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The Chemical Composition and the Antifungal Activity of the Essential Oil of *Origanum glandulosum* against *Neofusicoccum parvum*

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Abstract:

The essential oil (EO) extracted from the leaves and flower of the *Origanum glandulosum* was screened for its *in vitro* antifungal properties. Antifungal activity (AA) was determined using a disc diffusion method against *Neofusicoccum parvu*m (KF465685) phytopathogenic fungus attacking grapevine wood. The essential oil exhibited high reducing power (IM: 41%). These results suggest that the aerial part of *O. glandulosum* have significant antifungal activities. The chemical composition of essential oils isolated by hydro-distillation and analyzed by Gas Chromatography/mass spectrometry (GC-MS) showed presence of volatile compounds, representing 99.96% of the total oil. The oil was characterized by relatively-high amounts of monoterpene hydrocarbons, phenolic monoterpenes, and sesquiterpenes. The principal components identified included: carvacrol (48.42%), ã–terpinene (27.09%), Para-cymene (16.9%) and β-caryophyllene (3.45%).The results of this study showed that the toxicity of the oregano oil varies with increasing the applied doses on the one hand, and a relatively gradual efficiency versus time, which resulted in improved efficiency on the other hand. This is the first study of its kind on the use of the essential oils of *oregano* to control this tested fungus.

Keywords: Antifungal activity, Essential oil, *Neofusicoccum parvum*, *Origanum glandulosum.*

1. Introduction

The genus *Oregano* (*lamiaceae*) is distributed throughout the world; most of which are native to the eastern part of the Mediterranean area, Europe, Asia, and North Africa. Letswaart (1980) described forty-nine species belonging to ten different sections. The most popular include: *O. vulgare*, *O. floribodum*, *O. marjona*, *O. dictamus*, *O. glandulosum*, and *O. scabrum*. A crucial part of the flora of Algeria, the genus *Origanum* includes four main species, among these is the *O. glandulosum*, which is an endemic spontaneous plant growing in North Africa [Algeria and Tunisia] (Ben Hamida and Abdelkéfi, 2001). It is an herbaceous plant characterized by a pleasant flavour, and is largely used in traditional medicine for its sedative, antispasmodic, expectorant, and carminative properties. In addition, oregano oil also showed antibacterial, antifungal, antiparasitic, antimicrobial, and antioxidant properties.

Essential oils are volatile, natural, complex compounds characterized by a strong odor produced by plants as secondary metabolites. The essential oil composition is strongly influenced by intrinsic factors such as species, cultivar, clone, ecotype, and ecological factors including the geographical origin, climatic and soil conditions, biotic and technological factors, cultivation techniques, storage conditions of raw materials and processing technologies (Russo *et al*., 2012). In this study, the extraction of the essential oil of *O. glandulosum* was done through the hydro-distillation method. The chemical composition of the oil was analyzed by Gas Chromatography/mass spectrometry (GC-MS). Then an antifungal investigation against *N. parvum* was performed. This type of fungus colonizes wood tissues and causes Dieback. It is also among the responsible agents causing Black Dead Arm (BDA), one of the fatal diseases of arboreal, viticultural and forestry heritages. BDA caused by the *Botryosphaeria* family, eutypiosis and esca are the most destructive diseases causing decline and loss of the productivity of vineyards in world (Úrbez-Torres, 2011) and pose a real threat to the sustainability of vineyards, especially young ones. This has become a serious problem in most vine-growing regions. Algeria is among those regions affected by these diseases as confirmed in (Ammad *et al*., 2014 a; 2014. b).

It should be noted that no chemical control of these wood diseases has been provided since the prohibition of the synthetic chemical, sodium arsenite, in 2001, because of its carcinogenicity, high and acute residual toxicity, and

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other side effects on humans (Lingk, 1991; Unnikrishnan and Nath, 2002). For these reasons, prophylactic methods are mainly recommended in order to limit the development of those phytopathogenic fungi. Several studies have reported the antifungal effectiveness of the essential oils of some medicinal plants (Jayasena and Jo, 2013).These natural products have the potential to be safe fungicides to replace the synthetic ones; they are biodegradable in nature, non-pollutant and possess no residual or phytotoxic properties. Therefore, this study aims to investigate, for the first time, *in vitro* antifungal activities (AA) of *Origanum glandulosum* EO in the treatment of phytopathogenic fungi attacking the wood of grapevine trees. This study identifies the EO composition by GC/MS after being extracted by Hydro-distillation from the aerial parts of oregano. Results show that EO exerts a significant AA against studied fungi attacking the wood of the grapevine and can be also effective to control the fungal diseases in the agriculture sphere.

2. Materials and Methods

2.1. Plant Material

The leaves and flowers of *Origanum glandulosum* used for this study were collected during 2012 from Larabaa (Blida), located in the north of Algeria (36°33’ 55’’N, 3° 09’ 14’’). Botanical identification of this species was authenticated by using a determination key (Quezel and Santa, 1963).The plant material used for the extraction of the essential oil was air-dried in free air.

2.2. Extraction of the essential oils

The essential oils were extracted by the Hydro-distillation of dried plant materials (100 g of leaves and flowers in 500 mL of distilled water) using a Clevenger-type for 5 h. The Bottles of oil were covered with aluminum paper to protect them against any negative effects of light, and were stored in a refrigerator at a temperature of 4 ° C. The actual yield based on the dried weight of the sample was calculated.

2.3. Analysis of the essential oils

2.3.1. Gas chromatography–mass spectrometry analysis (GC-MS)

Chromatographic analysis was performed using a Gas Chromatography (GC) to separate the complex mixtures of the volatiles identified and quantified in a relatively short time (Sharp,1986). GC-MS was performed using a Perkin-Elmer Clarus 600 mass spectrometer with a silica capillary column of 50 m length, and 0.22 mm inner diameter of 50 µm film- thickness. Chromatogram was recorded with temperature ramp in a four-minute step at 40 °C then at a further increase of the temperature up to 250°C at a rate of 30°C/ min. Helium was used as the carrier gas at a rate of 1 mL/min. The oil sample (0.1 µL) was introduced directly into the source of the MS (mass spectrometry) via a transfer line (280° C) with a split ratio of 1:50. EO components were identified based on their retention indices (determined with reference), and were calculated using Biot’s law equation 1 as follows (Eqn. 1):

$$[\alpha] = \frac{\alpha}{C.l} \quad \text{(Eqn. 1)}$$

Where [α] is the specific rotatory power, (l) is the optical path length of the tank; α is the optical rotation; and C is the concentration of the solution in g/mL. The EO components were identified on the basis of their retention indices (determined with reference to a homologous series of normal alkanes), and also after comparing their masses, obtained by the different fragmentation patterns of the mass spectroscopic analysis, with the masses reported in the related literature (Adams, 2007).

2.4. Fungal material

One strain of the fungal material (*Neofusicoccum parvum*) meant for evaluating the effectiveness of the treatments using the tested essential oil was obtained by a personal collection. *Neofusicoccum parvum* was isolated from the infected wood of a grapevine (Ammad et *al.*, 2014 b), and was identified using a combination of morphological and cultural characters confirmed by molecular analysis, Internal transcribed spacer (ITS) and (β-tubuline primer). Culture of the fungi was maintained on potato dextrose agar (PDA) and was stored at 4 °C.

2.5. Antifungal activity assays

The effects of volatile essential oils were assayed by inoculating mycelia plugs in the center of a PDA Petri dish. The essential oil (EO) was dissolved in tween water solution (3%) and three doses of this essential oil were prepared (0.25, 0.50 and 0.75%). Sterile filter paper discs (7cm diameter), soaked in 30 µL of each dilution of the EO, were placed on the inner surface of the Petri-dish lid (Inouye *et al.*, 2006).The dishes were sealed with Para film and incubated upside-down at 25˚C. Measurements of colony radius (in cm) were made after five days. Three replicates per treatment were carried out, and each experiment was repeated at least twice. Data were expressed as percentage inhibition of mycelia growth, according to the formula of Pandey *et al*, (1982). Using the following formula: (P Ig = (DT-D)/DT) x 100), where P Ig is the percentage of inhibition growth, DT is the mean diameter of mycelial growth in control, and D is the mean diameter of mycelial growth in treatment. The estimation of mycelia growth was carried for ten days; three days following the treatment with the essential oil. For a better measure of the diameters of the mycelia growth, digital pictures of all plates were taken and treated with Image Tool software (3.1), three (03) measures were selected for each diameter.

2.6. Statistical analysis

The results of antifungal potency were treated with Excel and SYSTAT software (ver.12), SPSS 2009. The hypothesis of the antifungal efficacy of the essential oil was tested by the analysis of variance with the Global Linear Model (GLM).

3. Results

3.1. Chemical composition

The oil yield obtained through the Hydro-distillation of the *O. glandulosum* leaves and flowers was 0.98 %. The major components, representing 99.96% of the essential oil were identified by the GC technique. The main

components are presented in Table 1. The chemical composition of the *O. glandulosum* oil was dominated by monoterpene hydrocarbons fraction with a predominance of phenolic compounds and sesquiterpenes. Similar to the essential oil extracted from other *Origanum* species, this oil was characterized by high percentages of phenols; the major compounds of the oil were (carvacrol or its isomer the thymol) (48.56%). This oil can be classified as a carvacrol chemotype.

Table 1 . Principal chemical composition, retention time and percentage composition of the essential oil of *O. glandulosum* collected from Larabaa,

Pick	Compound	Formula	Réel Time (min)	Retention time	Percentage (%)
1	Carvacrol (Isothymol)	$C_{10}H_{14}O$	9.63	33.40	48.42
2	γ - Terpinene	$C_{10}H_{16}$	10.62	16.55	27.09
3	Para-Cymene	$C_{10}H_{14}$	17.10	14.44	16.01
4	β-Caryophyllene	$C_{15}H_{24}$	17.35	39.43	3.45
5	α-Terpinene	$C_{10}H_{16}$	9.33	13.91	2.55
6	Myrcene	$C_{10}H_{16}$	15.77	12.61	2.44

3.2. Antifungal activity of essential oils

The antifungal activities (AA) recorded in this study, which represents the inhibition of radial growth on solid medium, reveals that the EO of *O. glandulosum* possesses potential AA against *N. parvum* fungi. The effects of the EO dose with different concentrations are summarized in Table 2. The statistical analysis of variance revealed significant results (Figure. 1); all the tested concentrations inhibit the growth of fungus at all concentrations. At a concentration of 0.25%, *O. glandulosum* showed low toxicity at the beginning of its application to an average toxicity at the end of treatment. On the one hand, the two concentrations D3 (0.75%) and D2 (0.50%) showed more inhibitory effect compared to D1 (0.25%). On the other hand, no inhibition was registered even after ten days with the control (Figure 1 a). The different periods showed significant probability (Figure 1 b).

The results obtained in this section (AA) indicate that the volatile oils exhibited different degrees of inhibition on the growth of the tested fungi, the higher concentrations inhibited more efficiently than the diluted ones, and the duration of treatments showed interested effectiveness.

Table 2 . Antifungal Activity of the Essenti al Oil of *O. glandulosum.*

Fungi	Dilution/ time	D1 (0.25%)	D2 (0.50%)	D3 (0.75%)	Control
N.parvum	T3 /Days(05)	17.20	12.25	11.24	18.4
	T2/Days(10)	15,2	10.35	9,07	23.14
	T1/Days(15)	38.12	30.00	28	54.21

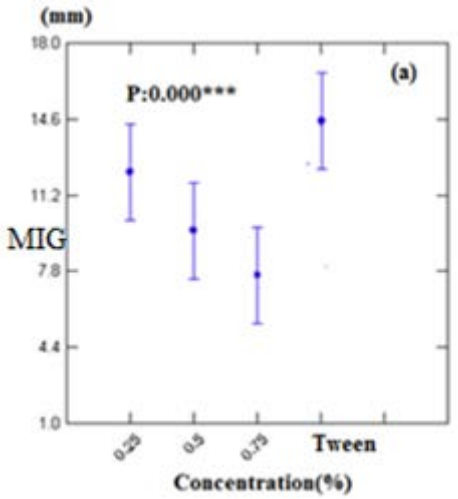

Figure1a. Variance Analysis (GLM) global linear model of time on the efficacy of essential oil against the pathogenic fungi MIG: Mycelia inhibition growth (mm), T1: 03 days, T2: 05 days and T3: 10 days P: probability , N.S.: non significant, * : significant Probability at 5 % ; ** : significant Probability 1 % ; *** : significant Probability at 0,1 %

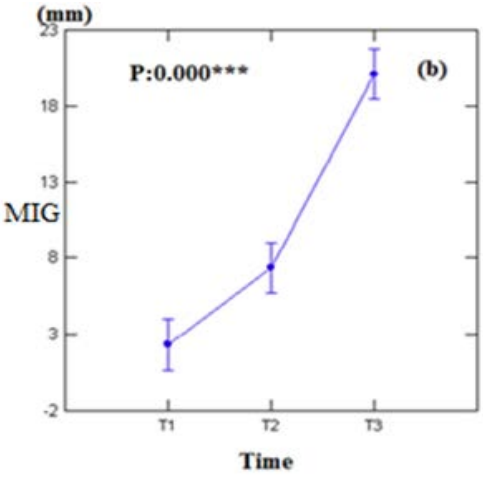

Figure1b. Variance Analysis (GLM) of dose on the efficacy of essential oil against the pathogenic fungi MG: Mycelia growth (mm), D1: 0.25% ,D2: 0.50%, D3: 0.75% s and D1: 1%, control: Dimethylsulfoxyde) solution (3%)(DMSO), P:probability , N.S.: nonsignificant, * : significant Probability at 5 % ; ** : significant Probability 1 % ; *** : significant Probability at 0,1 %

4. Discussion

Black Dead Arm (BDA), a disease of grapevines caused by *N. parvum*, can be a real nuisance to the economy. The absence of an effective treatment against these funguses has become a real problem**.** The present study is an evaluation of a treatment method which uses oregano as a protective safe chemical against *N. parvum***.**

In this study, the chemical results indicated that *O. glandulosum* EO was characterized by the high proportion of monoterpenes, notably cavracrol, which was found to possess a good biological activity against *E. coli* and *Bacillus subtilis*. This component has proven effective in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Pinto *et al*.,2006).

The essential oil of oregano has been investigated in earlier studies, which showed that carvacrol is the major component of the oil, but in different levels. Similar results by Bejaoui *et al.* (2013), concerning many essential oils extracted from *O. glandulosum* grown in some regions of Tunisia showed that the chemical composition is dominated by monoterpenes and that the highest proportion of carvacrol (68-83%) can be obtained from the oregano plant. The percentages of the components obtained in this study were different from other results concerning the essential oil of *O. glandulosum* reported by Ouled Lyiche and Djebel Megriss (Sétif) (East region in Algeria) (Ruberto *et al*, 2002). According to Maarse, (1974) and Bousbia (2004), these differences in the oil composition and yield can be attributed to several factors, including climatic and geographic conditions, time of collection, and extraction methods. According to Vaughn and Spencer, (1991) as well as Panizzi *et al*, (1993) and Caccioni and Guizzardi, (1994) the essential oils produced by different plant species belonging to the Meliaceae, Rutaceae, Asteraceae, Lamiaceae, Abiateae, and Canellaceae families are in many cases biologically active and have antimicrobial, allelopathic, antioxidant and bioregulatory properties. The antimicrobial properties can be related to the presence of active constituents, mainly attributed to isoprene's such as monoterpenes and sesquiterpenes and other hydrocarbons.

Apparently, these essential oils with their high phenolic are more effective, and have a broad spectrum of activity against filamentous fungi and insects (Cosentino, 1999). According to Dinan *et al*, (2001), the plant secondary compounds possess several modes of action against the fungal strain, but in general, their action takes place in three phases: the attack of the wall by the plant extraction, resulting in an increase permeability and losing of cellular constituents, the acidification of the inside of the cell blocking the production of cellular energy and synthesis of structural components, and finally the destruction of the genetic material leading to the death of fungi.

Several authors have showed that phenols were not the only compounds responsible for the activity. All substances of the chemical composition should be taken into account. In this regard, (Cosentino, 1999). Lahlou, (2004) and Klaric *et al*., (2006) reported that the activity of the essential oil is higher than that of the majority of its composition tested separately. The antifungal activity of the essential oil tested in this study was attributed to the presence of phenol, sesquiterpenes and monoterpenes, and the synergism between components which all play an important role. The chemical structure of the constituents of the EOs directly influences their activity (Guinoiseau, 2010).

5. Conclusions

In this study, *O. glandulosum* (EO) led to the growth inhibition of *N. parvum*. All tested concentrations were found to be lethal under the test conditions. Based on the present study, it could be concluded that EO of *O. glandulosum* possesses fungi toxic activities that can inhibit the growth of phytopathogenic fungi. Moreover, it was found that EO was characterized by a relatively-high content of phenol, showing a high yield of oils rich in carvacrol, p-cymene, and c-terpinene, which are known to possess an important antifungal activity. The data presented confirm the antifungal potential of the *O. glandulosum* essential oil. The EO tested represents an inexpensive source of natural antifungal substances for use in pathogenic systems. It would be beneficial to test the effectiveness of each component of this essential oil separately to be able to confirm the source of the oil's efficacy i.e. whether its effectiveness is related to all the substances in the oil or specific components. It is really significant to assess the morphological alterations and the modes of action of this oil on phytopathogenic fungi in further studies.

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