil samples. Turkish oil contains 24.8% of 1,8-cineole and that of Algeria contains just 15.79%.
In northern Morocco, common myrtle has two different chemotypes, the first is richer in α-pinene (42.6%) [34], while the second has a chemical composition similar to that of our samples with a dominance of 1,8-cineole (40.4 - 46.5%), and α-pinene does not exceed a concentration of 24.5% [35].
In Greece, Gardeli et al. [18] highlighted a new chemotype of myrtle, containing mertenyl acetate as the predominant compound with concentrations from 23.7 to 39.0%, followed by 1,8-cineole (13.5 to 19.6%) and α-pinene (10.9 -11.6%).
Studies published by Messaoud *et al.* [36] and Snoussi *et al.* [37] showed that essential oils of myrtle from Tunisia mainly contain α-pinene with levels of 19.20% and 48.9%, while 1,8-cineole concentrations not exceeding 15.96% and 15.3% respectively. In Iran, the myrtle has a high content of α-pinene (29.1%, 29.4% and 31.8%) [35 - 40]. Similarly, Bouzabata et *al*. [41] found in Algeria a chemotype characterized by its high concentration of α-pinene.
Results of the chemical composition of our *R. officinalis* samples are similar to those of Elamrani et *al*. [42 - 43]; Zaouali et *al.* [44] ; Yang et *al.* [45]; Tahri et *al.* [46] and Bomfim et *al.* [47] ; they reported that rosemary from Morocco, Tunisia, China and Brazil, respectively, generally produce a cineole essential oils. Miresmailli et *al.* [48] also obtained a 1,8-cineole (31.5%) chemotype against 20% of camphor and 17.5% of α-pinene and other monoterpenes.
However, the essential oils of *R. officinalis* from Algeria are dominated by α-pinene (19.70 - 23.1%) followed by camphor (14.5 - 12.56%); 1,8-cineole does not exceed 7.98% [49 - 50]. Similarly, in Europe, the essential oils of rosemary from France have α-pinene chemotype [51] while those from Northeast of Spain are characterized by a codominance of camphor and α-pinene [52].
In Iran, rosemary presents two chemotypes. The first is dominated by α-pinene (43.9 - 46.1%) [53]; the second is characterized by the abundance of piperitone (23.7%) followed by α-pinene and linalool, while the 1,8-cineole and camphor are less abundant compared to our results, they do not exceed 7.43% and 4.97%, respectively [54].
In South Africa, verbinone is the major component (17.43%) of rosemary; other components are camphor (16.57%), 1,8-cineole (11.91%) and α-pinene (11.47%) [55].
This variation in the compositions of essential oils could be related to the environmental factors (temperature, day length, nutrients), geographical origin, drying [43, 56], the vegetative stage of the plant [57], the level and time of cutting, the time of harvest [42, 58], extraction method.

3.2. Antifungal activity of essential oils

The essential oils of *M. communis* and *R. officinalis* exhibited inhibitory activity *in vitro* on apple rot fungi (Fig.1). The essential oils of myrtle has shown to be the most active; it has achieved complete inhibition of mycelial growth of *B. cinerea, A. alternata* and *P. expansum* at concentrations above 400, 600 and 1800 µl/l, respectively. While rosemary extract presented a lower activity; indeed, a concentration of 1800 µl/l was necessary for obtaining 89% growth inhibition of *P. expansum*, whereas *A. alternata* and *B. cinerea* required concentrations of 800 and 1200 µl/l to obtain a total inhibition of its growth. It was *P. expansum*, which exhibited the strongest resistance to the inhibitory effect of the two essential oils tested; it was followed by *A. alternata* that showed intermediate resistance, finally *B. cinerea* was the most sensitive (Fig.1, Fig.2).
These results confirm other studies that have highlighted the antimicrobial activity of essential oils of myrtle and rosemary [39; 54; 59 - 61].
Wilson et *al.* [62] obtained an inhibition of spore germination of *B. cinerea* by essential oils of *M. communis* and *R. officinalis* at concentrations higher than 50%.
Curini *et al*. [51] reported that the essential oils of myrtle slightly inhibits the growth of *Rhizoctonia solani*, *Fusarium solani* and *Colletotrichum lindemuthianum*. The percentages of inhibition of these molds at concentration of 1600 ppm are respectively 60, 15.59 and 21.41%
Soylu and coworkers [63] showed that the volatile fraction of rosemary essential oils has a fungicidal effect on *B. cinerea* at concentrations > 25.5 µg/ml; they also highlighted the effectiveness of the volatile fraction of the essential oils compared with direct contact. According to Bomfim *et al*. [47], the activity of the essential oils of rosemary on *Fusarium verticillioides* is due to its influence on the integrity and rigidity of the cell membrane, causing a blockage of cell growth.

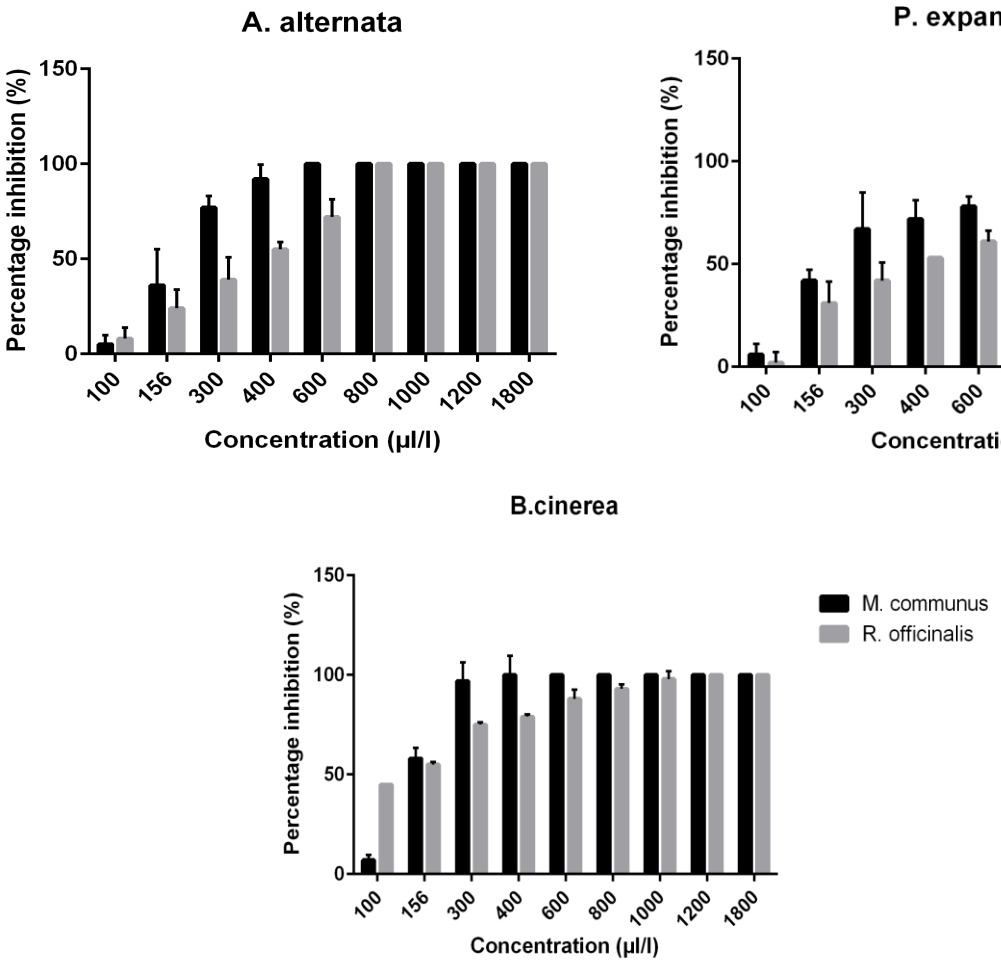

Figure 1: The effects of different concentration of volatile phse of essential oils of *M. communus* and *R. officinalis*

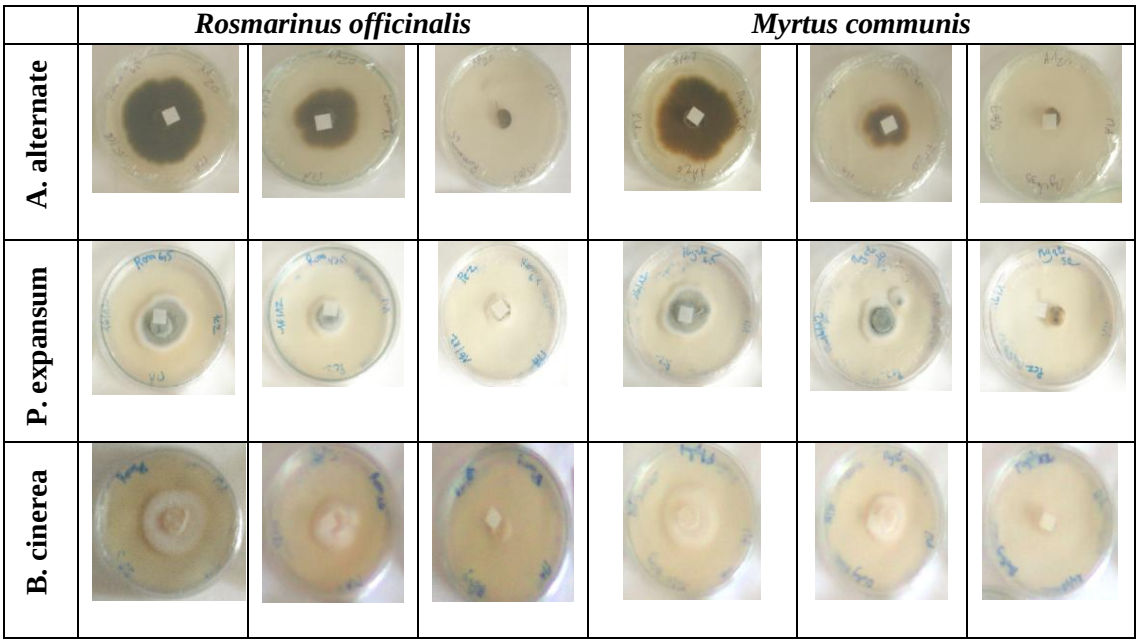

Figure 2: Growth of *A. alternate*, *P. expansum* and *B. cinerea* mycelium treated with *Rosmarinus officinalis* and *Myrtus communis* essential oils after 7 days at 25 °C

The antifungal activity of essential oils can be attributed mainly to their major compounds. Although 1,8-cineole is the major constituent of both essential oils, myrtle was more active than the rosemary; indeed, Vilela et *al.* [64] showed that 1,8-cineole alone has a low antifungal power, hence the importance of the synergistic effect of other components in the antifungal activity, especially that of α-pinene who has several biological activities. Mansouri *et al.* [65 - 66] have shown the strong antifungal activity of essential oils of *Juniperus oxycedrus* and *Juniperus phoenicea* rich in α-pinene, against *P. expansum*. Wilson *et al.* [62] tested the antifungal activity of 49 essential oils against *B. cinerea*; the most common constituents in essential oils having a high antifungal activity were the cineole, α-pinene, *d*-limonene, β-myrcene, β-pinene and camphor. Limonene and α-terpineol have also an antifungal power [67 - 68].

Conclusion

The essential oils of *M. communis* and *R. officinalis* are rich in constituents. 1,8-cineole is the major compound of the two essential oils with concentrations of 37.0% and 43.16% for the myrtle and rosemary respectively. The volatile fractions of these two oils showed an antifungal activity against *A. alternata*, *B. cinerea* and *P. expansum*. The essential oils of *M. communis* was the most active, while *P. expansum* was found to be the most resistant fungus. These results are the first step for the development of a biological control method based on essential oils; they must be supplemented by *in vivo* tests.

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