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Antimicrobial Activity and Inhibition of Aflatoxin B1 Production by Essential Oils *Origanum glandulosum* Desf

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ABSTRACT

Origanum glandulosum Desf., an endemic species of Algeria is used not only as a herb tea but as medicinal plant to cure several pains including cough and rhumatism. The oil was obtained from the aerial parts of the plant by hydrodistillation and analysed by GC and GC-MS. The main components of the oil were carvacrol (57%), thymol (7%), and their biogenic precursor's γ-terpinene (13%) and ρ-cymene (11%). The antibacterial activity of a serial dilution of the oil was evaluated against ten strains of bacteria using the disk diffusion test. *Escherichia coli* and *Acinetobacter boumanii* were the most susceptible to the oil, whereas *Pseudomonas aeroginosa* ATCC 27853 was the least one. The ability of the oil to inhibit *Aspergillus flavus* growth and aflatoxin B_1 (AFB_1) production was conducted on yeast extract sucrose (YES) medium. The minimal inhibitory concentration (MIC) for the growth of this fungus was at 400 ppm, whereas that for the AFB_1 production was 200 ppm. Carvacrol was used as control and the values for the two MICs were 180 ppm and 50 ppm, respectively.

Key Words: *Origanum glandulosum*; Essential oil composition; Aflatoxine B1; carvacrol; Antimicrobial activity; *Aspergillus flavus*.

INTRODUCTION

Origanum glandulosum Desf., synonymous of *O. vulgare* L. subsp. *glandulosum* (Desf.) Ietswart (Fam. Labiatae or Lamiaceae) is an endemic species of Algeria and Tunisia (Boulos, 1983). This plant is used in folk medicine to cure several diseases such as respiratory pains, rheumatism and colds (Belhattab *et al.*, 2006).

Numerous species of *O. vulgare* including *O.glandulosum* Desf. have been reported to possess antifungal properties (Sivropoulou et *al.*, 1996; Belhattab *et al.*, 2004), but there is no report on the inhibition of aflatoxin production by the essential oil of this species. Aflatoxins are secondary metabolites produced by toxinogenic strains of *Aspergillus flavus* and *A. parasiticus*. Aflatoxin B_1 (AFB_1) is well-known as a potent mutagen and hepatocarcinogen (Ueno, 1985and Scott, 1986).

The Gram positive bacteria, such as *Staphylococcus aureus* are mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte *et al.*, 1987). The Gram negative bacteria, such as *Escherichia coli* are present in human intestines and causes urinary tract infection, coleocystitis or septicemia (Singh *et al.*, 2000). The development of bacterial resistance to presently available antibiotics has necessitated the search for new antimicrobial agents.

Several works have been carried out on *O. vulgare* essential oils (Sivropoulou *et al.*, 1996; Karpouhtsis *et al.*, 1998). It should be more interesting to investigate their antimicrobial activities for a possible use in food preservation and in medical applications. This may encourage the use of natural products instead of chemicals to limit the growth of hazardous microbes in stored foods and infections.

This work reports the chemical composition of the essential oil of *O. glandulosum*, the antimicrobial activity and the inhibition of growth and aflatoxin B_1 production by *Aspergillus flavus*.

MATERIALS AND METHODS

Plant material

Plants were collected at the flowering period and dried in the shade at air temperature until use. A voucher specimen has been conserved in the herbarium of the Department of Biology, Faculty of Sciences at F.A. University, Setif, Algeria.

Isolation and qualitative evaluation of the oil

Essential oil was isolated from dry plant material by distillation-extraction for 3 h, using a Likens-Nickerson-type apparatus with distilled *n*-pentane as organic solvent and by hydrodistillation for 3 h, using a

Clevenger-type apparatus. The sample isolated by hydrodistillation was used to estimate the oil yield, and that isolated by distillation-extraction to determine its percentage composition, since the chance of an artefact is smaller when the latter method is used. Both isolation procedures were run at a distillation rate of 3mL/min (Belhattab *et al.*, 2005).

Gas chromatography: GC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with two FIDs, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30m x 0.25mm *i.d.*, film thickness 0.25μm) and a DB-17HT fused-silica column (30m x 0.25mm, film thickness 0.15μm). Oven temperature was programmed, 45°-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10min; injector and detector temperatures, 280°C and 290°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30cm/s. The samples were injected using the split sampling technique, ratio 1:50. The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as mean values of two injections from each oil, without using response factors.

Gas Chromatography-Mass Spectrometry: The GC-MS unit consisted of a Carlo Erba 6000 Vega gas chromatograph, equipped with a DB-1 fused-silica column (30m x 0.25mm, film thickness 0.25μm), and interfaced with a Finnigan MAT 800 Ion Trap Detector (ITD; software version 4.1). Injection volumes and oven temperature were as above; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30cm/s; split ratio, 1:40; ionization energy, 70eV; ionization current, 60μA; scan range, 40-300u; scan time, 1s.

The identity of the components was assigned by comparison of their retention indices, relative to C_8-C_{17} *n*-alkanes, and GC-MS spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from a home-made library.

Microorganismes

Aspergillus flavus was isolated from different substrates including cereals and from the collection of the department of Biology, Faculty of Sciences, F. A. University. They were identified upon microscopic characteristics.

All the isolates (15) were examined for aflatoxin B_1 production ability on the semi-synthetic medium yeast extract sucrose (YES). The following species of bacteria were used to assess the antibacterial activity of the oil: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *Acinetobacter boumanii, Serratia marcescens, Klebsiella pneumonia, enterobacter aglomerans, Proteus morganella morganii, Pseudomonas aeruginosa, Salmonella thyphimirium.* Bacteria were grown on nutrient broth and incubated at 37° C.

Assessment of aflatoxin B_1 production

Different isolates of *A. flavus* were inoculated in tubes containing 15 ml YES medium at a concentration of 10^6spores/ml and incubated for 12 days at 28°C in the presence or absence (control) of different concentrations of the oil. Tubes were prepared in triplicates.

Minimal inhibitory concentration (MIC) of growth corresponds to the lowest concentration in the tubes. The medium was filtered (Whatman N°4) to remove the mycelium and the filtrate was extracted twice with chloroforme. The organic layers were added together and concentrated under vacuum in a rotary-evaporator (Büchi B-490). An aliquot was separated by TLC beside a spot of AFB_1 standard to check the presence of the mycotoxin.

Antibacterial Assay (Disk Diffusion Assay)

Bacterial isolates were seeded on Muller–Hinton medium at a concentration of 10^6strain /ml. The filter paper disks (Whatman 3MM, 6mm diameter) containing 10μl of the oil were applied on the surface of the plates. Ethanol was used as control. The plates were incubated at the appropriate temperature for 24h, then the diameter of the zone of inhibition was measured (mm). Fifteen standard antibiotics were used as reference: AMX: amoxicilline (25μg); CTX: cefotaxine (30μg); GM: gentamicine (15μg); SXT: sulfamide (23,75μg); NA: Nalidixate (30μg); PIP: piperacilline (75μg); IPM: imipeneme (10μg); CAZ: cefazidine(30μg); CZ: cefozoline(30g): TIC: ticarcilline(75μg); ERY: erythromycine(15UI); OXA: oxacilline(5μg); VAN: vancomicyne(30μg); P: benzylpenicilline(6μg); OFX: ofoxicine(5μg);

Viability test

Plates containing nutrient broth agar were inoculated from the clear zone of inhibition obtained in the

previous assay to determine the effect of the oil on the bacteria. After an appropriate time of incubation the effect of the oil can be determined. If no growth occurred, the effect of the oil is bactericide , if the bacteria colonies appeared on the medium, the effect is considered as bacteriostatic.

Determination of the MICs for bacteria

MICs were determined using a series of dilution of the oil. The MIC corresponds to the lowest concentration (highest dilution) at which there is no growth of the bacteria. The following dilutions of the oil were used: 1/10, 1/20, 140, 1/50 (v/v).

RESULTS AND DISCUSSION

Chemical composition of the oil

The essential oil of *O. glandulosum* was obtained at a yield of 2,7%. Thirty one component were identified, amounting to 89,4% of the total oil. The identified components and their percentages are listed in Table (1), in the order of their elution from a DB-1 column.

The monoterpene fraction was the most representative in the oil, 97%. This fraction was dominated by the oxygen – containing compounds, carvacrol being clearly the main component (47%), γ-terpinene (13%), and *p*-cymene (11%) were the second and third main components of the oil. Thymol was present in a relatively high amount (7%).

The sesquiterpenes were dominated by β- caryophyllene (ca.1%). A fourth fraction of other components was only found in minor amounts (< 0,4%). Many studies explained in details the essential oil composition of several *Origanum* species, only two studies have reported the essential oil composition of *O. glandulosum* grown in cultivation areas in Italy (Lawrence, 2003) and four populations grown in Algeria (Ruberto *et al.*, 2002). The oil yield obtained int the present study (rich in carvacrol and poor in thymol subgroup) is in good agreement with that obtained by Ruberto *et al.*, (2002) for other Algerian populations but shows some differences with regard to the *p*-cymene and γ-terpinene amounts.

Antimicrobial activity

The essential oil was evaluated for antimicrobial activity against pathogenic strains of Gram positive and Gram negative bacteria using the disk diffusion test. It was found that the essential oil is active against all the bacterial species tested. The activity of the oil varied with its concentration and the bacteria. *Escherichia coli* and *Acinetobacter boumanii* were the most susceptible to the oil, whereas *Pseudomonas aeroginosa* ATCC 27853 was the least one. Ethanol used as control showed no effect on the different tested bacteria.

When ATCC 27853 transferred from the dilution 1/1 and 1/2 to the nutrient broth was *Pseudomonas aeruginosa*, visible growth was noticed which demonstrates a bacteriostatic effect of the oil against this strain, whereas no growth was observed with the other species of bacteria which confirmed a bactericide effect (Table 2).

The minimal inhibitory concentrations (MIC) were obtained with a dilution of 1/40 for *Escherichia coli* ATCC 25922, *Acinetobacter boumanii, Serratia marcescens, Enterobacter aglomerans, Salmonella thyphimirium, Staphylococcus aureus* ATCC25923 (Table 2) and 1/20 for *Klebsiella pneumonia, Proteus morganella morganii,* whereas it was obtained with the dilution 1/10 for *Pseudomonas aeruginosa* (Table 2).

Escherichia coli ATCC 25922, *Serratia marcescens, Proteus morganella morganii* and *Staphylococcus aureus* ATCC25923 exhibited sensitivity to both essential oil and reference antibiotics, Meanwhile *P. aeruginosa* was considered resistant to the essential oil but not to the reference antibiotics, because of the weak zone of inhibition. Although *Klebsiella pneumonia, Enterobacter aglomerans, Salmonella thyphimirium* and *Acinetobacter boumanii* were sensitive to the oil, they were considered resistant to the most corresponding reference antibiotics tested since no inhibition zone was observed (Table 3). Standard antibiotics revealed equivalent effects to the oil dilutions when used against *E. coli, S. marcescens* and *P. morganella morganii*, whereas they were less effective when tested against *A. boumanii, K. pneumonia* and *E. aglomerans* (Table 3).

Inhibition of aflatoxin B_1 production

The MIC of the oil for the fungus *Aspergillus flavus* was 400 ppm and for aflatoxin B_1 production was 200 ppm. The MIC values for carvacrol were 180 ppm and 50 ppm, respectively (Tab.4). Although few studies have been carried out using essential oils from *O. glandulosum* for screening antibacterial activity, no

Table (1): Constituents and percentage composition of the essential oils of *Origanum glandulosum* isolated by hydrodistillation from the aerial parts collected during the flowering phase

Constituants	Essential oil	
	RI[a]	%
α-Thujene	924	0.3
α –Pinene	930	0.7
Camphene	938	0.1
Octen-3-ol	961	0.2
3-Octanone	963	0.2
β-Pinene	963	0.2
β –Myrcene	975	1.6
α –Phellandrene	995	0.2
α –Terpinene	1002	1.5
***p*-Cymene**	1003	**11.2**
β –Phellandrene	1005	0.2
Limonene	1009	0.3
Cis- β –Ocimene	1017	0.1
Trans- β -Ocimene	1027	t
γ-Terpinene	1035	**13.4**
Terpinolene	1064	0.1
Linalool	1074	1.0
Borneol	1134	0.1
Terpinen-4-ol	1148	0.4
α –Terpineol	1159	0.5
Methyl thymol	1208	0.4
Methyl carvacrol	1224	0.1
Thymol	1275	**6.6**
Carvacrol	1286	**47.0**
β -Caryophyllene	1414	1.4
trans- α-Bergamotene	1434	0.1
α –Humulene	1447	t
Zingiberene	1492	0.1
β –Bisabolene	1495	0.3
Sesquiphellandrene	1508	0.7
β -Caryophyllene oxide	1561	0.4
Identification %		89.4
Grouped components		
Monoterpene hydrocarbons		29.9
Oxygen containing monoterpenes		56.1
Sesquiterpene hydrocarbons		2.6
Oxygen containing sesquiterpenes		0.4
Others		0.4
Oil yield (v/w)		2.7

[a] RI, retention index relative to C_9-C_{16} *n*-alkanes on the DB-1 column.
t = trace (<0.05 %).

Table (2): Effects of *O. glandulosum* essential oils on different species of bacteria and MICs.

Bacterial strains	Dilution				Effect	MICs
	1/1	1/2	1/5	1/10		
Escherichia coli ATCC 25922	39**	38	33	16	c	1/40
Pseudo*monas aeroginosa* ATCC 27853	13	10	09	-	s	1/10
Staphylococcus aureus ATCC25923	30	28	25	16	c	1/40
Acinetobacter boumanii	60	60	40	19	c	1/40
Serratia marescens	18	16	14	12	s	1/40
Klebsiella pneumonia	28	23	19	14	c	1/20
Enterobacter aglomerans	32	29	28	14	c	1/40
Proteus morganella morganii	51	38	19	14	c	1/20
Pseudomonas aeroginosa	18	10	09	-	c	1/10
Salmonella thyphimirium	38	35	27	13	c	1/40

*control= ethanol ; c: bactericide; s: bacteriostatic
**zone of inhibition (Ø=mm) are the mean values of triplicates

Table (3): Effects of antibiotics on the different species of bacteria*

Bacterial strains	Zone of inhibition, mm				
Tested antibiotics	AMX	CTX	GM	SXT	NA
Escherichia coli ATCC 25922	13	33	30	22	26
Serratia marescens	17	34	23	30	27
Klebsiella pneumonia	-	10	-	-	23
Enterobacter aglomerans	-	30	-	-	25
Proteus morganella morganii	18	35	24	27	23
Tested antibiotics	PIP	GM	IPM	CAZ	TIC
Pseudo*monas aeroginosa* ATCC 27853	20	17	10	22	22
Pseudomonas aeroginosa	21	18	-	22	25
Tested antibiotics	ERY	SXT	OXA	VAN	P
Staphylococcus aureus ATCC25923	35	-	28	20	-
Tested antibiotics	GM	AMX	OFX	CZ	NA
Salmonella thyphimirium	-	-	31	-	21
Tested antibiotics	PIP	AMX	GM	CAZ	TIC
Acinetobacter boumanii	8	-	12	-	11

*zone of inhibition (Ø= mm)

Table (4): Determination of the MICs (ppm) of *Aspergillus flavus* growth and aflatoxine B1 production by *O. glandulosum* essential oils and carvacrol from *O. glandulosum*.

Treatment	MICs for	
	Growth	Aflatoxin B_1 production
Essential oil	400	200
Carvacrol	180	50

attention was given to antifungal activity or inhibition of aflatoxin production. The strong activities demonstrated in this investigation would suggest that monoterpens such as carvacrol are responsible of them since they dominate the essential oil. Joseph *et al.* (2005) reported that *Garcinia* sp. extracts inhibited completely the production of AFB_1 and growth of *A. flavus* at 2000 and 3000 ppm, respectively. It has been shown that these activities are dose dependent when Rasooli and Owlia tested thymol (2005).

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الملخص العربى

النشاط ضد الجرثمي وتثبيط إنتاج الأفلاتوكسين B١ من قبل الزيوت الأساسية لـ *Origanum glandulosum* Desf

رشيد بلحطاب & العربي لعروس

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يستعمل fseD *munagirO musoludnalg* و هو نبات مستوطن بالجزائر، في علاج عدة أمراض منها السعال و الروماتيزم. تم الحصول على الزيوت الاساسية من الأجزاء الهوائية للنبتة عن طريق التقطير المائي و تحليلها بواسطة CG و CG– SM. من أهم المركبات المشكلة لها lorcavrca (٥٧%) و lomyht (٧%) ومولداتها البيولوجية γ– enenipret (١٣) و ρ–cneemy (١١%). تم إختبار النشاط المضاد للبكتيريا بإستعمال سلسلة من التخافيف للزيت على عشرة أنواع من البكتيريا وذلك بإعتماد تقنية الإنتشار حول القرص. من أكثر هذه البكتيريا تحسسا نجد *iloc* *aihcirehcsE* و *iinamuob* *retcabotenicA* وكانت بكتيريا *asonigorea* *sanomoduesP* أقل تحسسا. إن قدرة الزيت على تثبيط *suvalf* و إنتاج الأفلا توكسين B١ تمت متابعتها على وسط مستخلص الخميرة و السكروز (YES). قدر التركيز الأدنى المثبط لنمو *sulligrepsA* الفطر(MIC) ب ٤٠٠ـ mpp في حين قدر ذلك المثبط لإنتاج السم بـ ٢٠٠ ppm. أستعمل lorcavrac كشاهد و كانت نتائج الـ sCIM تقدر بـ ١٨٠ppm و ٥٠mpp على التوالي.