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Antifungal and Antioxidant Activity of *Asteriscus graveolens* subsp. *odorus* Essential Oil

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Abstract

The essential oil of air-dried *Asteriscus graveolens* subsp. *odorus*, plant of southern Morocco, obtained by hydrodistillation were analysed by capillary gas chromatography-mass spectrometry (GC-MS). Twenty eight compound accounting 69.14% of the total oil was identified. The oxygenated sesquiterpenes 6-oxocyclonerolidol (30.72%) and *epi*-α-cadinol (14.50%) were the main constituents. The essential oil was tested for antifungal activity through mycelial growth inhibition tests *in vitro* against three agricultural pathogenic fungi: *Penicillium digitatum*, *P. expansum* and *Botrytis cinerea*. The essential oil at 125, 150, 200, 250, 500, 1000 and 2000 ppm was highly effective against mycelial growth of *P. digitatum* with 100% inhibition from the first day of incubation. Complete inhibition was also observed at 150, 200, 250, 500, 1000 and 2000 ppm concentrations of the essential oil from the first day of incubation for *P. expansum*. The doses 500, 1000 and 2000 ppm showed a percentage inhibition of 100% from day one for *B. cinerea*. *Asteriscus graveolens* subsp. *odorus* essential oil was also tested at different concentrations on *Citrus* fruits (*Citrus reticulata* Blanco cv. Nules) inoculated with *P. digitatum* (10^5 conidia ml^{-1}, giving a fungistatic or fungicidal effect. In addition, the oil was subjected to screening for its possible antioxidant activity. For that, the *in vitro* assay based on the scavenging of the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used. The *Asteriscus graveolens* subsp. *odorus* essential oil gave a value of CI_{50} 0.2498 mg/ml. It inhibition percentage of free radical was slightly lower than that of BHT (the control compound) for all concentrations used. Our findings demonstrate that *Asteriscus graveolens* subsp. *odorus* essential oil possesses antioxidant and antifungal activities that might be a natural potential source of preservative and antifungal compounds used in food, in cosmetics and in pharmaceuticals products.

Keywords: Medicinal plant, antifungal activity, antioxidant activity, citrus fruits, natural product.

1. Introduction

Fruits and vegetables are often susceptible to be atracked by phytopathogenic fungi, which affect their quality. Green mold caused by *Penicillium digitatum* is one of the most important post-harvest diseases of *Citrus* fruits (Holmes & Eckert 1995). To prevent the development of this pathogen and limit losses in commercial fruit shipments, treatment with chemical fungicides is a widely used procedure. However, this treatment causes serious problems, such as fungicide residues remaining on the fruit (Cabras *et al*. 1999), the appearance of fungicide-resistant strains of *P. digitatum* (Ben-Yehoshua *et al*. 1994), and the accumulation of fungicides in human adipose tissue, posing a health threat (Suwalsky *et al.* 1999).

Various *Penicillium* spp., including *P. expansum* (Link) Thom, cause blue mold in stored apples (Rosenberger 1990; Sanderson & Spotts 1995). Post-harvest losses due to blue mold can be significant. *Botrytis cinerea* Pers: Fr. (grey mold rot) is an ubiquitous pathogen that causes severe pre and post-harvest damage in many fruits, vegetables and ornamental crops. Grey mold is particularly destructive on greenhouse crops (Elad 1997). Frequent applications of the most effective fungicides have resulted in the selection and predominance of fungicide resistant strains. Elad *et al.* (1992) showed that *Botrytis cinerea* develops resistance against specific fungicides (benzimidazoles, dicarboximides, diethofencarb and sterol biosynthesis inhibitors) within a relatively short time. Markets in industrialized countries obviously look chemical-free in both fresh or processed fruits and vegetables. To respond to this demand, several studies on the activity of essential oils against *B. cinerea* and

Penicillium spp. have been published (Wilson *et al*. 1987; Shimoni *et al.* 1993; Arras *et al*. 1995; Carta *et al*. 1996; Chebli *et al*. 2003a, 2003b; 2004; Behdani *et al*. 2012; Tabassum & Vidyasagar, 2013).

The essential oil composition of the aerial parts of *Asteriscus graveolens* subsp. *odorus* (family *Asteraceae*, species in Southern Morocco) was determined and evaluated *in vitro* as a friendly natural product to control three agricultural pathogenic fungi: *P. digitatum, P. expansum* and *B. cinerea*. Also *in vivo* assays have been carried out to control *P. digitatum*. In addition, the kinetics of extraction by hydrodistillation and the fungistatic or fungicidal effects of the essential oil has been conducted. Moreover, as many authors have reported antioxidant and radical-scavenging properties by essential oils (Maestri *et al*. 2006), the antioxidant activity of this essential oil compared to that of a reference antioxidant has been analyzed in this study.

2. Materials and methods

2.1 Plant material

The aerial parts of *Asteriscus graveolens* subsp. *odorus* (*Asteraceae)* were collected randomly from Agadir, in May 2005. The plant was taxonomically identified by B. Chebli. A voucher specimen was deposited at the herbarium of the laboratory of vegetable biotechnology (Faculty of Science, Ibn Zohr University, Agadir).

2.2 Isolation of the volatile oil

The aerial parts of the plant were air-dried in the laboratory at room temperature and subjected to hydrodistillation using a Clevenger-type apparatus according to the European Pharmacopeia (Council of Europe, 1975). In order to optimize the extraction time, we studied the kinetics of essential oil yield compared to dry matter.

2.3 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane-5% diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60°C during 5 min, with 3°C/min increases to 180°C, then 20°C/min increases to 280°C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30–500 range with an ionizing voltage of 70 eV. Kovat's retention index was calculated using co-chromatographed standard hydrocarbons. The individual compounds were identified by MS and their identity was confirmed by comparison of their RIs, relative to C_8-C_{32} *n*-alkanes, and mass spectra with those of authentic samples or with data already available in the NIST 2005 Mass Spectral Library and in the literature (Adams 2007).

2.4 Antifungal testing

2.4.1 In vitro trials

The essential oil was diluted serially using 2% Tween 80 in distilled sterile water which was also used as the control. Potato dextrose agar (PDA) was autoclaved and cooled to 40°C in a water bath. The oil prepared as described above was mixed with sterile molten PDA to obtain final concentrations of 0, 50, 100, 125, 150, 200, 250, 500, 1000 and 2000 ppm. Aliquots of 20 mL of solution were immediately dispensed to Petri dishes which were seeded with 6 mm diameter mycelium from the edge of 7-day-old *P. digitatum*, *P. expansum* and *B. cinerea*. The inoculated Petri dishes were incubated in the dark at 25°C. The percentage of growth inhibition was calculated using the following formula:

$$\% \text{ growth inhibition} = (C\text{-}T/C) \times 100 \quad (1)$$

Where C is the average of 3 replicates of mycelial growth (cm) of control Petri dishes ant T is the average of 3 replicates of mycelial growth (cm) of treated Petri dishes.

2.4.2. Transfer experiments

To distinguish between the fungistatic and the fungicidal effects of the essential oil on the target organism, a transfer experiment was done. Discs of fungi that had been 100% inhibited were transferred to fresh PDA to assess their viability after exposure to the essential oil at 25°C for 1, 3, 6 and 12 days. Fungal growth was determined by measuring the radial growth of the fungi.

2.4.3 In vivo trials

Essential oil of *Asteriscus graveolens* subsp. *odorus* was tested on Clementine fruits (*Citrus reticulata* Blanco cv. Nules). Fruits were uniform in size and free from physical damage or disease symptoms. They were placed in

1.5 L plastic containers (10 replicates per treatment) and dipped in a 10% sodium hypochlorite solution for 2 min, rinsed with tap water, and air-dried before being wounded.

Clementine fruits were wounded with a sterile puncher on their peel at the equatorial region, to make one wound 2 mm deep and 4 mm wide per fruit. Aliquots of 20 µL from 500, 1000 and 2000 ppm of *Asteriscus graveolens* subsp. *odorus* essential oil were pipetted into each wound. Control fruits were treated with 20 µL sterile distilled water. After 30 min, 20 µL of a conidial suspension of *P. digitatum* (10^5 conidia mL^{-1}) was added to each wound. The conidial concentration was determined using a Thoma slide. Treated Clementine fruits were stored at 25°C. Clementine fruits were observed daily for symptoms, and the percentage of decayed fruits was determined after 10 days.

2.4.4 Statistical analysis

Statistical analysis was performed by applying the ANOVA and Duncan tests to the statistical software (Statistical version 6.0).

2.5 Antioxidant activity

The antioxidant activity *in vitro* was assessed by measuring the scavenging power of free radical DPPH (2,2-diphenyl-1-picryhydrazyl) by the essential oils of *Asteriscus graveolens* subsp. *odorus*, with some modifications according to Chen *et al.* (2004) and Leitao *et al.* (2002).

Methanolic solution (500µL) of the essential oil of *Asteriscus graveolens* subsp. *odorus* tested at different concentrations (1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL) was mixed with 500 µL of methanolic solution of DPPH (0.004 %). After an incubation period of 30 minutes in the dark at ambient temperature, the absorbance is read at 517 nm wavelength. The inhibition of free radical DPPH by BHT (butylhydroxytoluene, reference antioxidant compound) was also analyzed with the same concentrations and the same conditions for comparison.

The inhibition of free radical DPPH percentage (I%) is calculated as follows (Leitao *et al.* 2002; Wang *et al.* 2002):

$$I\% = 100 \times (A_{control} - A_{test}) / A_{control} \quad (2)$$

Where $\mathbf{A_{control}}$ is the absorbance of the control (containing all reagents without the test product) and $\mathbf{A_{test}}$ is the absorbance of the test compound (containing all reagents and the test product).

The value IC_{50} is the concentration of the essential oil which reduces the initial DPPH concentration by 50% and it is used to characterize the antioxidant activity of the essential oil. All tests were performed in triplicate for each concentration.

3. Results and discussion

3.1 Kinetics of extraction of essential oil by hydrodistillation

Figure 1 shows that the extraction yield increases gradually up to 4 h to reach a plateau. Thus, we proposed to fix the optimum time of *Asteriscus graveolens* subsp. *odorus* hydrodistillation to 4 hours.

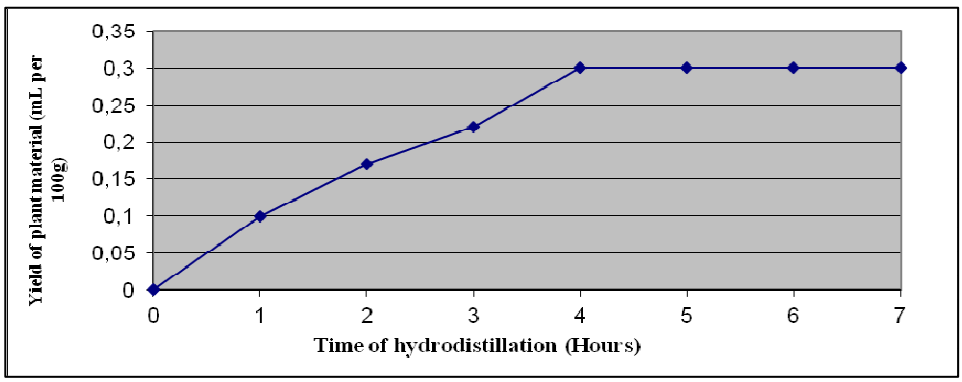

Figure 1: Kinetics of extraction by steam distillation of essential oil *Asteriscus graveolens* subsp. *odorus.*

3.2 Chemical composition of the essential oil of Asteriscus graveolens *subsp*. odorus

Aerial parts of *Asteriscus graveolens* subsp. *odorus* produced greenish yellow oil, with a yield of 0.3 mL from 100 g.

Twenty eight compounds were identified by capillary GC/MS analysis. Compounds are listed as homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated

sesquiterpenes and others (Table 1). Oxygenated sesquiterpenes was the main fraction (56.05%) due to the main compounds 6-oxocyclonerolidol (30.72%) and *epi*-α-cadinol (14.50%). Other oxygenated sesquiterpenes, humulene epoxyde II (3.50%), α-bisabolone oxide (3.56%) and bisabolone (3.50%), reached percentages higher than 3.0%. Between the monoterpene hydrocarbons only α-pinene (4.22%) was found in large amount, being *trans*-chrysanthenyl acetate and myrtenyl acetate with percentages of 2.00 and 2.54, respectively, the main compounds among the oxygenated monoterpenes.

Table 1. Chemical composition of essential oil extracted from the aerial part of *Asteriscus graveolens* subsp. *odorus*

Compound[a]	**RI**[b]	**RT**[c]	**Peak Area (%)**
Monoterpene hydrocarbons			**4.45**
α-Pinene	939	7.04	4.22
Camphene	954	7.60	0.03
Sabinene	975	8.65	tr[d]
β-Pinene	979	8.69	0.03
Myrcene	991	9.51	0.11
p-Cymene	1025	11.04	0.04
Limonene	1029	11.23	0.02
Oxygenated monoterpenes			**4.99**
1,8-Cineole	1031	11.33	0.02
α-Camphenol	1122	15.99	0.04
trans-Verbenol	1145	16.88	0.09
Myrtenol	1196	19.39	0.30
trans-Chrysanthenyl acetate	1238	22.52	2.00
Myrtenyl acetate	1327	25.41	2.54
Sesquiterpene hydrocarbons			**3.58**
β-Caryophyllene	1419	29.37	0.25
α-Humulene	1452	30.86	2.41
allo-Aromadendrene	1460	31.11	0.14
Dehydro-Aromadendrene	1462	31.26	0.03
γ-Cadinene	1514	33.30	0.35
δ-Cadinene	1523	33.69	0.40
Oxygenated sesquiterpenes			**56.05**
α-Ionone	1428	30.27	0.06
6-Oxocyclonerolidol	-	35.91	30.72
Humulene epoxyde II	1604	37.15	3.50
epi-α-Cadinol	1640	38.40	14.50
α-Bisabolone oxide	1699	41.14	3.56
Zerumbone	1732	41.58	0.21
Bisabolone	1742	42.21	3.50
Others			**0.07**
6-Methyl-5-hepten-2-one	979	9.36	0.03
Benzene acetaldehyde	1042	12.01	0.04
Total			**69.14**

[a] Components listed in order of elution from a HP OV-17 column

[b] RI: Retention Index values are calculated from retention times relative to that of *n*-alkanes on the non-polar HP OV-17 column

[c] RT: Retention time on a HP OV-17 column in minutes

[d] tr: trace (<0.03)

3.3 Antifungal activity

The essential oil was highly active against all tested fungi. For all fungi tested, antifungal activity increased with increasing concentrations of the oil. Complete inhibition was observed at 125, 150, 200, 250, 500, 1000 and 2000 ppm concentrations of the essential oil from the first day of incubation for *P. digitatum* (Figure 2). For the same fungus, the doses 50 and 100 ppm begin to take effect until after the third day to reach an inhibitory power of only 21.43% and 75.82% respectively (Table 2).

Moreover, complete inhibition was observed at 150, 200, 250, 500, 1000 and 2000 ppm concentrations of the oil from the first day of incubation for *P. expansum*. At 125 ppm, the antifungal effect of this fungus that begins on the third day with an inhibition of 67.53% (Figure 3 and Table 2). At 50 and 100 ppm, the essential oil does not show a significant effect on *P. expansum*. This effect does not exceed 2.21% for the 50 ppm dose and 12.92% for the dose 100 ppm (Table 2).

A significant difference was found between the magnitude and evolution of the effects of different doses tested on *Botrytis cinerea* (Figure 4 and Table 2). The intensity of this effect depends strongly on the dose used and the incubation period. Thus, complete inhibition was observed for doses 500, 1000 and 2000 ppm on the first day. At the other concentrations the essential oil had moderate activity ranging from 16.48% to 71.65%.

P. digitatum discs were transferred from the 2000 ppm concentration trial to fresh PDA. The results showed that mycelial growth returns after the disc has been exposed to the essential oil for 1, 3, 6 and 12 days. Thus, *Asteriscus graveolens* subsp. *odorus* essential oil against *P. digitatum* has fungistatic effect.

The statistical study showed that different concentrations of *Asteriscus graveolens* subsp. *odorus* essential oil have an effective antifungal activity against all the fungi tested: *Penicillium digitatum, Penicillium expansum* and *Botrytis cinerea*. Its effectiveness might be due to its high levels of oxygenated sesquiterpenes. Previous studies showed that essential oils with large amount of phenolic compounds (thymol and carvacrol) have strong antifungal activity against *Botrytis cinerea (*Bouchra *et al.* 2003).

A wealth of 6-oxocyclonerolidol in *Asteriscus graveolens* subsp. *odorus* representing 30.72% of the total amount of essential oil could explain its antifungal activity. Indeed, Znini *et al.* (2011) showed that *Asteriscus graveolens* essential oil has a fungicidal effect on *Penicillium expansum* at 80 µL; in this study 6-oxocyclonerolidol represented 66.7%. Another study presented by Melekmi *et al.* (2006) showed the antibacterial effect of essential oils *Bubonium graveolens* (Forsk.).

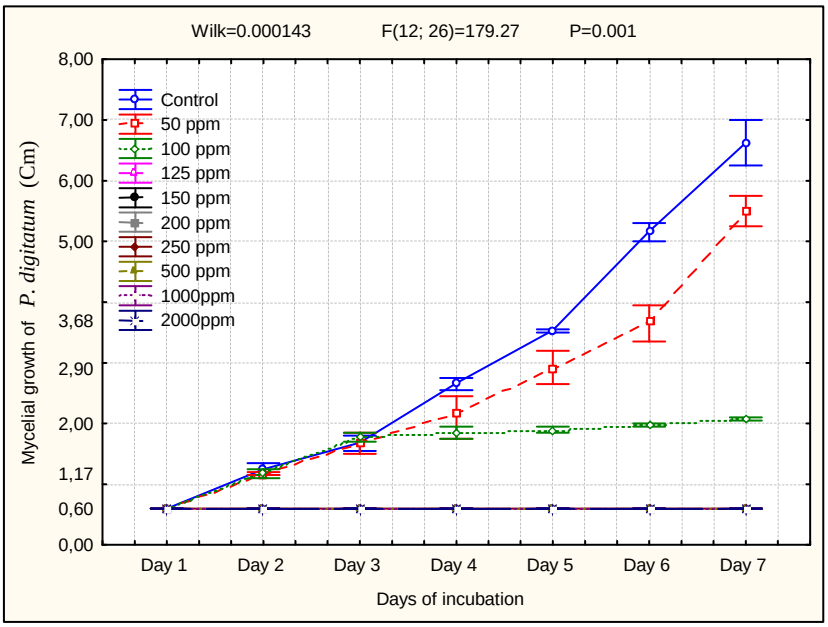

Figure 2. Mycelial growth of *Penicillium digitatum* measured daily during seven days of incubation with different concentrations of *Asteriscus graveolens* subsp. *odorus* essential oil.

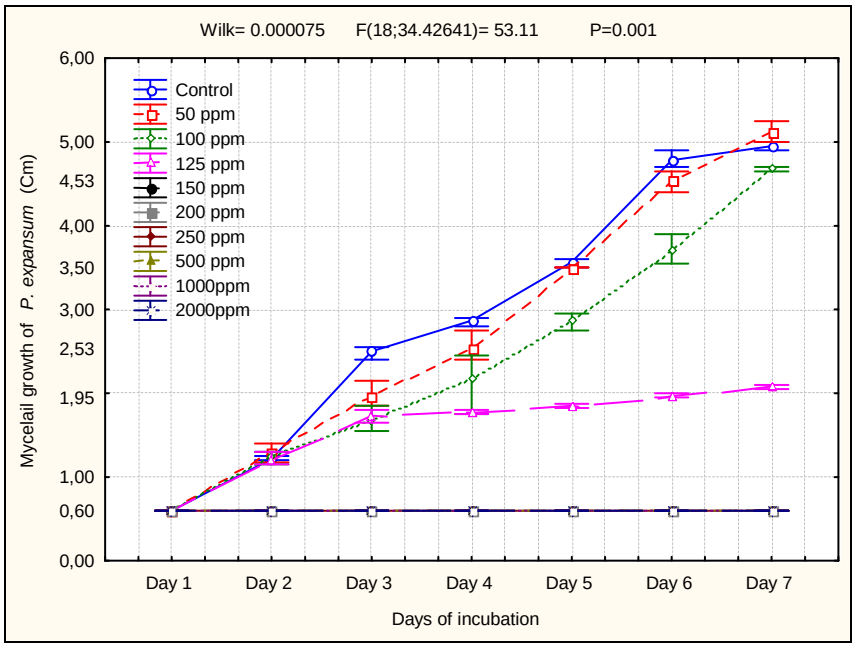

Figure 3. Mycelial growth of *Penicillium expansum* measured daily during seven days of incubation with different concentrations of *Asteriscus graveolens* subsp. *odorus* essential oil.

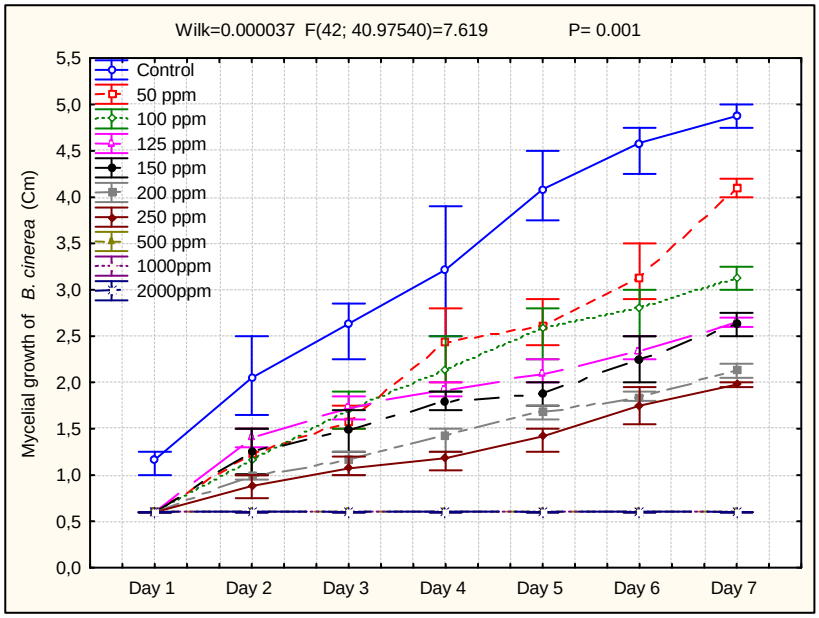

Figure 4. Mycelial growth of *Botrytis cinerea* measured daily during seven days of incubation with different concentrations of *Asteriscus graveolens* subsp. *odorus* essential oil.

Table 2. Percent of inhibition of radial growth of *Penicillium digitatum*, *P. expansum* and *Botrytis cinerea* on PDA medium with *Asteriscus graveolens* subsp. *odorus* essential oil added at different concentrations.

Fungal species	Essential oil concentration								
	50 ppm	100 ppm	125 ppm	150 ppm	200 ppm	250 ppm	500 ppm	1000 ppm	2000 ppm
B. cinerea	16.48%	43.68%	52.11%	52.49%	66.67%	71.65%	100%	100%	100%
P. digitatum	21.43%	75.82%	100%	100%	100%	100%	100%	100%	100%
P. expansum	2.21%	12.92%	67.53%	100%	100%	100%	100%	100%	100%

3.4 Antifungal effect in vivo of Asteriscus graveolens *subsp.* odorus *essential oil against* Penicillium digitatum

The *in vivo* effect of *Asteriscus graveolens* subsp. *odorus* essential oil on clementine fruits inoculated with *P. digitatum* spores was evaluated. Figure 5 shows a significant reduction of spores of *P. digitatum* on clementines ($p < 0.001$) in comparison with the control for 10 days of incubation. A significant increase during the 10 days of incubation was observed for the control. The inhibitory effect of *Asteriscus graveolens* subsp. *odorus* essential oil was in fact effectively higher after 7 days of storage, with a percentage of infected Clementine fruits of 77.50%, 85.83% and 93.33% with concentrations 500, 1000 and 2000 ppm. Moreover, after 10 days of incubation, a reduction of the percentage inhibition of *P. digitatum* was noted. The percentage of this inhibition was estimated at 75.83%, 77.50% and 92.50%, respectively. Indeed, Alilou et *al.* (2008) showed that *Asteriscus imbricatus* presents a significant reduction of infected fruit for three doses (500, 1000 and 2000 ppm) compared with the control during 7 days of incubation.

This decrease in the percentage of inhibition could be explained by the fungistatic effect of *Asteriscus graveolens* subsp. *odorus* essential oil on *P. digitatum*. Their effectiveness could be due to the oxygenated sesquiterpenes in the essential oils. Several authors have shown that essential oils and their constituents have significant potential as antimicrobial agents and antifungal agents in several industrial and medical (Baser *et al.* 2002; Dorman & Deans 2000). Another study by Wilson *et al.* (1997) showed that fungitoxic compounds of essential oils could be an alternative to methyl bromide as a fumigant in the soil.

In conclusion, further studies will be carried out to determine the effect of this essential oil on the germination of *P. digitatum* spores, in order to evaluate its potential as a preventive treatment.

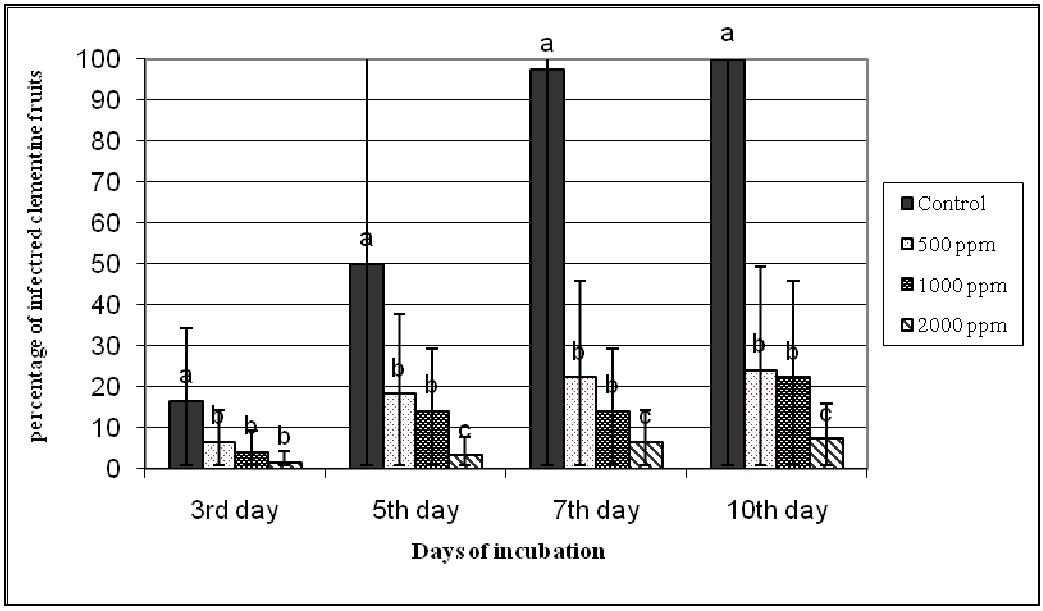

Figure 5. *In vivo* antifungal activity of different concentrations of *Asteriscus graveolens* subsp. *odorus* essential oil against *Penicilium digitatum* spores on infected Clementine fruits 3, 5, 7 and 10 days after incubation.

3.5 Antioxidant activity

The antioxidant activity of *Asteriscus graveolens* subsp. *odorus* essential oil was assessed by antioxidant DPPH assay (Blois and Marsden,1958), i.e. evaluating the H-donating or radical-scavenging ability of the essential oil using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as reagent. From the absorbance values obtained, we calculated the percentage of DPPH-scavenging using the formula given in material and methods section (antioxidant activity). The results obtained during the test measurement of the percentage of inhibition of DPPH are recorded in Figure 6. It shows that this percentage inhibition increases with increasing concentration for each essential oils or BHT (reference antioxidant compound). The percentage inhibition of free radical for essential oils studied is slightly lower than that of BHT for all concentrations used. For example, a concentration of 0.25 mg/mL of *Asteriscus graveolens* subsp. *odorus* essential oil, showed a percentage inhibition of 50.74 ± 3.94% while BHT showed a percentage inhibition of 72.91 ± 8%.

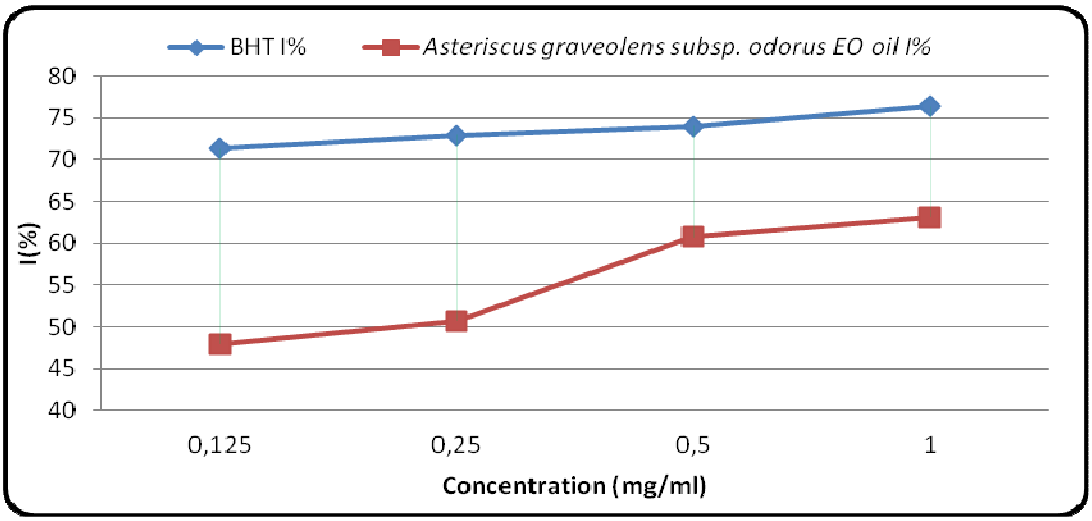

Figure 6. Inhibition percentage for essential oil and BHT

We determined graphically the concentration corresponding to 50% inhibition of DPPH (IC_{50}), which represents the antioxidant activity of the essential oil studied. The essential oil of *Asteriscus graveolens* subsp. *odorus* gave a value of IC_{50} = 0.2498 mg/mL and for the reference compound BHT was 0.1714 mg/mL (Table 3). These results established that BHT has only 1.5 times more antioxidant capacity than the essential oil studied.

Table 3. DDPH*-Scavenging activity (IC_{50DPPH}) of the *Asteriscus graveolens* subsp. *odorus* essential oil and the reference antioxidants BHT

Samples	*Asteriscus graveolens* subsp. *odorus*	*BHT*
IC_{50DPPH} (mg/mL)[a]	**0.2498**	**0.1714**

[a] Values are means (n=3).

The antioxidant activity detected in *Asteriscus graveolens* subsp. *odorus* essential oil may be due to major compounds, 6-oxocyclonerolidol and epi-α-cadinol, alone or together with other minority compounds that could act synergistically (Villaño *et al.* 2007; Singh *et al.* 2006). However, before any hasty conclusion, it is suggested that the antioxidant activity of this essential oil should be also evaluated by other methods and also to evaluate the antioxidant activity of these components separately.

Comparison with synthetic antioxidants witnesses revealed the importance of natural products and the high possibility of replacing synthetic antioxidants by natural products. Although the safety of natural antioxidants is no totally reliable, is encouraging to know that a lot of aromatic plants have been used in traditional medicine practical or in pasturage. The essential oils of these plants have a great interest for their antioxidative effect for the preservation of the foods and other important uses, given that the reactive oxygen species are involved in important disorders.

Conclusion

To our knowledge, this is the first study on the chemical composition, antifungal and antioxidant activity of the essential oil of aerial parts of *Asteriscus graveolens* subsp. *odorus* from southern Morocco.

In conclusion, our results indicated that the major components of *Asteriscus graveolens* subsp. *odorus* essential oil were 6-oxocyclonerolidol (30.72%) and *epi*-α-cadinol (14.50%).

The essential oil demonstrates stronger antifungal activity against *Penicillium digitatum*, *Penicillium expansum* and *Botrytis cinerea* and a remarkable antioxidant activity.

The results obtained in this study show that the essential oil of *Asteriscus graveolens* subsp. *odorus* may be a new potential source of natural antifungal and antioxidants agents for the food industry and fruit postharvest. However, further studies need to be conducted to understand the mechanism of the activity and obtain more information on the safety and toxicity of the essential oil.

Acknowledgments

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