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In Vivo Antifungal Activity of the Essential Oil of *Bupleurum gibraltarium* against *Plasmopara halstedii* in Sunflower

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The antifungal activity of the essential oil of the aerial parts of *Bupleurum gibraltarium* was evaluated against *Plasmopara halstedii*. Fungus spores were inoculated in sunflower seedlings, previously treated with several essential oil solutions, and the sporulation percentage was measured after an 11-day treatment. The oil at a concentration of 5.0 mL/L clearly inhibited the fungus sporulation. The contact between fungus sporangia and essential oil was minimized, so it seems that the oil pretreatment could activate the defense response of the sunflower seedlings against the pathogen invasion. The main compounds in the oil were sabinene (31.1%), α-pinene (15.6%), and 2,3,4-trimethylbenzaldehyde (10.9%), among a total of 65 components identified.

KEYWORDS: ***Plasmopara halstedii*; mildew; sunflower; *Bupleurum gibraltarium*; Apiaceae; essential oil composition; antifungal activity; sabinene; α-pinene; 2,3,4-trimethylbenzaldehyde**

INTRODUCTION

It seems that a perceptible tendency toward the utilization of alternative methods for pest and disease control in agriculture has taken place during recent years. Research on plant extracts that might contribute to the development of new agrochemicals, less damaging to the environment and human health, is an interesting field that offers potential beneficial uses in agriculture (*1*). Within the diverse existing phytochemical groups, essential oils have widely proved their important antimicrobial properties (*2*). We herein report on the chemical composition and antifungal activity of the essential oil of *Bupleurum gibraltarium* Lam. (Apiaceae) against *Plasmopara halstedii* (Farl) Berl & de Toni, a common pathogen of sunflower, a culture of great economical importance in the world.

B. gibraltarium is a small evergreen shrub native to central and southern Spain and part of Morocco (*3*, *4*). The composition of the essential oil has been studied on a few occasions (*5*−*7*), and other volatile compounds from a leaf extract (*8*) and several saikosaponins from root extracts have been isolated (*9*, *10*). Rural inhabitants of the areas where the plant grows wild use it in folk remedies (*11*, *12*) and, hence, the antiinflammatory activity of essential oils and extracts (*6*, *9*, *11*) and the in vitro antimicrobial activity of an oil sample (*13*) have been investigated.

Sunflower downy mildew caused by *P. halstedii* is an important disease of this plant, capable of causing losses of >80% of production. It is an obligate parasite with physiological races that attacks a variable range of sunflower genotypes and which, unlike other downy mildew fungi, infects seedling roots rather than foliage to initiate a systemic infection. Several races have been identified in Spain; among them is race 6, occurring in the south, the main sunflower-growing region of the country (*14*). In the United States, the protection afforded by the fungicide Metalaxil against *P. halstedii* (seed pretreatment) is quite impressive and controls this disease at rates as low as 50 ppm. Unfortunately, many fungi have been able to develop tolerance to Metalaxil, and it is also likely to happen with *P. halstedii*. Although there are other effective commercial compounds (*15*) and novel synthetic chemicals (*16*, *17*) have recently been tested for their antifungal activity, it seems that seeking new alternatives to control this fungus is an interesting target.

MATERIALS AND METHODS

Plant Material. The sample of *B. gibraltarium* was collected in September 1996 during the late flowering period near the city of Jaén in southern Spain (30S VG3278). The plant material consisted of the whole aerial parts of several plants formed by stems, leaves, and flowering umbel rays. A voucher specimen (950910) was deposited at the Jaen Herbarium (Universidad de Jaén) and authenticated by us.

Essential Oil Isolation. Air-dried plant material (146.45 g) was ground and hydrodistilled for 8 h using a circulatory Clevenger-type apparatus (*18*). The essential oil obtained was recovered with diethyl ether, dried over anhydrous sodium sulfate, concentrated under a gentle

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Table 1. Chemical Composition of the Essential Oil of Aerial Parts of *B. gibraltarium*

RI[a]	component[b]	% RA[c]	method of ID[d]
856	unknown[e]	0.1	
915	α-thujene	0.4	A, B
923	α-pinene	15.6	A, B
938	camphene	0.1	A, B
938	unknown[e]	0.1	
955	sabinene	31.1	A, B
968	myrcene	2.3	A, B
987	δ-phellandrene	0.1	A, B
997	δ-3-carene	0.1	A, B
1001	α-terpinene	0.7	A, B
1003	unknown[e] + *p*-cymene	0.5	A, B
1012	limonene + 1,8-cineole	7.3	A, B
1012	(*Z*)-β-ocimene	0.5	A, B
1025	(*E*)-β-ocimene	t	A, B
1036	γ-terpinene	1.4	A, B
1047	*cis*-linalool oxide (furanoid) [f]	t	A, C
1064	*trans*-linalool oxide (furanoid) [f]	t	A, C
1067	terpinolene [f]	0.3	A, C
1099	*cis*-*p*-menth-2-en-1-ol [f]	0.1	A, C
1113	*trans*-*p*-menth-2-en-1-ol + *cis*-verbenol	0.1	A, B, C
1117	*trans*-verbenol	0.3	A, B
1117	unknown	0.4	
1124	safranal [f]	0.1	A, C
1137	unknown	0.6	
1148	terpinen-4-ol	2.4	A, B
1157	unknown[e]	0.1	
1169	*trans*-piperitol [f]	0.1	A, C
1180	*trans*-carveol	t	A, B
1188	myrtenyl acetate[f]	t	A, C
1198	citronellol	0.1	A, B
1205	methyl thymol[f] + cuminaldehyde[f]	0.2	A, C
1231	*trans*-chrysanthenyl acetate[f]	0.2	A, C
1238	*cis*-chrysanthenyl acetate[f]	0.1	A, C
1277	2,3,4-trimethylbenzaldehyde[f]	10.9	A, C
1314	citronellyl acetate + 2,3,6-trimethylbenzaldehyde	1.9	A, B, C
1324	unknown[e]	0.5	
1334	α-copaene[f]	0.1	A, C
1360	β-cubebene[f]	0.2	A, C
1393	α-gurjunene[f]	t	A, C
1399	β-caryophyllene	0.4	A, B
1418	α-guaiene[f]	t	A, C
1418	aromadendrene[f]	t	A, C
1427	α-cadinene[f]	t	A, C
1431	α-humulene	0.4	A, B
1438	*allo*-aromadendrene	0.1	A, B
1449	γ-muurolene[f]	0.1	A, C
1455	cadinene isomer[f,g]	0.7	A, C
1455	unknown	0.2	
1470	germacrene B [f]	1.0	A, C
1470	unknown[e]	0.2	
1479	β-bisabolene[f]	0.5	A, C
1487	γ-cadinene[f]	0.8	A, C
1493	δ-cadinene[f]	1.1	A, C
1502	cadina-1,4-diene[f]	0.1	A, C
1513	elemol [f]	1.4	A, C
1530	(*E*)-nerolidol	0.4	A, B
1546	caryophyllene oxide	0.1	A, B
1564	guaiol [f]	3.6	A, C
1579	10-*epi*-γ-eudesmol [f]	0.4	A, C
1596	1-*epi*-cubenol [f]	0.3	A, C
1596	γ-eudesmol [f]	0.7	A, C
1604	cubenol [f]	0.7	A, C
1611	α-muurolol [f]	0.4	A, C
1611	β-eudesmol [f]	0.2	A, C
1617	valerianol [f]	0.3	A, C
1617	guaiol isomer[f,g]	1.2	A, C
1630	bulnesol [f]	1.3	A, C
1639	guaiol isomer[f,g] + isoferulyl isovalerate[f]	0.6	A, C

[a] Temperature-programmed retention indices (RI) referred to *n*-alkanes, determined on methyl silicone capillary column according to ref *23*. [b] Constituents listed in order of increasing RI. [c] Relative area (peak area relative to total peak area); t (traces) indicates percentage values of <0.05%. [d] Method of identification: A, by comparison of the mass spectrum with those of the computer mass libraries; B, by comparison of RI with that of an authentic sample; C, by comparison of RI with those from the literature. [e] Unknown compounds with RI 856, 938, 1003, 1157, 1324, and 1470 could be tentatively identified, according to only the mass spectrum, as 2-methylcrotonic acid,[g] dehydrosabinene, trimethylbenzene,[g] 2,3-dimethylbenzaldehyde, methyl 2,3,6-trimethylbenzoate, and dihydroagarofuran isomer,[g] respectively. [f] Tentatively identified according to only the mass spectrum (method of ID = A) and by comparison of RI with the literature (method of ID = C). [g] Correct isomer not found.

stream of nitrogen, and stored under nitrogen at −0 °C until analyzed (2.79 g, 1.91% yield).

Chemical Analyses. Qualitative data were determined by gas chromatography (GC) and gas chromatography−mass spectrometry (GC-MS).

GC analysis was carried out on a Hewlett-Packard 5890 series II gas chromatograph fitted with a methyl silicone HP-1 capillary column (25 m × 32 mm × 0.17 μm). The oven temperature was programmed from 50 to 200 °C at 3 °C/min. Carrier gas was He with a flow rate of 1.2 mL/min. Temperatures of the injector and FID detector were 200 and 250 °C, respectively. The oil sample was injected using the on-column mode.

GC-MS analysis was carried out on a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5971A mass spectrometer. The GC unit was equipped with a methyl silicone PTE-5 capillary column (30 m × 0.25 mm × 0.25 μm). The oven temperature was programmed from 70 °C (2 min) to 250 °C at 2 °C/min. Carrier gas was He with a flow rate of 1.5 mL/min. The injector temperature was 250 °C, and the oil sample was injected using split mode injection (1:50 ratio). The ion source temperature of the MS unit was 260 °C, and mass spectra were recorded at an ionizing voltage of 70 eV. Quantitative data were determined from the GC peak areas without correction factors. Most constituents were identified by comparison of their mass fragmentation patterns with those found in the built-in spectrometer libraries Wiley.L and NBS54 K.L. Retention indices and authentic standards were also used for further identification. When authentic standards were not available, retention indices compared with the literature values were used (*7*, *19*−*21*).

Antifungal Assays. *Pathogen.* In this work, race 6 of *P. halstedii*, the causal agent of downy mildew, was used.

Plant. In all experiments, Peredovick sunflower seeds were used.

Asepsis and Germination of Seeds. Sunflower seeds were surface sterilized in 70% ethanol for 4 min, rinsed with distilled water, then immersed in 10% household bleach (0.5% sodium hypochlorite) for 20 min, rinsed twice with distilled water, and, finally, rinsed a third time with sterile distilled water. For germination, seeds were rolled up in wet double filter paper, then covered with aluminum foil, and kept at 30 °C for 36 h.

Sunflower Inoculation and Pathogen Maintenance. Inoculation was made following the whole seedling immersion method (*22*) during 3.5 h, with an inoculum density of 90000 zoosporangia/mL. Seedlings were sown in wet perlite and grown in a growth chamber, with 16 h of light/8 h of darkness at 20 °C. After 9 days, pots were covered with plastic bags for 2 days to allow fungus sporulation. Plant cotyledons were cut and stirred in 20 mL of distilled water for several minutes. The concentration of sporangia was calculated using a Neubauer chamber. Sporangia collected from Peredovick plants were used as inoculum source for further treatment of the seedlings.

Pretreatment of the Seedlings with Essential Oil. Essential oil solutions from *B. gibraltarium* were prepared in distilled water with 0.5% v/v Tween 20 added at concentrations of 0 (control), 0.05, 0.5, and 5.0 mL/L. After 36 h, four groups of 60 seedlings each were immersed for 12 h with the four essential oil solutions. After treatment, sunflower seedlings were rinsed several times with distilled water, to eliminate the essential oil, then inoculated with *P. halstedii*, sown in three pots/treatment with 20 seedlings/pot, and cultured as described

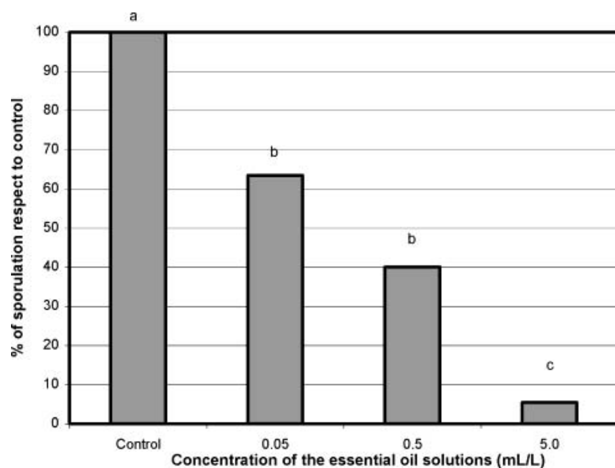

Figure 1. Percentage of *P. halstedii* sporulation after treatment of sunflower seedlings with *B. gibraltarium* essential oil. Bars with different letters denote a statistically significant difference ($p < 0.01$, Neuman–Keul's test).

previously. Nine days later, pots were covered with plastic, and after 2 days, plant cotyledons of every pot were cut and stirred in 20 mL of distilled water for several minutes, and the concentration of sporangia was calculated.

Experimental Design. Three independent experiments were done. Each experiment consisted of three essential oil treatments and a control. Every treatment and control included three different pots with 20 seedlings each.

Statistical Analysis. Data were analyzed using Neuman−Keul's test.

RESULTS AND DISCUSSION

The essential oil used in this work was obtained in a yield of 1.91% by hydrodistillation of the aerial parts of *B. gibraltarium*. The chemical composition is given in **Table 1**, where the constituents are listed in order of their elution from a DB-1-type phase capillary column, and their identification has been carried out by means of GC and GC-MS analyses in combination with retention indices. Sixty-five constituents accounting for 93.9% of the total oil composition were identified. None of the unidentified compounds accounted for >0.6% of the total area. Fourteen monoterpene hydrocarbons (59.8%), 16 sesquiterpene hydrocarbons (5.5%), 18 oxygenated monoterpenes (5.0%), 14 oxygenated sesquiterpenes (10.3%), and 3 diverse components were identified in the oil. The major constituents were sabinene (31.1%), α-pinene (15.6%), 2,3,4-trimethylbenzaldehyde (10.9%), limonene plus 1,8-cineole (7.3%), and guaiol (3.6%). This composition strongly differs from that described by other authors (*5*, *6*). The main components, sabinene, 2,3,4-trimethylbenzaldehyde, and guaiol, have not been described by them. On the contrary, the main components found by them, δ-3-carene/β-pinene (21.1−37.4%), have little presence in the oil studied by us. In addition, ∼60 constituents have been described in our study for the first time in *B. gibraltarium* essential oil.

Three aqueous solutions of this oil sample were used to treat three groups of germinated sunflower seeds. Spores of *P. halstedii* were then inoculated in these seedlings, and the fungal growth inhibition was measured after 11 days.

Results Expression. The essential oil effect on intensity of infection was measured as percentage of sporangia collected from all plants of each pot in relation to those found in plants of control pots (percent sporulation). According to Neuman−Keul's test, all concentrations of essential oil employed significantly reduced (at the 99% confidence level) percent sporangia with respect to untreated control. The essential oil of *B. gibraltarium* at a concentration of 5.0 mL/L practically eliminates fungus sporulation (**Figure 1**), whereas lower concentrations only reduce it. Pairwise comparisons among concentrations of essential oil have also been carried out. As can be seen in **Figure 1**, with the exception of only 0.05 and 0.5 mL/L, the increase of essential oil concentration reduces percent sporangia production with respect to control to different degrees according to the concentration employed.

The most probable hypothesis for sunflower protection given by the essential oil against *P. halstedii* is a direct fungicide effect. Nevertheless, it is worth noting that after essential oil treatment and before inoculation with *P. halstedii*, seedlings were rinsed with distilled water until the odor of the essential oil could not be appreciated, so contact between fungus sporangia and essential oil was minimized. In other words, perhaps it is possible that some chemical component of the essential oil activates, at present unknown, defense responses. This last hypothesis cannot be confirmed with present data but opens a new line of investigation.

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