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Chemical Composition and Antibacterial Activity of two Essential Oils of rosemary Against *Erwinia*amylovora, the causal agent fire blight

L. Doukkali [1], A. Tahiri [2], B. Tazi [3], F. Guenoun [1]

[1]*Laboratoryof Biactives,molecules and environment, Université Moulay Ismail Faculté des Sciences, B.P 11201–, Meknes, Morocco*

[2] *Laboratory of Phytopathology, Department of the Protection of the Plants &Environment; Ecole Nationale d'Agriculture, BP S/40 Meknès, Morocco.*

[3]*Laboratory of Chemistry, Department of the Basic Sciences; Ecole Nationaled'Agriculture, BP S/40 Meknès, Morocco*

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elaazizadoukkali@yahoo.fr ; Phone:+2120644221175;

Abstract

To deal with resistance and toxicity problems caused by the massive use of chemical synthetic substances, and with the fact that regulation organizations are questioning about the use of antibiotics in agriculture, it is particularly urgent to find alternatives to provide efficient protection of crops against plant diseases. Therefore, this study aimed at evaluating the antibacterial activity of essential oils (EOs) extracted of two species of *Rosmary (Rosmarinus officinalis* and *Rosmarinus eriocalyx)* against *Erwinia amylovora,* the causal agent of fire blight disease. The extraction was carried out by water distillation Clevenger's type apparatus. The yields of extracted EOs are about 1.6% and 2.1% respectively for *R.officinalis and R. eriocalyx.* The chemical compositions of EOs were investigated by using a gas chromatography/mass spectrometry (GC-MS) and showed that EO of *R.officinalis* was characterized by the presence of 1,8-cineole (40,43%), alpha-pinene (8,84%), and camphor (22,23%) as the main chemical components. The EO of *R.eriocalyx* is formed by1,8-cineole (45,01%), alpha-pinene (11,29%) and camphor (18,43%). The in vitro antibacterial activity against *E.amylovora* was evaluated using the method of aromatogram. The results revealed that EO of *R.officinalis* is the most active against *E. amylovora.*

1. Introduction

Fire blight caused by *E.amylovora* (Burrill), is a highly destructive and economically important bacterial disease affecting awide variety of landscape plants in the rosaceous family including apple, pear, pyracantha etc. [1].*E. amylovora*,which is a kind of necrogenic, phytopathogenic gram-negative bacteria, infects the host through natural openings inflowers[2] or wounds occurring in young organs, often following hail storms.

Fire blight is the most important bacterial disease of Maloideae.The major economic impact of the disease has been reported in 40 countries [1]. This bacterium have been reported to be the causative pathogen of early outbreaks in the 20th century in Japan and New Zealand [1]around 1960, and since then the disease has widespread to Europe and countries of the Mediterranean region.

The pathogen infects all parts of plant, including blossoms, fruit, leaves, shoots, limbs, and trunks. The bacteria colonize the intercellular spaces of bark, causing the death of the plant cells associated with distortion of cell walls and the formation of lysogenic cavities. In the susceptible, succulent shoots, the necrosis spreads downwards from the apex with browning of the tissues [3].

Fire blight symptoms result inextended necrosis of infected tissues, as a consequence of the massive colonization of parenchyma intercellular spaces. Insusceptible hosts, bacteria can spread through the apoplast leading to systemic infection. The death of woody tissue leads to the formation of cankers, where bacteria can eventually over winter [4].

The chemical control of *E. amylovora* relies primarily on copper compounds. Antibiotics, such as streptomycin, can also be very effective. However, copper compounds can be toxic to plants at higher doses, and can cause fruit rust that negatively affects fruit finish [5]. Moreover, the intensive and repeated use of antibiotics against the diseaseofthe fire blight might lead to the development of resistant strains [6-7]. Similarly, the current trend of consumers to seek for more natural products prompted the research, development and application of new natural products with antibacterial activities in order to use them as alternative to synthetic products.
Medicinal and aromatic plants (MAP) were traditionally used for flavoring and extending the shelf life of foods[8]. Most of their properties are due to EOs produced by their secondary metabolism [9]. These oils are of growing interest for industries and scientific research because of their antioxidant activity, antibacterial and antifungal properties [10].
EOs extracted of two species from (MAP) of the family of Lamiaceaeare known for their antimicrobial activities [11]. Accordingly, two plants from this botanical family were chosen and used for in vitro antibacterial effect of EOs on *E.amylovora.*These includes EO of both MAP plants *R. officinalis*and *R. eriocalyx.* In this context, our study investigated the first extraction by steam distillation of water from *R. officinalis, R. eriocalyx* and identification of the various components used in their chemical compositions to the study of the *in vitro* antibacterial activity against *E.amylovora*.

2. Materials and Methods

2.1. Plant material

The samples of *R. officinalis* were harvested from the native growing area of Agadir, while those of *R. eriocalyx* were harvested from the area of Tetouan. The obtained biomass of both plants was then subjected to extraction in the purpose of obtaining EO.
EOs extraction was performed by the drive technology to water vapour using a Clevenger-type apparatus, according to the following protocol: The plant biomass was soaked in a flask containing sterile distilled water. The whole was boiled, after the appearance of the first drop of distillate at the exit of the steam condensation tube; EO was then driven by the steam. It is then condensed through a condenser and recovered using a syringe. The time of this extraction is about four hours.Both EOs recovered were dried with anhydrous sodium sulphate and subsequently stored at a temperature of 4°C in amber glass vials sealed to preserve against the air and light.

2.2.Microorganisms

E. amylovora were obtained from the Laboratory of Phytopathology; National School of Agriculture, Meknès, Morocco (ENA-Meknes). The bacterium had been isolated from Levan culture medium. The identity of the bacterium *E. amylovora* was confirmed by PCR assays using the protocol described by Llop et al (1999) [12].
The pathogenicity test had been carried out using the method developed byDoolotkeldieva andBobusheva (2016)[13]. For log-term storage at least 3 months, the strain of 24 hours were subcultured to Levan culture medium and stored at 4 °C.

2.3.Chemical characterization of EOs

The chemical characterization of EOs: *R. officinalis*and *R. Eriocalyx* was performed on a gas chromatograph Varian THERMO ELECTRON: Trace GC Ultra type, equipped with a polar capillary column BP-5 (30 m x 0.25 mm, film thickness 0.25 µm), a FID detector fed with a mixture of H_2/air, and a *split-splitless* injector, The injection mode was split splitess (split ratio: 1/50, flow rate 66 ml/min) and the injected volume was about 1 µl. The carrier gas used was nitrogen with a flow rate of 1 ml/min.The column temperature was programmed from 50 to 200°C at a heating rate of 4°C $min^{-1}$for 5 min. The apparatus was controlled by a computer system.
The chemical identity of the various components was carried out by gas chromatography coupled to mass spectrometry (GC/MS); allowing a qualitative and quantitative determination of compounds there off. The apparatus used is the following Gas chromatograph (Trace GC Ultra) coupled to a mass spectrometer (Polaris Q ion trap MS), ionization was effected by electron impact (70 eV). The column used was DB-5MS (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas is helium whose flow is fixed at 1.5 ml/min. The injection mode was split (split ratio: 1/70, flow rate112 ml/min). The column temperature was programmed from 50 to 200 °C at a heating rate of 4 °C min^{-1} for 5 min. The identification of the components was based on the comparison of their respective mass spectra (GC/MS) with spectra of the library (NIST 98), and on the basis of calculation of Kovats indices.

2.4.Microbiological Procedures

This effect was tested using aromatogram method as described previously by [14-15] with small modifications. Briefly, 15 ml of Levan medium was cooled into Petri dish. After solidification of the culture medium, 100 µl of the bacterial suspension under test (1×10^7 CFU/ml) were plated on the surface and left until total desiccation under aseptic conditions. Sterilized whatman paper discs (6 mm in diam(3 disc/box) were deposited on the dried agar inoculated beforehand with the bacterial suspensions. These paper discs wereloaded with increasing volumes of EOand antibiotic streptomycin as a positive control (2, 6 and 10 µl). There were 3 repetitions for each treatment combination. To allow the diffusion of EO into the culture medium, the Petri dishes are kept at 4 °C for 2 hours. Petri plates free of EOwere used as negative controls. Finally, Petri plates were incubated at 28°C for 72 hours until the growth in the control Petri dishes reaches the edges. The antibacterial activity was determined by measuring diameters of inhibition zones (mm) around the discs.

Sensitivity to oil was rated based on the diameter of inhibition zones according to Ponce and al. (2003) [16]: non sensitive (-) for diameters less than 8 mm; sensitive (+) for diameters from 8 to 14 mm; very sensitive (+ +) for diameters from 15 to 19 mm and extremely sensitive (+ + +) for diameters over 20 mm.

3. Results and discussion

3.1.Yields and Chemical composition of EOs

The yields of extracted EOs are about 1.6 % and 2.1 % respectively for *R. officinalis* and *R. eriocalyx*. Such result demonstrated that our species are highly EOs-rich compared to the same species from other countries such as *R.officinalis* from Algeria (0.8%) [17]. The species from Tunisia yielded 1.17 % for *typicus* breed and 2.7 % for the *troglodytorum* breed [18]. The results also showed that *Eriocalyx* species is very high in EO than *Officinalis* species. Chemical composition of *R. officinalis* and *R. eriocalyx* EOs are described in **Table 1**.

Table1: Chemical composition of EOs of *R.officinalis*and *R.eriocalyx*.

N°	Identified compound	Percentages% *R. officinalis*	Percentages% *R. eriocalyx*	Formula
1	α-pinene	**8.87**	**11.29**	$C_{10}H_{16}$
2	Camphene	4 .40	4.19	$C_{10}H_{16}$
3	β -pinene	5.93	6.08	$C_{10}H_{16}$
4	1,8-Cineole	**40.34**	**45.01**	$C_{10}H_{18}O$
5	δ-2-Carene	0.69	0.51	$C_{10}H_{16}$
6	Camphor	**22.23**	**18.43**	$C_{10}H_{16}O$
7	Borneol	6.95	5.14	$C_{10}H_{18}O$
8	Terpinen-4-ol	1.09	0.81	$C_{10}H_{18}O$
9	Terpineol	3.36	3.19	$C_{10}H_{18}O$
10	Bornylacetate	1.91	0.88	$C_{12}H_{20}O_2$
11	Caryophyllene E	0.89	1.06	$C_{15}H_{24}$
12	Caryophyllene oxide	0.03	0.11	$C_{15}H_{24}O$
13	Spirolepechinene	-	0.39	$C_{15}H_{24}$
	Monoterpenes %	19.89	22.07	
	Oxygenated Monoterpenes%	75.88	73.46	
	Sesquiterpenes%	0.89	1.45	
	Oxygenated Sesquiterpenes%	0.03	0.11	

Chemical analysis identify 12 compounds, representing 96.69 % for *R. officinalis* EO with major compounds: 1,8-cineole (40,43 %); camphor (22.23 %); alpha-pinene (8.87 %); borneol (6.95 %); beta-pinene (5.93 %). Regarding *R. eriocalyx*EO, 13 compounds were identified, representing 97.09 % with the following major

compounds: 1,8-cineole (45.01 %), camphor (18.43 %), alpha-pinene (11.29 %), beta-pinene (6.08 %), borneol (5.14 %) (**Table1**).

We concluded that the 1,8-cineole was the major constituent of both EOs. Therefore, both species were considered 1,8-cineole chemotype. Both EOs were appeared to be qualitatively similar, but were quantitatively different. We noticed also that the oxygenated monoterpenes fraction was the most abundant with 73.97 % and 72.58 % for *R. officinalis*and *R. eriocalyx* respectively.

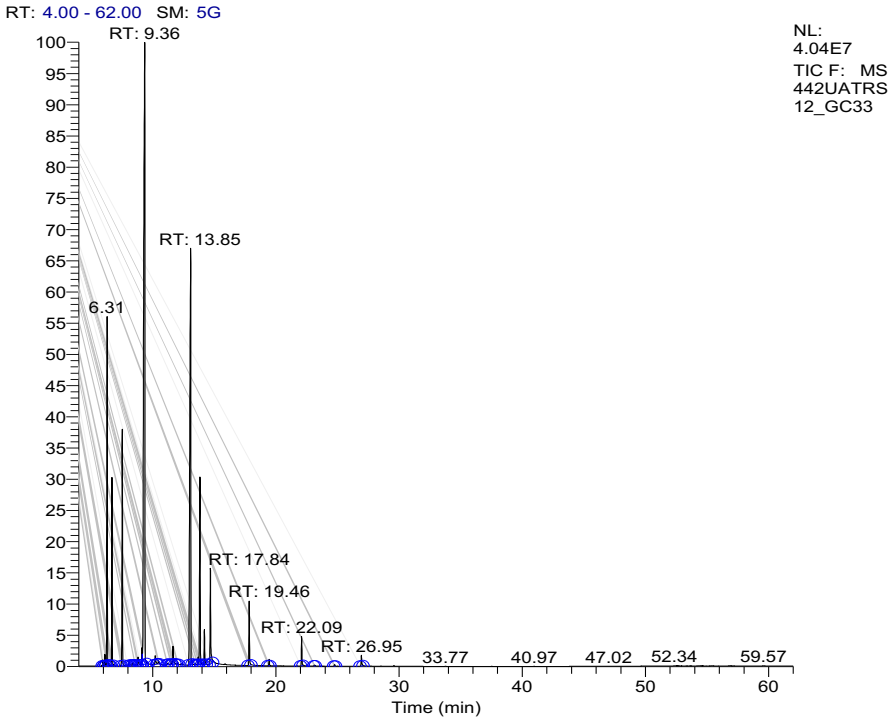

Figure1: Rosmarinusofficinalis' essential oil chromatogram

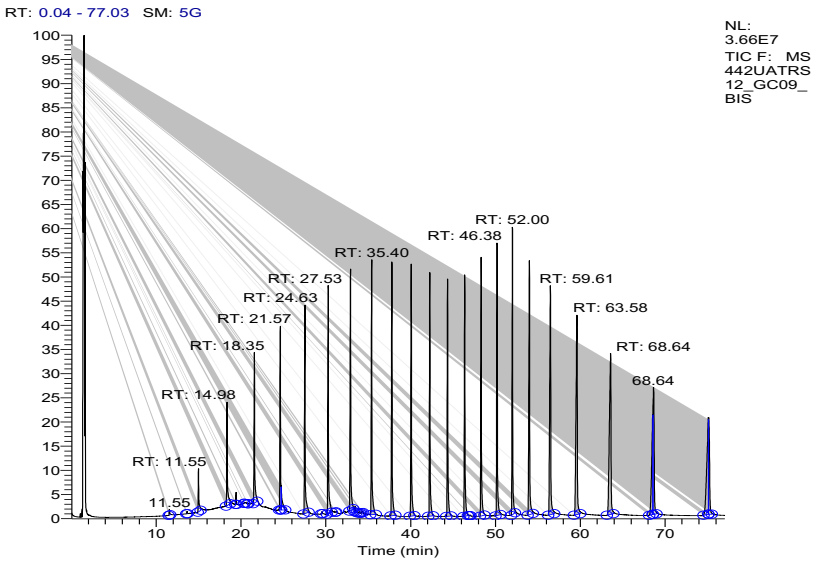

Figure2: Rosmarinuseriocalyx' essential oil chromatogram

Our results showed substantial quantitative differences in the chemical composition of these both medicinal plants when comparing to the available datasets on the same plant species.

Comparing our results with those of other researchers, we found that Rosemary's OE from Errachidia, Morocco is characterized by 1,8-cineole (42 %), camphor (13.99 %), α-pinene (11.92 %), borneol (3.57%), and β-pinene (7.71 %) [19], whereas the sample from Tunisia is mainly composed of 1.8-cineole (40%), camphor (17.9%)

and α-pinene (10.3%) [18]. In addition, Rosemary from North East of Spain presents an EO containing camphor and α-pinene as main constituents [20], while Rosemary harvested in Portugal is rich in myrcene (25%), 1,8-cineole, and camphor [21]. Furthermore, EO of Lebanese Rosemary is characterized by 1,8-cineole (20%) and α-pinene (18.8 – 38.5%) [8]. The major compounds of *R.officinalis* EO from Eastern Cape Province in South Africa are : verbenone (17.43%), camphor (16.57%), 1,8-cineole (11.91%), α-pinene (11.47%), borneol (5.74%) and camphene (5.70%) [22]. However, the chemical composition obtained from the EO *R. eriocalyx* wasapproximately the same as that reportedby Fadili et al (2014) [23] and in which the EO composition of *R. eriocalyx* was largely dominated by 1,8-cineole (45.01%); camphor (18.74%); α-pinene (11.29%); β-pinene (6.08%) and borneol (5.14%). Many factors can affect yield and chemical composition of EOs such as drying, harvesting period, harvesting region, extraction technique, and the developmental stage of the plant [24-25].

3.2.Antibacterial Activity of Essential Oils

The table 2 showed the result of antibacterial activity of EOs*R. officinalis*and *R. eriocalyx* on *E.amylovora.* This antibacterial activity increased with increasing volume of EO. Indeed, it was observed that the zones of inhibition were greater with a greater volume of EO. This is may due to the presence of more active compounds in a higher volume of EO.

This antibacterial activitycan be attributed ofthe antibacterial properties of some compounds such as: 1.8-cineole [26], the camphor [27-28], α-pinene [29-30], borneol [27].

For disc-diffusion method,the obtained results underlined that EO of *R. officinalis* displays a higher antibacterial activity compared to *R. eriocalyx*. However, inhibition zone diameter induced by *R. officinalis* and *R. eriocalyx* essential oils against *E. amylovora* was lower relative to that of streptomycin.

Our results have some similarities with those of Kokošková and Pavela (2007) [31] since they found that *R.officinalis*is active against *E.amylovora*. But, differed from those obtained by Yakoubi et al (2014)[15], who studied the antibacterial activity of *R.officinalis* and found inhibition zone diameters lesser than 5 mm.

Table 2: Diameters of the inhibition halos (mm) produced by the two EOs against *E.amylovora*.

	Treatments	negative control	2 µl	6 µl	10 µl
E. amylovora	*R. officinalis*	0	8 (-)	12 (+)	20(+++)
	R. eriocalyx	0	8 (-)	10 (+)	16 (++)
	Streptomycin	0	20(+++)	27(+++)	29(+++)

(-): inactive
(+): active
(++): very active
(+ + +): extremely active

Furthermore, both essential oils are known to be very rich in oxygenated monoterpenes and monoterpenes, which are well well-recognized for their antimicrobial activities[32-33] .These results demonstrated that EOs of the investigated species can be used as a natural potential antimicrobial agent in preventing and treatment of fire blight disease.

Conclusion

In this work, chemical composition of EOs from two *Rosmarinus* species and their subsequent antibacterial activities against *E. amylovora* were investigated. Qualitative and quantitative analyses of both EOs revealed 12 and 13 compounds for *R. officinalis*and *R.eriocalyx*respectively. Both species are considered 1,8-cineole chemotypes.

The results of biological antibacterial assay showed *in vitro* efficiency of *R. officinalis*and *R. eriocalyx*in suppressing *E. amylovora* with higher antimicrobial activity for *R. officinalis.* Therefore, the results presented in this study might contribute to the good knowledge of antibacterial potential of these species and the possibility

of using them further in integrated control strategy against this devastating bacterial disease. Moreover, other studies involving the use of plant extracts of these species in controlling this disease are needed in order to compare their efficiency to that obtained with their EOs.

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