

INHIBITION OF *FUSARIUM OXYSPORUM* F. SP. *ALBEDINIS* BY ESSENTIAL OILS OF FLOWERS AND STEMS OF *RHANTERIUM ADPRESSUM*

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Abstract

In the present study, we evaluated the antifungal activity of essential oils extracted from the stems and flowers of *Rhanterium adpressum*, a spontaneous plant growing in North Africa. The plant samples were collected from four regions in the Algerian desert. The antifungal activity of the essential oils was tested against *Fusarium oxysporum* f. sp. *albedinis*, the causal agent of the fungal vascular wilt of date palm. The GC/MS analysis allowed the identification of forty-two chemical components in the essential oil samples. The results of the antifungal tests showed an inhibitory effect from the concentrations of 1.8 and 2 µl/ml. The fungicidal concentration corresponds to 10 µl/ml. In order to validate the results of the antifungal activity of the studied essential oils, a bioinformatics simulation was performed to compare the influence of the chemical composition of the oils on the antifungal activity.

Key words: *Rhanterium adpressum*, *Fusarium oxysporum* f. sp. *albedinis*, antifungal activity, bioinformatics simulation, GC/MS analysis.

Introduction

Used by locals as antidiuretic and within tanning, *Rhanterium adpressum* Coss. & Durieu (Asteraceae) is a spontaneous plant; endemic in arid regions of North Africa. It is a subshrub, multicaulis, with alternate, small, whole and toothed leaves. The plant has many right twigs, tight in tufts, scales closely applied in pericline, obtuse, plan receptacle. It flowers from April to June. The Fruit is a narrow cylindrical achene with 4-5 ribs. *Rhanterium adpressum* is found in north algerian desert, starting from Aïn Sefra in the West, going through Mزاب, Laghouat, Bou-Saâda, arriving to Biskra in the East [1, 2]. *Rhanterium adpressum* shows a very interesting biological potential: antimicrobial [3, 4] and antioxidant activities [4, 5]. However, the botanical and ecological characteristics of the plant are still poorly studied, as well as its biological and chemical properties. For over a century, the palm groves of Morocco and Algeria were devastated by an imperfect Ascomycete fungus commonly found in soils, *Fusarium oxysporum* f. sp. *albedinis* (*F.o.a.*). This fungus, which causes a rapid withering of the date palm, is a serious threat to grove palms of North Africa. It is the agent of the *Fusarium* wilt of palm trees, commonly called Bayoud.

The disease affects particularly the best producing varieties of dates. However, its impact goes beyond the economic aspect. Indeed, the date palm occupies a key position in the oasis ecosystem and in the social organization of the peoples of the Saharan regions. All chemical treatment trials have failed because of the deep location of the internal parasite, out of reach of even the systemic fungicides [6, 7, 8, 10].

F. o. a. is on the list of quarantine organisms. Indeed, only the felling of infected palms and their incineration currently helps fighting the spread of infection. Facing the growing threat of the infection, and the degrading health of palm groves, research was undertaken for the development of a method of control using natural product from plants, such as essential oils to uncover the extent of contamination and avoid the felling of palm trees [6,7, 8, 9] Valorization of the antifungal potential of *Rhanterium adpressum* against *F. o. a.* will be the subject of this study. We primarily aimed to quantify and know the whole essential oils components of *Rhanterium adpressum* extracts. Afterwards, we tested the inhibitory power and the antifungal activity of the extracts from this plant, which was not evaluated before.

Materials and Methods

Preparation of samples

Vegetal material

The samples of *Rhanterium adpressum* were collected from four different locations in the Algerian desert:

1. Laghouat, 400 Km south of Algiers (38°48'00.00"N, 2°52'00.00"E);
2. Zelfana, 670 Km south of Algiers (32°23'46.70"N, 4°13'34.40"E);
3. Ouargla, 800 Km south-east of Algiers (31°57'46.72"N, 5°20'31.17"E);
4. El-Goléa, 900 Km south-west of Algiers (30°35'45.99"N, 2°52'54.73"E).

The freshly collected samples were dried at room temperature and away from light and humidity. The plant was then divided into two parts: the flowers and the stems. Each part was manually ground with a mortar.

Preparation of the fungal strain

We isolated *Fusarium oxysporum* f. sp. *albedinis* from a palm infected with Bayoud in the region of Ghardaia (Regional Station of Plant Protection-Ghardaia, South of Algiers). The Isolates were grown on PDA medium for 5-8 days at 25 °C. We then performed a series of successive purifications to obtain a pure mycelial mat. The fungal species was re-identified based on macroscopic characteristics in a solid medium culture, and the mycelial structure by microscopic observation.

Extraction and quantification of essential oils

Hydrodistillation was performed for the extraction of volatile compounds from the dried plant samples using a Clevenger type apparatus. The extraction process lasted 7 hours. We dried the obtained oils with anhydrous sodium sulfate. Afterwards we stored them at +4 °C until use.

The content of the essential oil, in percentage, is defined as the ratio between the mass of the extracted oil and the biomass of the plant. It is calculated by the following formula [11]:

$$T\% = (m_1/m_0) \times 100$$

- m_1 : the essential oil mass in grams;
- m_0 : the mass of the treated plant material in grams;
- T: is the content of EO.

The content of the essential oil, in ml/kg, is defined as the amount of the extracted EO (ml) from a Kilogram of plant. This content is calculated by the formula [12]:

$$T' = v/m$$

- v : volume of the extracted EO (ml);

- m: the mass of plant material subjected to extraction (Kg);
- T': the content in EO expressed in ml/kg.

GC/MS analysis

A Varian CP-3800 gas chromatograph was used to analyse the extracted essential oils. It was equipped with a UB-Wax fused capillary column (60m × 0,32mm, film thickness of 0.25 µm) and flame ionization detector. The oven temperature was programmed from 50 to 250°C at a rate of 3°C/min, then maintained on 250°C for 10 min. The carrier gas was Helium at a flow rate of 1ml/min (constant flow). The retention indices of constituents were calculated relative to C8-C25 alkane standards.

The analysis of the volatile constituents were run on an Agilent HP-6890 coupled to 5973 mass spectrometer. The fused-silica HP-5 MS capillary column (30 m 0.25 m; film thickness 0.25 µm) was directly coupled to the MS, provided with quadrupole detector (70 eV). Helium as carrier gas (1ml/min), column temperature was programmed from 60°C for 2 min, then increased gradually to 125°C for 2 min, and finally carried to 220°C for 2 min. The transfer line and injector temperatures were fixed to 220 and 250°C, respectively. 1µl of diluted oil in ethanol solution (1:100, v/v) was injected manually in a splitless mode.

The identification of the EO components was based on comparison of their RI (calculated in relation with series of linear alkanes) and mass spectra with those of authentic samples and/or the Wiley and NIST libraries spectra.

Antifungal activity assay

1/10th to 1/1500th dilutions of EO were prepared in an agar solution (2%). In tubes each containing 13.5 ml of sterile PDA medium (45°C), we added 1.5 ml of each dilution to obtain final concentrations ranging from 1/100 to 1/150000 (v/v). The tubes were then shaken and poured in petri plates. Witnesses, containing the medium and agar solution without EO, are prepared and used as a control. We inoculated the petri plates by putting an agar disc containing mycelium taken from the periphery of the thallus derived from a *Fusarium* culture of 7 days on PDA. A culture on PDA media without extract served as a control.

We incubated the cultures in the dark at 25 ± 2 °C for 7 days. We determined the antifungal activity by comparing the mycelial growth after treatment, with the mycelial growth of the control, using the following equation [13]:

$$I (\%) = (D_k - D_0 / D_k) \times 100$$

Where:

- D_k: diameter (mm) of the mycelial growth in the witness;
- D₀: diameter of the mycelial growth in experience;
- I: The rate of inhibition of mycelium growth (percentage).

Bioinformatics simulation

We performed the Simulation of the inhibitory activity with SimBiology of Matlab MathWorks. We created a model according to Nielsen et al. [14], modified and arranged with the overall conditions of this study. It is a sigmoid E_{max} PK/PD model, where E_{max} is the maximum effect possible (which refers to the minimal fungicide concentration: MFC). The MIC is the concentration that causes 50% of E_{max} which are related as [15]:

$$\text{Effect} = (E_{\max} \cdot C^{\gamma}) / (\text{MIC}^{\gamma} \cdot C^{\gamma})$$

Where:

- C: the variation of extract concentrations from 0.5 to 10 µl/ml,
- γ: the sigmoidicity factor, which defines the shape of the concentration-effect relationship.

The inhibition effect was included in this model as an additive part to the natural death rate of the fungi. Growth progress curves in the presence of different extracts concentrations were fitted to Boltzmann sigmoid function with Origin 8 Fit Sigmoidal Analysis, with which we can check the model results by obtaining parameters Stats. This model describes the concentration-effect relationship over a range of concentrations. With this model, we can establish and evaluate dose-concentration-response relationship, describe and predict the effect time courses resulting from extract dose.

Results and Discussion

Quantification and identification of extracted essential oils

After the extraction of EO from different samples, quantification of these obtained oils (Table 01) enabled analysis of different yields between regions and extraction parts. More or less close contents between extraction parts (1.5 to 4 ml/kg) were influenced by environmental parameters of the collection regions. The average value of the highest content of EO is that of the region of Zelfana (4.00 ml/kg for the flowers), while this region has the same contents in the stems with the region of El-Goléa to which the contents of Ouargla samples are more closer. Moreover, we found a distinct difference in the content of EO from the stems and flowers for the

sample of Laghouat. The dependence between flowers yields with those of stems has not been reported, so this difference or similarity is highly variable from one species to another [16, 17].

Chemical composition of essential oils of *Rhanterium adpressum* has been determined using gas chromatography coupled to mass spectrometry GC/MS. This analytical method has allowed us to identify a total of forty-two chemical components (Table 2).

The EO Samples are qualitatively close to each other (Table 02), insofar as they are all characterized by the same dominant compounds. Components present in the majority percentages of *Rhanterium adpressum* EO are: α -Pinene (3.99 to 14%), Camphene (from 3.18 to 11.75%), β -Myrcene (3.55 to 13.4%), Linalool (from 3.96 to 11.12%), α -Terpinene (3, 48 to 13.5%), bicyclo-germacrene (1.47 to 6.69%), α -cadinol (1.12 to 7.96%), α -eudesmol (1.02 to 5.78%) and β -Eudesmol (from 3,12 to 7.71%). Other components present in lesser amounts have been identified, namely; β -pinene (1.88 to 3.66%), sabinene (1.62 to 4.7%), limonene (1.47 to 2, 73%), terpinen-4-ol (1.79 to 4.79%), geraniol (1.71 to 4.34%) and Spathulenol (1.01 to 2.06%). Significant recorded levels differ, more or less, with the results of other studies reflecting a remarkable variability influenced by region environment and the season of collection [5, 18, 19].

Overall, we can divide our essential oil to four classes of compounds (Figure 01):

1. Monoterpene hydrocarbons (10.8 to 43.72%) presented the highest percentages;
2. Oxygenated monoterpenes (12.11 to 30.32%) are less represented;
3. The lowest percentages are those of oxygenated sesquiterpenes (5.42 to 19.22%);
4. Sesquiterpene hydrocarbons (from 3.29 to 12.91%).

All of these compounds give this oil a strong odor, and characterized by a pleasant and yellowish-green color. The histogram shows that the samples are qualitatively close, regardless of the sampling site. However, the proportions of dominant components vary considerably from one region to another and from one organ to another for the same region. This variation could be due to exogenous factors such as sunlight (climatic conditions of the sample collection season), photoperiod, temperature, nature and components of the soil, water, altitude, as well as endogenous factors such as the physiology of the plant and the genetic composition of individuals. The attacks of

insects and microorganisms may also influence the composition of EO [20].

Antifungal activity results

From the results (Figure 02), we can notice that *Fusarium oxysporum* f. sp. *albedinis* is sensitive towards *Rhanterium adpressum* EO, for all tested samples. We can also notice that with the increase of the concentration of EO, colony diameter decreases up to a lack of germination disk (MIC). Moreover, the MIC will vary slightly between EO samples especially for stems extracts. They seem to have the same tendency. It is noteworthy, that the essential oil acts on the mycelial growth. Figure 02 clearly elucidates the activity of essential oils at different doses on *Fusarium oxysporum* f. sp. *albedinis*. We noticed that the antifungal potency of the different samples of EO varies little according to the sampling area. The same observation was made for extracts of stems and flowers. The inhibition percentages of our oils vary from 84.5 to 92.4%. We noticed in the case of Laghouat and Zelfana, flowers EO of Zelfana showed low activity compared to those of Ouargla. While the sample of Laghouat showed significant activity despite its chemical components percentages is fairly low compared to other EO samples, a fact which recalls the very important biologic role of minority chemical compounds in essential oils which might act synergistically [21, 22, 23]. However, by comparing the activity of EO of Laghouat and Zelfana, we found that extracts from Laghouat have shown more important activities than those of Zelfana, despite the fact that the chemical components percentages of these are larger than those of Laghouat. The difference in activities between extracts from these two regions and even from the others reveals the presence of chemotypes in populations of *R. adpressum* [24, 25, 26]. Our results of antifungal activity showed a great antifungal power with low inhibitory and fungicide concentrations compared to other studies [27, 28, 29, 30, 31] on the same fungus. These studies tested other chemical components extracts from other plants, essential oils from various plant parts and/or regions. It is important to note that despite the variability in percentage of the chemical compounds and the differences in the samples inhibitory actions, this antifungal activity remains high.

Bioinformatics simulation results

With the results of fungal growth model in the presence of different extracts concentrations, we were able assess several parameters affecting the growth rate, such as the growth constant ($0,23 \text{ h}^{-1}$),

the death constant ($0,088 \text{ h}^{-1}$) and F_{max} ($8 \cdot 10^{14}$ CFU/ml) which is the fungal concentration in the system at stationary phase. Growth simulation Results of *Fusarium oxysporum* f. sp. *albedinis* in the presence of different extracts concentrations validated the results of the antifungal activity (Figure 03). In the case of stems extracts, we can notice a similar inhibitory effects ranging from concentrations of 1.8 to 2 $\mu\text{l/ml}$ for the MIC (reducing the population to $3\text{-}1.5 \cdot 10^7$ according to simulation results) and Rates from 10 $\mu\text{l/ml}$ for MFC for all the samples. In the case of floral extracts (Figure 03), we noticed a difference of inhibition action for the region and for the extracts part. The greatest inhibitions were recorded for the sample from the region of Ouargla, followed by El-Goléa, Laghouat and Zelfana. The interval of the CMI is a little wider ranging from 0.5 to 2 $\mu\text{l/ml}$ (reducing the fungal population to $6\text{-}2 \cdot 10^7$, noticing an accelerated decline starting from the CMI for all regions). Fungicidal concentrations do not differ greatly from CMF of stems extracts.

The significant decrease of the growth of *Fusarium oxysporum* f. sp. *albedinis* with the increase of concentrations added to the culture media is completely dependent (Figure 04). Analysis of this figure confirms the previous results. We also noticed, in the case of the stem samples, a remarkable dependency between samples from all regions, which refers to slight differences previously shown. The significant inhibition of EO flowers samples was confirmed too, especially with the samples of Ouargla and Laghouat. The curves were perfectly fitted with Boltzmann function. Coefficients as R squared and adjusted R2 (0.99 for each) indicate that the regression line fits perfectly the data. This allows us to conclude that the predictive power of the model is strong.

Starting from a concentration of 2 $\mu\text{l/ml}$, it appears that the antifungal activity differs less and less while approaching the concentration of 10 $\mu\text{l/ml}$ in function of time. Indeed, with high concentrations, the fungal population gets closer to a stationary phase where it gradually touches its limits. Testing the effect of other concentrations, such as 7 and 8 $\mu\text{l/ml}$, would be very important in studying the behavior of *Fusarium oxysporum* f. sp. *albedinis* towards concentrations, only in this phase and according to time.

Conclusion

The study of essential oils from the aerial part of *Rhanterium adpressum* collected in four different regions enabled us to reveal their chemical

composition and to evaluate their antifungal potential. This allowed us to make a comparative study. Our experimental results show that the difference in the composition of essential oils is probably related to bioclimatic zones from which the plant samples were collected (altitude, longitude, latitude and climate).

The bioactivity of our essential oils could be attributed to only majority compound, or to a synergy between the various components of the oil. Finally, we can say that the variable chemical composition of essential oils of *Rhanterium adpressum* could induce the observed antifungal properties. Moreover, this variation affects directly the antifungal activity of oils given the different percentages of chemical compounds it contains. Despite the composition variation, most extracts showed high percentages of inhibition.

A bioinformatics simulation confirmed the *Fusarium oxysporum* f. sp. *albedinis* sensitiveness toward the essential oils of this plant. According to our results, it would be very interesting to know the action mode of our oils and the molecular level at which they operate. It would also be interesting to study the synergistic mechanism of their minority components.

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Table 1. The variation of essential oils content for different samples.

		Content in % (m/m)	Content (ml/Kg)
Laghouat	Stems	0,22	2,50
	Flowers	0,11	1,25
Ouargla	Stems	0,18	2,00
	Flowers	0,20	2,25
Zelfana	Stems	0,28	3,25
	Flowers	0,35	4,00
El-Goléa	Stems	0,29	3,25
	Flowers	0,20	2,25

Table 2. Chemical composition of essential oils of *Rhanterium adpressum* in the regions of Laghouat, Ouargla, Zelfana and El-Goléa.

N°	Constitute	Composition (%)								LRI*
		Laghouat		Ouargla		Zelfana		El-Goléa		
		Stems	Flowers	Stems	Flower	Stems	Flowers	Stems	Flowers	
1	α -pinene	3,99	3,3	6,06	6,64	4,54	7,47	9,05	6,92	1033
2	Camphene	3,18	0,75	7,59	5,71	4,31	6,55	1,8	0,49	1074
3	β - pinene	1,17	0,57	2,82	1,67	1,19	2,08	3,15	1,88	1117
4	Sabinene	1,62	0,51	3,02	0,82	0,88	0,59	4,47	2,14	1131
5	β - myrcene	4,25	3,23	3,98	4,17	10,49	3,04	13,38	11,37	1179
6	α -Terpinene	0,15	0,08	0,37	0,23	-	0,4	0,53	0,21	1202
7	Limonene	0,89	0,56	1,67	1,63	1,5	2,63	1,9	1,47	1220
8	γ - terpinene	0,34	0,21	0,75	0,55	-	0,76	1,04	0,13	1264
9	p-Cymene	0,16	0,21	1,3	1,52	-	0,6	0,71	0,9	1292
10	α - terpinolene	0,24	0,19	0,38	0,29	-	-	0,53	0,46	1302
11	Linalool	9,06	4,28	11,12	4,95	10	6,94	3,96	1,02	1577
12	β - caryophyllene	0,36	0,88	-	0,59	-	0,44	0,43	1,42	1625
13	Terpinene-4-ol	2,35	2,44	0,2	3,14	1,79	4,09	4	4,8	1638
14	α - humulene	0,68	1,27	0,5	1,63	-	0,63	0,22	0,49	1658
15	α - amorphene	0,23	0,26	-	0,33	-	0,2	0,22	0,16	1675
16	1,8 menthadien-4,ol	0,23	0,5	-	0,24	-	0,14	0,1	0,77	1718
17	Germacrene D	0,49	0,57	0,4	1,24	0,66	1,32	0,47	0,49	1726
18	α -Terpineol	7,33	4,36	6,11	4,29	13,5	7,76	7,08	3,48	1737
19	Bicyclo-germacrene	2,76	5,51	0,53	1,47	1,17	2,83	1,68	6,69	1741
20	δ - cadinene	1,15	1,18	0,45	0,65	1,63	2,19	0,4	1,09	1766
21	Nerol	0,3	0,27	0,32	0,6	0,69	0,2	0,13	0,16	1822
22	Germacrene B	0,75	0,65	1,47	-	1,29	0,76	0,57	0,38	1840
23	Trans-carveol	0,3	0,38	-	0,31	-	0,13	0,1	0,14	1864
24	p-cymene 8 ol	0,35	0,53	-	0,65	-	0,74	0,23	0,41	1878
25	Geraniol	4,02	2,01	-	2,41	4,34	2,09	1,71	0,78	1888
26	Napthalene	0,63	0,62	-	0,59	0,68	0,78	0,72	0,57	1894
27	Trans β - ocimene	0,28	0,31	-	0,4	-	0,22	0,14	0,18	1959
28	Caryophyllene oxide	0,37	0,39	0,23	0,68	-	0,41	0,56	0,19	2031
29	Trans-2-carene	0,73	0,88	0,19	0,29	-	0,46	0,12	0,36	2041
30	Methyl eugenol	0,26	0,14	0,41	0,78	-	0,69	0,53	1,66	2061
31	Globulol	0,49	0,43	0,24	0,24	-	-	0,61	0,31	2090
32	Viridiflorole	0,6	0,41	0,26	0,43	-	0,28	0,43	0,63	2104
33	p-cymene α - ol	0,32	0,46	-	-	-	-	0,2	0,55	2111
34	Spathulenol	1,01	1,97	0,89	-	1,69	0,14	0,77	1,56	2123
35	Bisabolol oxyde	-	-	0,27	-	-	1,61	-	-	2126
36	Dihydro-cis- α - 8, ol	0,25	0,48	0,19	0,35	-	0,41	0,2	0,5	2153
37	α - cadinol	-	3,53	7,19	5,31	7,96	-	5,06	2,24	2173
38	Isospathulenol	0,41	0,43	0,28	0,3	-	0,23	0,15	0,74	2247
39	α - eudesmol	0,61	0,41	5,78	5,14	5,02	0,47	0,24	0,38	2275
40	β - eudesmol	4,97	5,25	4,56	6,34	3,53	2,9	0,26	5,33	2284
41	Myristic acid	0,73	0,2	0,73	0,56	0,81	0,32	0,32	0,38	2764
42	Palmitic acid	0,73	3,86	0,59	1,36	0,79	0,33	0,31	1,93	2962

Compositions percentages were obtained by FID peak-area normalization.

*LRI: is the linear retention indices relating to homologous series of n-alkanes C8-C30 obtained on UB-Wax column.

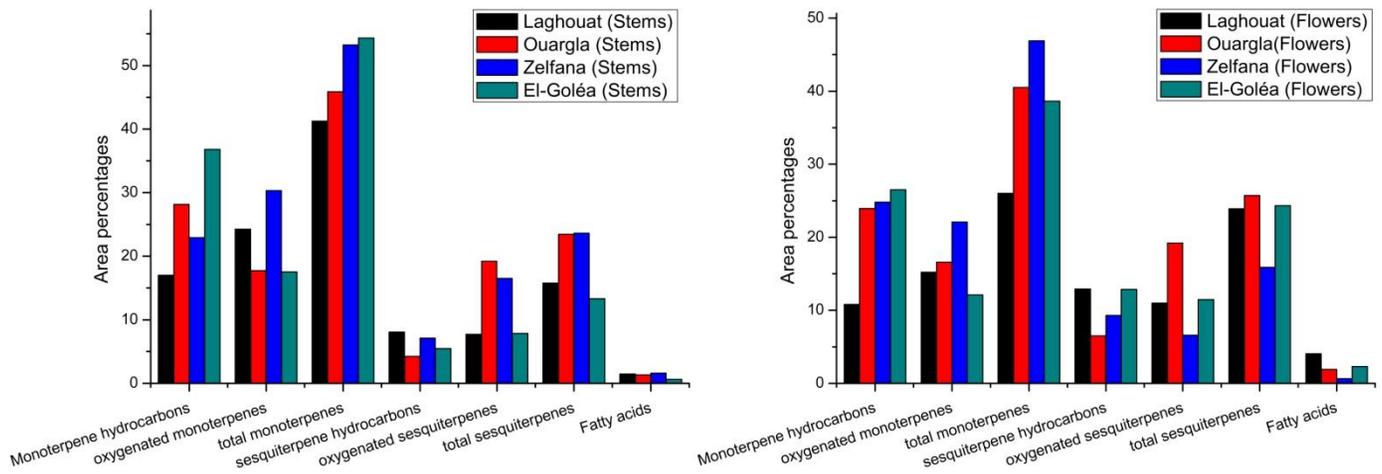


Figure 1. The variation of contents of different chemical classes by region and extraction part.

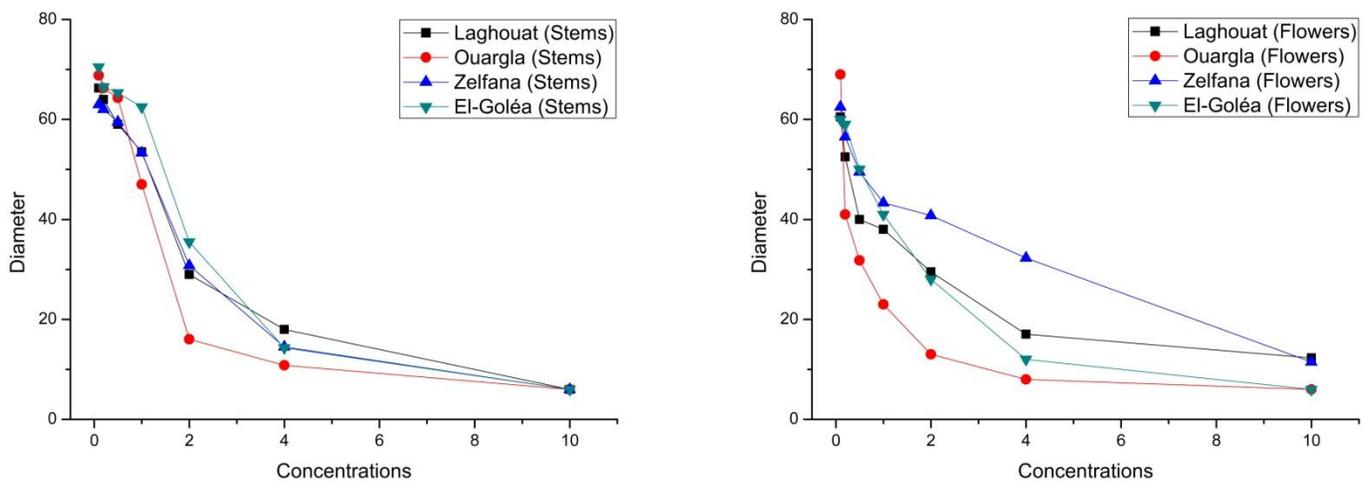


Figure 2. Presentation of *Rhanterium adpressum* EO effect on diameter (mm) of the mycelial colonies of *Fusarium oxysporum* f. sp. *albedinis*.

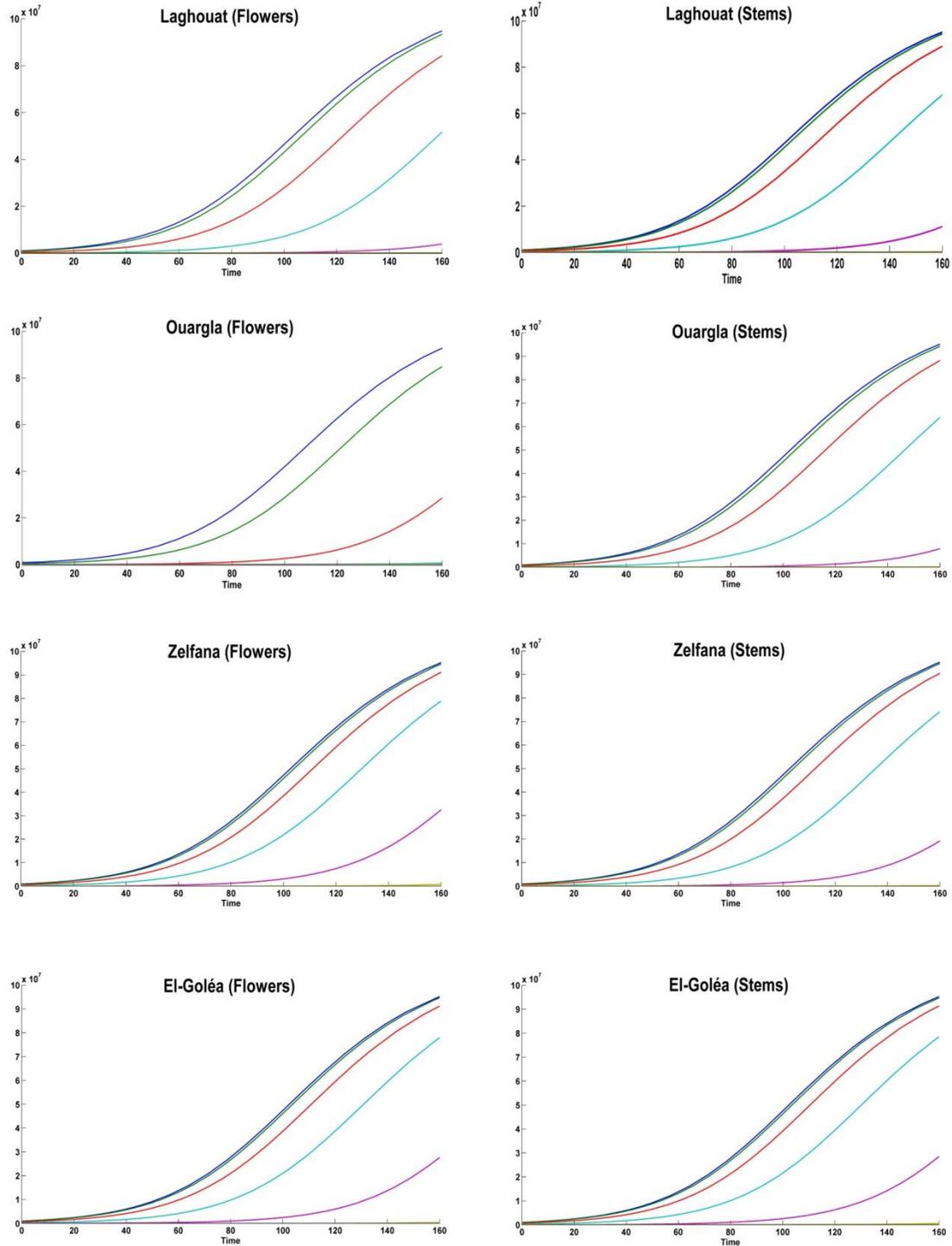


Figure 3. Growth simulation progress of *Fusarium oxysporum* f. sp. *albedinis* in the presence of different extracts concentrations (0.1 µl/ml to 10 µl/ml).

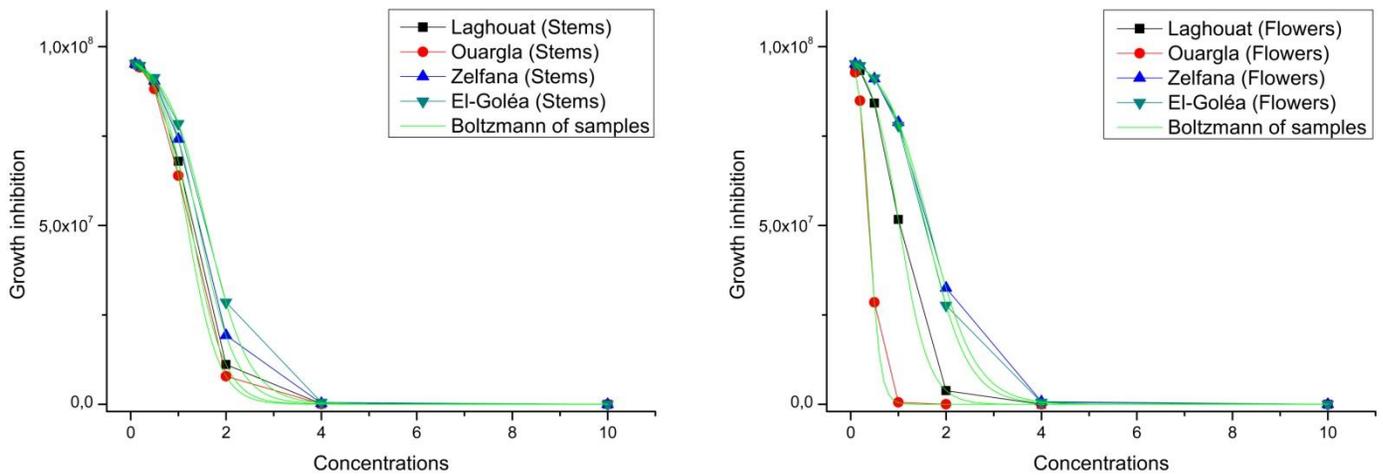


Figure 4. Representation of the growth inhibition of *Fusarium oxysporum* f. sp. *albedinis* following increased concentrations depending the region and the extracts parts.