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RESEARCH PAPERS

Chemical composition and antifungal activity of *Bubonium imbricatum* volatile oil

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Summary. The chemical composition of the essential oil extracted from the aerial parts of *Bubonium imbricatum*, an endemic plant of southern Morocco, was analysed. GC-MS showed 51 compounds, representing 60.2% of the total oil. Thymol isobutyrate (18.3%) and 2,5-dimethoxy-*p*-cymene (16.2%) were the major constituents of the oil. The oil was tested for antifungal activity in mycelial growth inhibition tests *in vitro* against three agricultural pathogenic fungi: *Penicillium digitatum*, *P. expansum* and *Botrytis cinerea*. The oil at 1000 ppm was highly effective against mycelial growth of *P. digitatum* with 99% inhibition. For *P. expansum* and *B. cinerea* the percentage of mycelial growth inhibition at 1000 ppm was respectively 87.2% and 87.8%. At 2000 ppm the oil was 100% effective in controlling mycelial growth of all three fungi. *B. imbricatum* 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}). The oil had a fungistatic or fungicidal effect on *C. reticulata*.

Key words: medicinal plant, *Penicillium digitatum*, *Penicillium expansum*, *Botrytis cinerea*, citrus fruits.

Introduction

Research on natural products (including plant extracts) which can replace agrochemicals or lead to the development of new agents for pest control is extremely important (Stanger *et al*., 2002; Mark *et al*., 2005). Many natural substances play a role in the host plant/pathogen relationship. The essential oils produced by many plants are biologically active, and have antimicrobic, allelopathic, antioxidant and bio-regulatory properties. Essential oils also have properties as fungicidal agents (Caccioni and Guizzardi, 1994; Alvarez-Castellanos *et al*., 2001; Gurdip *et al.,* 2005, 2006; Gurdip and Sumitra, 2006; Neri *et al.,* 2006).

Fruits and vegetables are often susceptible to attack 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 and Eckert, 1995). To prevent the development of this patho-

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gen and limit losses in commercial fruit shipments, treatment with chemical fungicides is a widely used procedure. However, such treatment causes serious problems, with 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 and 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 application of the most effective fungicides have resulted in the selection and predominance of fungicide-resistant strains. Elad *et al*. (1992) showed that *B. cinerea* develops resistance against specific fungicides (benzimidazoles, dicarboximides, diethofencarb and sterol biosynthesis inhibitors) within a relatively short time. Markets in industrialized countries obviously look for fresh or processed fruits, and vegetables that are chemical free. To respond to this demand, several studies on the activity of essential oils against *B. cinerea* 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).

The essential oil of the aerial parts of *Bubonium imbricatum* (family *Asteraceae*), an endemic species in Southern Morocco was determined and evaluated *in vitro* as an agent to control three agricultural pathogenic fungi: *P. digitatum, P. expansum* and *B. cinerea* and it was tested *in vivo* on *P. digitatum*. The fungistatic or fungicidal effect of this oil was also studied.

Materials and methods

Plant collection and isolation of essential oil

Plant material

The aerial parts of *Bubonium imbricatum* (*Asteraceae)* were collected randomly from Cap Ghir, 60 km from Agadir, Morocco, 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, Morocco).

Isolation of the volatile oil

The aerial parts of the plant were air-dried in the laboratory at room temperature and subjected to hydrodistillation for 5 h using a Clevenger-type apparatus according to the European Pharmacopoeia (Anonymous, 1975).

GC-MS analysis was done on a thermo mass spectrometer (Model trio 1000; Warrington, UK); combined with a thermo gas chromatograph (Model 8000, Fisons Instruments, Rodano, Italy). An OV-17 capillary column (25 m long×0.25 mm Hewlett-Packard Ltd., Stockport, UK) was employed for the analysis. The column temperature program was 60°C for 6 min, with 5°C increases per min to 150°C, which was maintained for 10 min. The carrier gas was helium at a flow rate of 2 ml min^{-1} (splitless mode). The detector and injector temperature were maintained at 250 and 225°C respectively. The quadrupole mass spectrometer was scanned over the range 28–400 amu at 1 scan. s^{-1}, with an ionizing voltage of 70 eV, and an ionization current of 150 μA.

The individual compounds were identified by MS and their identity was confirmed by comparing their Kováts Index (KI) and their mass spectra and retention times with those of authentic samples or with data already available in the NIST library and in the literature (Adams, 2001).

The KI of a compound is a number obtained by interpolation, relating the adjusted retention time of the sample compound to the adjusted retention times of two standards (in our analysis C_8 and C_{32}) eluted before and after the peak of the sample compound.

The NIST employed was a compilation of 191,000 spectra, 163,000 chemical structures, 121,000 Kováts retention indices and 5200 MS/MS spectra.

Antifungal testing

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:

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

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

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 oil at 25°C for 1, 3, 6 and 12 days. Fungal growth was determined by measuring the radial growth of the fungi.

In vivo trials

Essential oil extracted from *B. imbricatum* 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 *B. imbricatum* 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.

Statistical analysis

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

Results and discussion

Chemical composition of the essential oil of *B. imbricatum*

The aerial parts of *Bubonium imbricatum* produced a clear yellow oil, with a yield of 0.16 ml from 100 g of plant.

Fifty-one compounds were identified by GC/MS analysis (Table 1). Twenty-four were hydrocarbons and the other 27 belonged to the oxygenated fraction. The oxygenated monoterpenes were at the highest quantities (75%), mainly thymol isobutyrate (18.32%), 2,5-dimethoxy-*p*-cymene (16.21%) and *cis*-chrysanthenyl acetate (8.22%). The monoterpene hydrocarbons (with the exception of α-pinene 5.53%) were found at lower quantities (0.02–0.16%). Twenty sesquiterpenes were identified; Δ-cadinene (1.20%) and its corresponding alcohols *epi*-α-cadinol (2.53%) and α-cadinol (1.54%), reached percentages greater than 1%. Twelve oxygenated compounds (M^+ 220–280 *m* / z) representing 35% of the oil were also detected.

Antifungal activity

The essential oil was highly active against all tested fungi. For all fungi, antifungal activity increased with increasing concentrations of the oil (Fig. 1, 2 and 3). Complete inhibition was observed when the oil was tested at the 2000 ppm concentration of the oil for *P. digitatum* and *P. expansum*. A 1000 ppm concentration of the oil was also highly effective, with an inhibition of 99% against *P. digitatum* and 89.1% against *P. expansum* (Table 2). At the other concentrations the oil had moderate activity: 51.4, 48.9, 42.9, 36.7, 35.3, 30.4, 27.6% against *P. digitatum* for respectively 500, 250, 200, 150, 125, 100 and 50 ppm. For these same concentrations, the inhibition percentages of *P. expansum* were respectively 67.1, 52.3, 49.8, 39.7, 29.9, 27.4 and 24.3. The percentage inhibition of mycelial growth of *B. cinerea* was 97, 87.8, 71.7, 71.3, 67.1, 53.6, 51.3, 47.8 and 42.5% for 2000, 1000, 500, 250, 200, 150, 125, 100 and 50 ppm concentrations respectively. At 1000 ppm the oil was more effective against *P. digitatum* than against the other two fungi after 7 days of incubation (Table 2).

Table 1. Chemical composition of essential oil extracted from the aerial part of *Bubonium imbricatum*.

Compound[a]	KI[b]	Total identified compounds (%)
α-Pinene	939	5.53
Camphene	954	0.13
Benzaldehyde	960	0.02
Verbenene	968	0.04
Sabinene	977	0.05
β-Pinene	980	0.16
β-Myrcene	992	0.06
α-Phellandrene	1006	0.05
□[3]-Carene	1010	0.04
α-Terpinene	1019	0.02
Limonene	1032	0.02
γ-Terpinene	1063	0.02
Linalol	1100	0.02
Nonanol	1102	0.02
cis-p –Meth-2-en-1-ol	1124	0.12
α-Campholenal	1128	0.02
trans-Pinocarveol	1140	0.02
cis-Verbenol	1142	0.09
p-Menth-1,5-dien-8-ol	1170	0.12
Terpinen-4-ol	1177	0.02
α-Terpineol	1192	0.02
Thymol, methyl ether	1235	0.90
Carvotanacetone	1247	0.12
cis-Chrysanthenyl acetate	1265	8.22
Bornyl acetate	1289	0.35
Thymol	1290	0.08
Carvacrol	1302	0.02
α-Copaene	1380	0.08
β-Bourbonene	1388	0.02
Methyl eugenol	1404	0.02
β-Caryophyllene	1419	0.17
2,5-dimethoxy-*p*-Cymene	1427	16.21
cis-Muurola-3,5-diene	1452	0.10
9-epi-β-Caryophyllene	1466	0.11
cis-Muurola-4(14), 5-diene	1467	0.18
trans-Cadina-1(6), 4-diene	1477	0.16
γ-Muurolene	1481	0.11
Germacrene D	1486	0.38
Thymol isobutyrate	1492	18.32
epi-Cubenol	1495	0.43
α-Muurolene	1500	0.77
α-Cubenol	1515	0.02
δ-Cadinene	1525	1.20
α-Cadinene	1539	0.10
α-Calacorene	1546	0.08
Nerolidol	1566	0.08
ar-Tumerol	1583	0.42
epi-α-Cadinol	1644	2.53
α-Muurolol	1648	0.40
α-Cadinol	1654	1.54
Manoyl oxide	1998	0.11
Total		59.82

[a] Components listed in order of elution from a HP OV-17 column.
[b] KI, Kováts indices calculated against C8-C23 n-alkanes on the HP OV-17 column

At 2000 ppm the oil completely inhibited the growth of these three fungi after 7 days. Furthermore, at 2000 ppm the essential oil of *B. imbricatum* was sufficient to ensure inhibition of *P. digitatum* and *P. expansum* growth from the first day.

P. digitatum and *P. expansum* discs were transfered from the 2000 ppm concentration trial to fresh PDA. The result showed that the inhibition of *B. imbricatum* essential oil against *P. digitatum* and *P. expansum* was fungicidal in nature.

The oil was very effective against all the fungi tested. Its effectiveness might be due to its high levels of thymol isobutyrate, 2,5-dimethoxy-*p*-cymene, *cis*-chrysanthenyl acetate and α-pinene. The fungi tested are sensitive to the phenolic compounds, being carvacrol and thymol, present in minor quantities in *B. imbricatum* essential oil.

The antimicrobial activity of thymol has been the subject of several studies (Kim *et al.*, 1995; Curtis *et al.*, 1996; Chebli *et al.*, 2003a, 2003b). Otherwise, these compounds act synergistically, as was suggested by Filipowicz *et al.*, 2003, so that their synergetic effect might also be responsible for the antifungal activity of *B. imbricatum* essential oil.

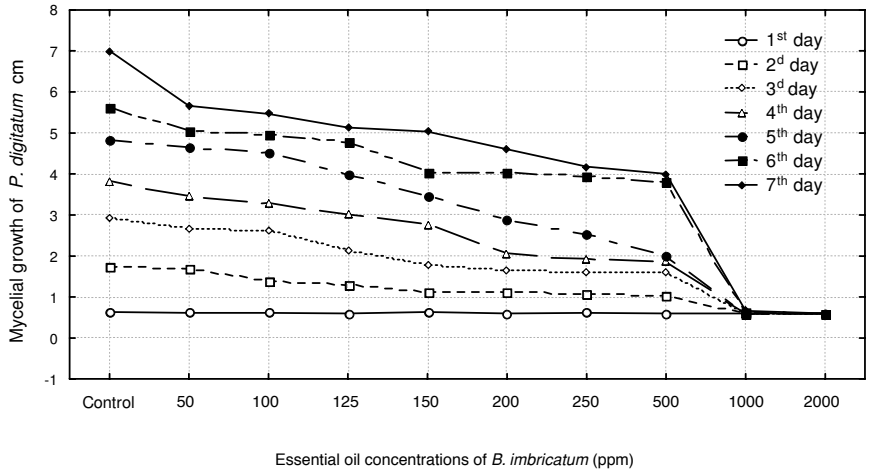

Fig. 1. Mycelial growth of *Penicillium digitatum* measured daily during seven days of incubation with different concentrations of *Bubonium imbricatum* essential oil.

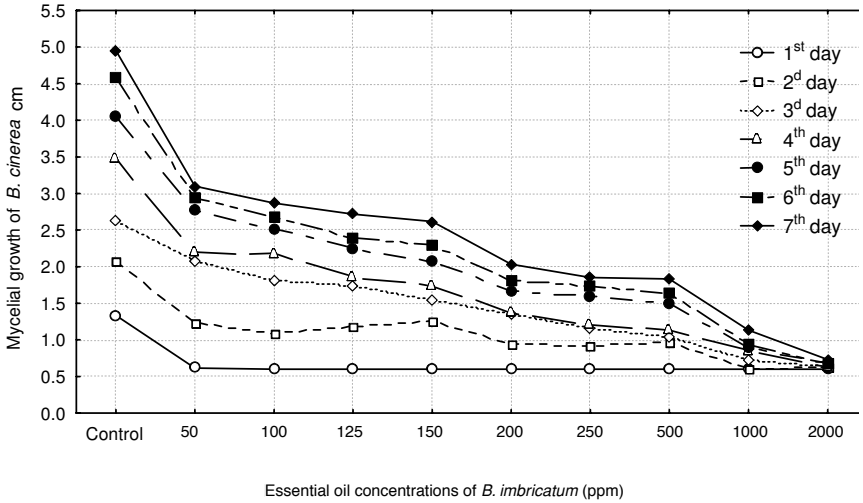

Fig. 2. Mycelial growth of *Botrytis cinerea* measured daily during seven days of incubation with different concentrations of *Bubonium imbricatum* essential oil.

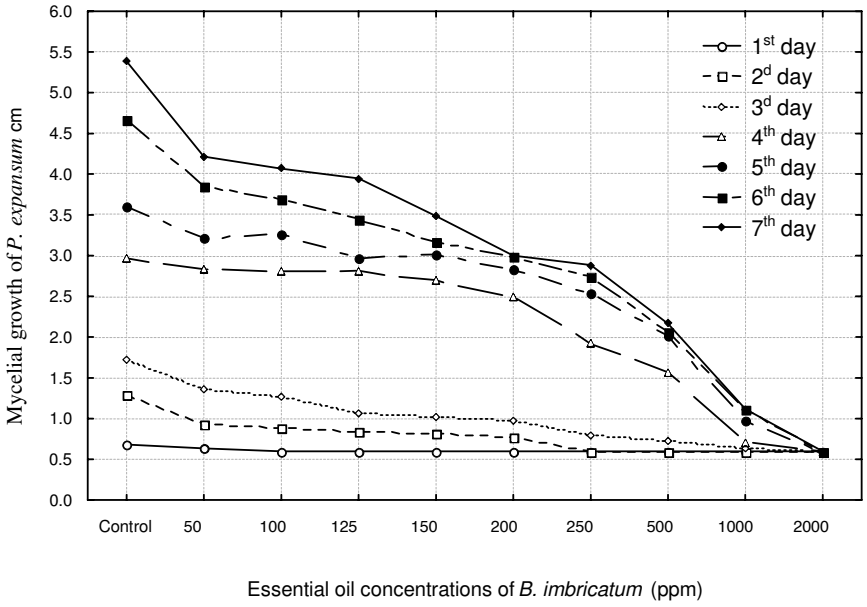

Fig. 3. Mycelial growth of *Penicillium expansum* measured daily during seven days of incubation with different concentrations of *Bubonium imbricatum* essential oil

Table 2. Percent inhibition of radial growth of *Penicillium digitatum, P. expansum* and *Botrytis cinerea* on PDA medium with *Bubonium imbricatum* essential oils added at different concentrations.

Fungal species	Essential oil concentration (ppm)								
	50	100	125	150	200	250	500	1000	2000
P. digitatum	27.57	30.43	35.29	36.71	42.86	48.86	51.43	99.00	100
P. expansum	24.27	27.41	29.92	39.75	49.79	52.30	67.15	89.12	100
B. cinerea	42.53	47.82	51.26	53.56	67.13	71.26	71.72	87.82	97.01

Antifungal effect *in vivo* of *B. imbricatum* essential oil against *P. digitatum*

The *in vivo* effect of *B. imbricatum* essential oil on Clementine fruits inoculated with *P. digitatum* spores was evaluated. Figure 4 shows that symptoms on both treated and control fruits appeared after 3 days of storage, except with the 2000 ppm concentration, where fungal infection was only observed after 5 days. A significant increase in decay was seen in the control from the 3rd to the 7th day. Furthermore, the inoculated *P. digitatum* spores on Clementine fruits were significantly ($P<0.001$) reduced in number as compared with the control during the 10 days of incubation (Fig. 4). The inhibitory effect of *B. imbricatum* essential oil was in fact effectively higher after 10 days of storage, at 50%, 73,3% and 81,7% with concentrations of 500 ppm, 1000 ppm and 2000 ppm respectively. The effect of *P. digitatum* was already strongly decreased after 7 days of storage. Percent inhibition was 53.8%,

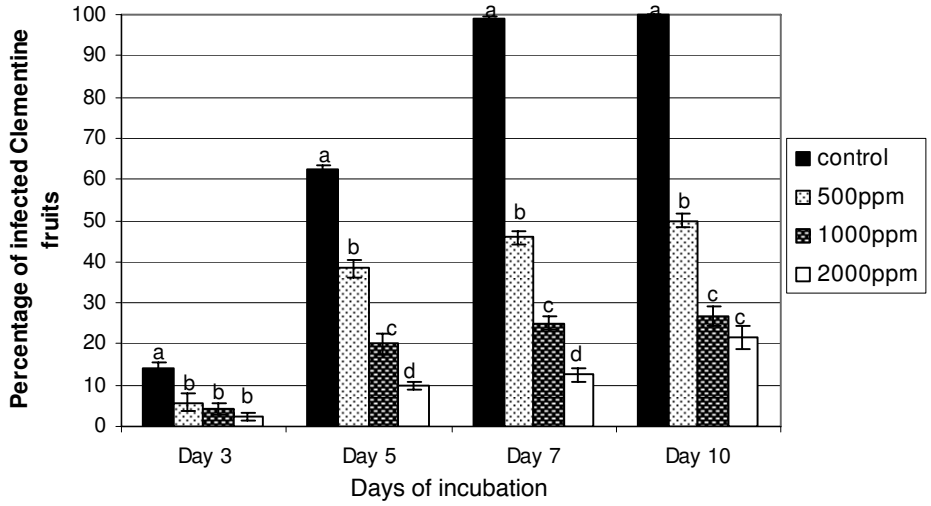

Fig. 4. *In vivo* antifungal activity of different concentrations of *Bubonium imbricatum* essential oil against *Penicillium digitatum* spores on infected Clementine fruits 3, 5, 7 and 10 days after incubation.

74.8% and 89.9% with concentrations of 500 ppm, 1000 ppm and 2000 ppm respectively.

B. imbricatum essential oil contained many monoterpenes (85% of identified compounds). Monoterpenes isolated from members of the *Asteraceae* possess a wide spectrum of biological activity (Marles *et al*., 1995) and seem to play a role in plant defence mechanisms. Wilson et *al*. (1997) reported that fungitoxic compounds from the essential oil of plants can be used to control post-harvest diseases of fruits and vegetables.

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

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