Nat. Volatiles & Essent. Oils, 2021; 8(3): 44-55
DOI: 10.37929/nveo.910331

NVEO
Natural Volatiles & Essential Oils

RESEARCH ARTICLE

Essential oil of *Rosmarinus officinalis* L. from West Highlands of Algeria: Chemical characterization and *in vitro* antifungal activity against *Fusarium oxysporum* f. sp. *albedinis*

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Submitted: 05.04.2021; Accepted: 14.07.2021

Abstract

Rosmarinus officinalis is a well-studied species; however, *R. officinalis* essential oil (EO) from West highlands of Algeria was not investigated chemically and biologically. In this context, chemical composition of *R. officinalis* EOs obtained from leaves and stem were determined by GC/MS analysis and their antifungal activity against *Fusarium oxysporum* f. sp. *albedinis* (Foa) were evaluated. The GC/MS analysis indicated that monoterpenes were the dominant class of compounds in both leaves and stem (oxygenated 84.9%, 52.4%) and hydrocarbons (12.2%, 8.4 %), respectively. Among them, 1,8-cineole was the main component (leaves: 54.4%, stem: 29.7%), which classifies these EOs as 1,8-cineole chemotype. The *in vitro* antifungal activity of Eos was evaluated through micro-atmosphere and direct contact methods. Best inhibitory activity against Foa was determined after 7-day incubation using direct contact method by relative growth reduction (RGR= 0.398; RGR=0.383) with EOs from leaves and stem, respectively.

Keywords: Essential oil, *Rosmarinus officinalis*, antifungal, fusariosis, GC-MS analysis

Introduction

The worldwide interest in the use of medicinal plants has been growing, and its beneficial effects being rediscovered for the development of new drugs (Andrade, et al., 2018). Essential oils (EOs) are known to have various bioactivities (antibacterial, antifungal etc.). Consequently, studies on their biological activities have become important (Shaaban, et al., 2012).

Rosmarinus officinalis species (syn.: *Salvia rosmarinus* Schleid, *R. angustifolius* Mill., *R. communis* Noronha) from Lamiaceae family, commonly known as 'Lazir' is an evergreen plant typical of the Mediterranean region. It is widely used in the Algerian ethnopharmacopoeia as a spice and for the treatment of various diseases such as digestive troubles, gallbladder disorders, cephalic pains, headaches, migraines, colic, diarrhoea, cough and broncho-pulmonary infections (Cheriti, et al., 1995; Cheriti, 2000).

Despite the huge number of studies on *R. officinalis*, the most focused on phytochemistry then on biological activities. The studied biological activities were interested principally to human health (antimicrobial, insecticidal, antioxidant, aromatherapy) (Andrade, et al., 2018; Durak, et al., 2016; Mekonnen, et al., 2016; Isikber, et al., 2006); less was concerned by *Fusarium oxysporum* (phytopathogen) (Mekonnen and Manahile, 2017; Ozkan and Chalchat, 2008). *R. officinalis* EOs from Algeria were found to be rich in 1,8-cineole and camphor. In addition, these EOs presented many biological activities such as antibacterial effect against human pathogenic bacteria (Boutekedjiret, et al., 1998; Djeddi et, al., 2007; Boutabia, et al., 2016).

Fusarium oxysporum f. sp. *albedinis* (Foa) is the causal agent of lethal disease of date palm called Bayoud. We have studied Bayoud disease for many years (Boulenouar, et al., 2009; Boulenouar, et al., 2011; Boulenouar, et al., 2012; Boulenouar, et al., 2014; Belhi, et al., 2020; Ghazi, et al., 2020); but till now there

DOI: 10.37929/nveo.910331

is no efficient treatment. The aim of this study is the investigation of antifungal effect of *R. officinalis* EO on phytopathogen fungus Foa. To the best of our knowledge, this is the first time that EO of *R. officinalis* from Algerian West highlands was investigated on phytochemical level and as potential antifungal agent against Foa.

Materials and Methods

Plant material and essential oil extraction

Aerial parts of *R. officinalis* were collected from West highlands (El-Bayadh, Algeria, Latitude: 33° 40′ 49″ N; Longitude: 1° 01′ 13″ E; Altitude: 1313m) during March 2018. The species was identified, and a voucher specimen is kept at Phytochemistry and Organic Synthesis Laboratory under N° CA99/11. The leaves and stem were separated, washed, dried in shade, then grinded until obtaining a fine homogenous powder.

The *R. officinalis* essential oil was obtained from dry plant material (100 g) by hydrodistillation using a Clevenger apparatus for 3 h, in accordance with the 3rd Edition of the European Pharmacopoeia cited by Bruneton (1999). The process was repeated five times to get a sufficient amount of EO for antifungal tests and chemical analysis. The obtained oil was dried over anhydrous sodium sulphate and stored in hermetically closed small vials at 4°C until use.

GC-MS analysis

Gas chromatographic (GC) analysis was performed on a Perkin Elmer Clarus 680 gas chromatograph equipped with an FID and fitted with a fused-silica Rtx-5MS capillary column (30 m x 0.25 mm, ID 0.25 µm film thickness). The analytical conditions were: Carrier gas was He (1.0 mL/min), injector and detector temperature were 280°C. The temperature program used was 4 min isothermal at 70 °C, increased to 180 °C at a rate of 4 °C/min, then increased to 240 °C at a rate of 10 °C/min and ending with a 10 min at 300°C. Samples were injected by splitting and the split ratio 1:5. The relative amounts of the individual components found in the oil are based on the GC peak areas obtained (FID response).

The GC/MS analysis was performed on Perkin Elmer Clarus 680 gas chromatograph, interfaced with Clarus SQ 8T mass spectrometer, operating at electron impact of 70 eV with an ion source temperature at 250°C, scan mass range of 30-300 m/z at a sampling rate of 0.5 scan/s. A fused-silica Rtx-5MS capillary column (30 m x 0.25 mm, ID 0.25 µm film thickness was used under the same conditions as those used for gas chromatography analysis as described above. The EOs component identification from the GC/MS spectra was confirmed by comparison of mass spectral fragmentation patterns with the computer library (NIST MS Library), and verified by comparison of their retention indices, determined relatively to the retention times of a *n*-alkanes homologous series (C4–C40) of the identified compounds with literature (Adams, 2007; Babushok, et al., 2011; Benabed, et al., 2017; Boukhobza, et al., 2020).

Antifungal test

Fungal strain

The fungal strain used in this study is *Fusarium oxysporum* f. sp. *albedinis*. It was obtained from the Technical Institute for Development of Saharan Agronomy (TIDSA), Adrar, Algeria. It was identified and a voucher specimen was stored at Phytochemistry and Organic Synthesis Laboratory under N° Foa-POSL/2011/01. Preparation of Foa culture was realized as described by Boulenouar et al. (2012).

DOI: 10.37929/nveo.910331

Procedure

Micro-atmosphere method. A 10mm diameter mycelial disc taken from 7 days culture of Foa was deposited upside-down in the center of PDA medium. Four volumes of EO (10, 20, 30, 40µL) were spread on the lids of the Petri dishes (85mm diameter). PDA inoculated with Foa and without EO was used as a negative control. The observation of the results was carried out after incubation for 7 days and 10 days at 25±1 °C. The tests were performed with three repetitions (n=3) (Stupar et al., 2014).

Direct contact method. Four volumes of the EO (10, 20, 30, 40µL) were spread on PDA medium (85mm diameter Petri dishes). A 10mm diameter mycelial disc taken from 7 days Foa culture was placed upside-down in the center of the PDA medium. The negative control was performed with PDA medium inoculated with Foa and without EO. The observation of the results was carried out after incubation for 7 days and 10 days at 25±1 °C. The tests were performed with three repetitions (*n*= 3) (Ozkan and Chalchat, 2008).

Evaluation of antifungal activity

Antifungal activity was evaluated using: growth rate (GR), percentage of growth inhibition (Inhib%) and relative growth reduction (RGR). GR represents the speed of mycelium growth as millimeter per day (mm/day) (Kibar and Piksen, 2011). Inhib% was calculated using the following formula: Inhib% = [(DC-DT)/DC] × 100. Where DC and DT are the average diameters (mm) of fungal growth from control and treatment samples, respectively (Ozkan and Chalchat, 2008). RGR (%.mm^{-1}.μL^{-1}) evaluation takes in consideration the size of inoculums and quantity of analysed substance in the antifungal activity. It was calculated using the following formula: RGR = Inhib% / (In × V). Where: "Inhib%" is the percentage of growth inhibition (%) calculated as cited above, "In" is the diameter (mm) of mycelial inoculum from fungi culture, "V" is the volume (µL) of EO used in treatment. No substance has been reported effective on *Fusarium oxysporum* f. sp. *albedinis* to be used as positive control. The negative control was test passed all protocol without using essential oils.

Experimental design and data analysis

The experimental design used in this study was factorial experiment. All experiments were conducted in triplicate. The significance of activities had been analysed with ANOVA test. Correlation between different factors was tested. The probability "*P* value" less than 0.05 was considered significant (α= 5%).

Results and Discussion

Chemical composition of *R. officinalis* EO

The green yellowish EO yield from leaves was significantly higher (2.17±0.02 %) than from stem (0.98±0.01 %), which is in accordance with the limits cited by Jawad et al. (2018). The extraction yield can be affected by many factors (seasonal and geographic conditions, distillation technique, harvest period…) (Rao, et al., 2014; Singh, et al., 2014; Kumar, et al., 2016). A total of 21 and 19 compounds representing 98.8% and 68.3% of the total EO were identified in leaves and stem, respectively. The chemical composition of the EOs were presented in Table 1. The components were listed in order of their elution on the Rtx-5MS capillary column.

The oxygenated monoterpenes were the dominant class of compounds in both leaves and stem (84.9%, 52.4%), respectively. The 1,8-cineole (54.4%, 29.7%) was the main constituent, followed by camphor (10.1%, 7.9%), α-terpineol (7.6%, 5.6%) and borneol (5.5%, 4.0%), respectively.

The EOs of *R. officinalis* were found to contain six monoterpenes hydrocarbons (leaves: 12.2%, stem: 8.4 %), of which α-pinene (leaves: 5.5%, stem: 4.0%) was the most represented component. In addition, EOs from both parts contains lower amounts of sesquiterpene (leaves: 1.4%, stem: 7.4%). When compared with leaves EO, higher amount of caryophyllene oxide (5.4%) was measured in the stem EO. It's probably due to change of cytological, biochemical and physiological activities within organs (Zaouali, et al., 2013), and the importance of leaves as a centre of volatile compounds production in the plant (Boix, et al., 2011). In accordance with the richness of leaves in terms of volatile compounds, in this study, yield of leaves EO (98.8%) was detected higher than that of stem EO (68.3%) (Boix, et al., 2011).

Table 1. Chemical composition of the *R. officinalis* essential oil

N°	Compounds	RI[a]	RI[b]	Content (%)	
				Leaves	Stem
1	α-Pinene	931	932	5.5	4.0
2	Camphene	946	946	2.6	1.8
3	β-Pinene	976	974	3.1	2.0
4	α-Terpinene	1016	1014	0.3	tr[c]
5	1,8-Cineole	1030	1026	54.4	29.7
6	γ-Terpinene	1059	1054	0.5	0.4
7	Sabinene hydrate	1068	1065	0.1	-
8	α-Terpinolene	1089	1086	0.2	0.2
9	Linalool	1103	1095	4.0	2.8
10	Camphor	1144	1141	10.1	7.9
11	Borneol	1170	1165	5.5	4.0
12	Terpinen-4-ol	1180	1174	1.6	1.3
13	α-Terpineol	1193	1186	7.6	5.6
14	Verbenone	1212	1204	0.5	0.4
15	Bornyl acetate	1289	1284	1.2	0.8
16	Carvacrol	1306	1298	0.1	0.1
17	Methyl eugenol	1405	1403	0.1	-
18	β-Caryophyllene	1421	1417	0.8	1.9
19	(*E*)-Geranylacetone	1453	1451	0.1	0.1
20	α-Humulene	1455	1454	0.2	0.1
21	Caryophyllene oxide	1589	1583	0.5	5.4
			Monoterpene hydrocarbons	12.2	8.4
			Oxygenated monoterpenes	84.9	52.4
			Sesquiterpene hydrocarbons	1.0	2.0
			Oxygenated sesquiterpenes	0.5	5.4
			Others	0.2	0.2
			Total	**98.8**	**68.3**

[a]Retention indices on Rtx-5MS column. [b]Retention Indices obtained from literature (Adams 2007, Babushok et al. 2011). [c]Trace amount < 0.01

The qualitative composition of EO from both parts was similar, but a marked quantitative difference was observed, which may be attributed to the growth phase of the plant, as it's well known that chemical variability may be related with different vegetative phases of the plant (Barra, 2009). Many studies have

been carried out on the chemical composition of different samples of *R. officinalis* from different Mediterranean geographical regions revealing that chemical composition and percentage vary depending upon the plant parts, vegetative phases, extraction methods and environmental and growing conditions (e.g. seasonal and geographical variations, soil composition) (Carvalho, et al., 2005; Figueiredo, et al., 2008; Zaouali, et al., 2013; Andrade, et al., 2018).

According to previous studies, 1,8-cineole ranging from 57.7 to 11.0%, camphor from 36.7 to 7.9% and α-pinene from 24.7 to 5,7%, are the most represented components in all samples of *R. officinalis* EO from Mediterranean region : Algeria (Fellah, et al., 2018), Egypt (Fadel & El-Massry, 2000), France (Chalchat, et al., 1993), Italy (Napoli, et al., 2010; Serralutzu, et al., 2020), Lebanon (Diab, et al., 2002), Morocco (Chalchat, et al., 1993; Rahmouni, et al., 2019), Portugal (Mata, et al., 2007), Spain (Chalchat, et al., 1993; Salido, et al., 2003), Tunisia (Hcini, et al., 2013; Zaouali, et al., 2013) and Turkey (Celiktas, et al., 2007; Ozcan & Chalchat. 2008).

The EO extracted from *R. officinalis* collected from El-Bayadh (Algerian West highlands) was characterized by high content of 1,8-cineole, which classifies it as 1,8-cineole chemotype according to the classification of Napoli et al. (2010). It has been reported that altitude affects the chemical composition of EOs in many plant species (Barra, 1990). Concerning *R. officinalis* EOs, Sabbahi et al. (2020) has demonstrated that only the major constituent (1,8-cineole) has a significant relationship with altitude. However, the effect of endogenous and exogenous factors on secondary metabolites biosynthesis –at the same time- makes the evaluation of altitude effect difficult.

Evaluation of antifungal activity

Among plant extracts, essential oils are the most difficult to be analyzed for antimicrobial activity, because of their limited yield, less-stability, less-solubility in media and their complex composition (Lahlou, 2004). Antimicrobial activities reported in the literature have been evaluated with diverse sets of methodologies, degrees of sensitivity, amount of test-compounds and microbial strains, often difficult to compare (Valgas, et al., 2007). Many laboratories have modified antimicrobial evaluation methods for specific samples, such as essential oils and non–polar extracts and these modifications became impossible to directly compare results (Scorzoni, et al., 2007).

It's well known that inoculum size and antimicrobial quantity influence the efficacy of antimicrobials (Cerero, et al., 2010; Xie, et al., 2017). The effect of inoculum density is observed to be strain dependent (Bedenic, et al., 2001). It has been concluded that the extent of antifungal effect varied depending on the levels of EO used in the experiment (Ozkan & Chalchat, 2008). Thus, the use of RGR principle in relation to Inhib%, mycelia inoculum and EO volume gives more opportunity to compare EO effect on filamentous fungi.

The principle of radial growth calculation is based on the diameter of control growth covering the Petri dish, in our case after 10 days. However, we preferred to evaluate the effect after 7-day incubation as well, to calculate the progression of antifungal effect through two periods of time.

Antifungal activity of *R. officinalis* EO on Foa

The date palm fusariosis caused by Foa presents a serious problem for desertic regions, especially in Algeria and Morocco. The development of efficient treatment is more than necessary to protect the oases.

DOI: 10.37929/nveo.910331

Table 2. Effect of essential oils from *Rosmarinus officinalis* L. on *Fusarium oxysporum* f. sp. *albedinis* expressed as GR, Inhib% and RGR using the micro-atmosphere and direct contact methods.

Method	EO[a] part	Incubation (days)	EO volume (µL)	GR[b] (mm/day)	Inhib %[c]	RGR[d] (%.mm^{-1}.µL^{-1})
Micro-atmosphere	Leaves	7	10	8.57±0.17	6.61±1.80	0.066
			20	7.95±0.05	13.36±0.52	0.067
			30	7.19±0.13	21.66±1.37	0.072
			40	6.90±0.13	24.77±1.37	0.062
		10	10	7.90±0.06	6.51±0.68	0.065
			20	7.63±0.09	9.66±1.04	0.048
			30	7.30±0.12	13.61±1.37	0.045
			40	7.20±0.12	14.79±1.37	0.037
	Stem	7	10	8.62±0.13	6.09±1.37	0.061
			20	8.05±0.05	12.32±0.52	0.062
			30	8.00±0.08	12.84±0.90	0.043
			40	7.28±0.08	20.62±0.90	0.052
		10	10	7.80±0.06	7.69±0.69	0.077
			20	7.70±0.06	8.88±0.69	0.044
			30	7.60±0.06	10.06±0.69	0.034
			40	7.37±0.09	12.82±1.04	0.032
Direct contact	Leaves	7	10	5.52±0.17	39.82±1.87	0.398
			20	4.76±0.17	48.12±1.87	0.241
			30	3.28±0.38	64.20±4.11	0.214
			40	3.19±0.13	65.24±1.38	0.163
		10	10	6.30±0.06	25.44±0.68	0.254
			20	5.57±0.12	34.12±1.42	0.169
			30	4.93±0.20	41.62±2.40	0.139
			40	4.37±0.18	48.32±2.09	0.121
	Stem	7	10	5.67±0.17	38.26±1.87	0.383
			20	3.95±0.42	56.95±4.61	0.285
			30	4.14±0.16	54.87±1.80	0.183
			40	3.19±0.37	65.24±4.05	0.163
		10	10	6.40±0.06	24.26±0.68	0.243
			20	5.63±0.12	33.33±1.42	0.167
			30	4.87±0.24	42.41±2.84	0.141
			40	4.00±0.36	52.66±4.27	0.132

[a]Essential oil, [b]Growth rate, [c]Percentage of growth inhibition (Inhib % values were calculated referred to radial growth of negative control. The radial growth of negative control was: 64.25±0.75mm (7 days of incubation); 84.50±0.29mm (10 days of incubation), [d]Relative growth reduction.

Best inhibitory activity of Foa was observed for 10µL of EOs after 7 days incubation using direct contact method (leaves: RGR=0.398; stem: RGR=0.383). The comparison of Inhib% values between the two periods

DOI: 10.37929/nveo.910331

shows a significant decrease from 7 to 10 days ($p<0.05$). This effect may be explained by the development of resistance mechanism through production of metabolites or enzymes by the fungus to detoxify the antifungal compounds in EO. Ozcan and Chalchat (2008) showed that EO from *R. officinalis* leaves inhibits *F. oxysporum* after 7 days but no significant inhibition was observed after 10 days. When we link this result with the EO quantity, the increase of EO volume in contact with Foa gave more brake to Foa growth. Farooq et al. (2002) present this phenomenon of detoxification by plant pathogenic fungus. (Table 2)

The antifungal activity of our essential oil might be related to their monoterpenes components which constitute dominant class of compounds in both leaves and stem, with oxygenated monoterpenes (84.9%, 52.4%) and monoterpenes hydrocarbons (12.2%, 8.4%) respectively. Besides a variety of biological activities of monoterpenes, EOs containing high amounts of oxygenated monoterpenes have also been reported to be important antifungal agents (Burt, 2004; Farooq, et al., 2002; Dias, et al., 2017; Danielli, et al., 2019). In addition, it is possible that antifungal activity of *R. officinalis* EO is due to cell membrane disruption by lipophilic compounds (Cowan, 1999).

Inhibition activity can be related to presence of aromatic ring and OH group (present in the minor compounds such as carvacrol and methyl eugenol) that is known to be reactive forming hydrogen bonds with enzymes causing their inhibition (Velluti, et al., 2003). Thus, antifungal activity may be related to these minor constituents. The synergism between EO constituents is among probable antifungal effect observed in this study.

Lee et al. (2007) demonstrated that *F. oxysporum* is inhibited by commercial EOs with Inhib% values 57 to 76% using micro-atmosphere principle. However, the inoculum size was presented as plugs without specification of diameter. Therefore, it is not possible to compare our results with this study.

Analysis of variance in micro-atmosphere method revealed that effects of all factors on GR and RGR are significant ($p<0.05$). For GR values, the highest antifungal effect was observed for 7 days of incubation, leaves and EO volume equal to 40µL (6.90±0.13mm/day). For RGR values, maximum inhibitory activity was observed with stem EO 10µL (RGR=0.077).

The link between EO amount used and antifungal activity can be explained through two principles. First, for GR and Inhib%, the increase in volume permit the increase in EO components responsible for antifungal effect, so 40µL is more efficient than 10µL. Second, for RGR, the increase of volume is not related directly to antifungal effect but other parameter is engaged which is fungus quantity (culture diameter). On the other hand, decrease of effect between 7 and 10 days reflects a detoxification phenomenon as explained previously.

The highest effects were observed for direct contact technique. This may be due to the fact that micro-atmosphere technique permits to only highly volatile compounds to act on Foa (Stupar, et al., 2014). Thus, the antifungal effect using this method is underestimated. However, the micro-atmosphere method has a positive point related to low contamination risk because there is no direct contact between EO and medium.

The results presented in (Table 2) showed a strong correlation among couples RGR/method (r=0.80), Inhib%/method (r=0.86), GR/method (r=-0.88) and Inhib%/GR (r=-0.99). A moderate correlation was observed among couples RGR/Inhib% (r=0.62) and RGR/GR (r=-0.60). No correlation was observed among others.

The effect of part used (leaves, stem) was more significant in direct contact method than micro-atmosphere one ($p<0.05$). This may be due to the specificity of direct contact method which facilitates the contact between EOs components and Foa.

If we use only Inhib% values, the best effect was observed for 40 µL EO from stem and leaves using direct contact method after 7-day incubation (65.24% for both). However, using RGR values, the efficient effect was observed for 10µL EO from leaves and stem using direct contact method after 7-day incubation (0.398 and 0.383, respectively). Among the problems related to antifungal effect evaluation is the high assay dosage that may lead to overestimation (Scorzoni, et al., 2007). Thus, the evaluation of antifungal effect without referring to dose used is not sufficient to talk about efficacy. The RGR values reflect the effectiveness of 1µL of EO in the presence of 1mm of mycelial inoculums. Therefore, RGR is more suitable to reflect the effect of this EO on Foa. (Table 2)

Its common knowledge that *R. officinalis* is rich in natural products with interesting biological activities. This is the first report on *R. officinalis* EO from Algerian West highlands as source of potential antifungal compounds against Foa.

The GC/MS analysis has demonstrated the richness of this EO in monoterpenes in both leaves and stem. Eucalyptol (1,8-Cineole) was the main component which classifies this EO as 1,8-cineole chemotype.

R. officinalis EOs has presented an important antifungal effect against Foa in first stage. Best inhibitory activity against Foa was determined after 7-day incubation using direct contact method (RGR= 0.398; RGR=383) with EOs from leaves and stem, respectively. Antifungal effect is probably due to the major compounds in the EO (oxygenated and hydrocarbons monoterpenes) or to synergism between constituents. However, it seems that Foa has developed a kind of resistance mechanism. This resistance may be related to detoxification phenomenon. RGR evaluation allows focusing on the efficient dose to avoid insignificant higher doses and work on other parameters to increase effect.

To the best of our knowledge, this is the first report of antifungal activity of *R. officinalis* EO (collected from West highlands of Algeria) on the causal agent of Bayoud disease. The obtained results shed the light on the possibility to use *R. officinalis* EO as source of treatment against Foa by proceeding with further advanced studies.

Conclusion

Upcoming new insights may focus on major compounds in this EO, especially 1,8-cineole, to develop efficient treatment. Further *in vivo* experiments are needed to be performed.

ACKNOWLEDGMENT

The authors would like to thank DGRSDT (MESRS, Algeria) for financial support.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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