Antifungal Activity of the Carrot Seed Oil and its Major Sesquiterpene Compounds

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Z. Naturforsch. **59 c**, 791–796 (2004); received July 22/August 8, 2004

Carrot seed oil is the source of the carotane sesquiterpenes carotol, daucol and β-caryophyllene. These sesquiterpenic allelochemicals were evaluated against *Alternaria alternata* isolated from the surface of carrot seeds cultivar Perfekcja, a variety widely distributed in horticultural practise in Poland. *Alternaria alternata* is one of the most popular phytotoxic fungi infesting the carrot plant. The strongest antifungal activity was observed for the main constituent of carrot seed oil, carotol, which inhibited the radial growth of fungi by 65% at the following concentration.

Key words: Carrot Seeds Oil, Carotol, Daucol, Antifungal Activity

Introduction

Nowadays there is a clear tendency towards the utilisation of natural products, especially allelochemicals, as alternative compounds for pest and plant disease control, safe for humans and environment. Therefore, the search of new natural products including plant extracts, which might substitute synthetic agrochemicals or contribute to the development of new agents for pest control, seems to be important. It is well recognised that among other plant products essential oils, rich in terpenoids and non-terpenoid compounds, possess various and interesting allelopathic properties. Their insecticidal action against specific pests and fungicidal action towards some important plant pathogens have been recently reviewed (Isman, 2000).

Over the past decade a large volume of data documenting the defence abilities of various seeds with a long period of dormancy were accumulated (Halloin, 1983; Harman, 1983; Kremer *et al.*, 1984; Ceballos *et al.*, 1998; Özer *et al.*, 1999). Among them antimicrobial activity of chemicals exuded from seeds and acting on soil-rhizosphere interface was reported (Helsper *et al.*, 1994). Quite surprisingly, there is little information about the role and importance of allelochemicals, which are primary constituents of seeds. The question whether these compounds might play any role during the storage, period of dormancy or at the very beginning of seedling development and emergency still remains unanswered. The fungal infections might be considered as the main factor influencing the health of plants at these early stages of their growth and development. Therefore, it is not surprising that many of the sesquiterpenoid compounds are known from their antifungal activity against plant pathogenic fungi, including the most popular *Alternaria* sp. (Alvarez-Castellano *et al.*, 2001; Skaltsa *et al.*, 2000). For example the sesquiterpene fulvoferruginin shows strong activity against Gram-positive bacteria and significant antifungal activity towards *Paecilomyces varioti.* Another carotane sesquiterpene, rugosal A, isolated from *Rosa rugosa*, which is accumulated in the leaf trichomes, shows antifungal activity against *Cladosporium herbarum* (Ghisalberti, 1994).

Large varieties of compounds synthesised in carrot tissues are known also for their allelopathic activity. These include asarones (Guerin and Städler, 1984; Jasicka-Misiak and Lipok, 2000), chlorogenic acid (Cole *et al.*, 1988) and *trans*-2-nonenal (Guerin and Ryan, 1980). In previous reports we described phytotoxic activity of carrot seed oil and its main terpenoic components (Jasicka-Misiak *et al.*, 2002).

To the best of our knowledge, the chemical composition and *in vivo* antimicrobial activities of

0939–5075/2004/1100–0791 $ 06.00

carrot seed oil were investigated several times (Guerin and Reveillere, 1985; Dwivedi *et al.*, 1991; Kilibarda *et al.*, 1996;, Friedman *et al.*, 2002). Although, the information about allelopathic activity of terpenes at all is massively accumulated in the literature, the biological role of carrot seed sesquiterpenes is still poorly defined, especially in the context of the low sensitivity of carrot seeds to fungal infections.

The structures and chemical properties of two most specific carrot seed sesquiterpenes, carotol and daucol, were described (Sykora *et al.*, 1961; Hashidoko *et al.*, 1992; Platzer *et al.*, 1987; Bülow and König, 2000), but nothing could be traced in the literature about their antifungal activity. The limited knowledge concerning the biological activity of daucol and carotol is surprising if considering the fact that they are present in carrot seed oil in high amounts. Moreover, the oil is relatively cheap and commercially available therefore it can be treated as a valuable source of antifungal substances.

In this work the novel, improved procedure of isolation of carotol and daucol is reported and the antifungal activity of these compounds against *Alternaria alternata*, the most popular phytotoxic fungus, was examined.

Materials and Methods

Starting material

Carrot seed oil was purchased from Augustus Oils Ltd., London. β-Caryophyllene was purchased from Aldrich, Poland. The commercially available fungicide Funaben T containing thiuram (45%) and carbendazim (20%) was produced by Chemical Corporation Organika-Azot s.a., Poland. Seeds of *Daucus carota* L. var. Perfekcja, collected in 2001, were purchased from Torseed Co., Torun, Poland.

Isolation of carotol and daucol

Carotol. A portion (10 g) of the carrot seed oil was subjected to silica gel (Merck Kieselgel 60; 0.063–0.2 mm particle size; 650 × 25 mm ID) column chromatography. The column was eluted with CH_2Cl_2, followed by 1% MeOH in CH_2Cl_2 and eluates were collected as 62 × 20 ml fractions. Each fraction was analysed by TLC (plate 5553, Merck; solvent system benzene/EtOAc, 19:1 v/v, developed by spraying with 0.5% anise aldehyde in MeOH, heating at 105 °C for visualisation), and fractions 9–20 having one spot of R_f 0.86, characteristic for carotol, were combined. The fractions were evaporated to dryness (3.35 g) yielding a pale yellow oil, which was identified by means of GC-MS and ^{1}H NMR spectroscopy.

Daucol. It was obtained by a series of silica gel column chromatographic steps. The first step was the same as during carotol isolation. The fractions were controlled by TLC, and fractions 46–55, showing a similar spotting patterns zone (R_f between 0 and 0.32), were combined. After evaporation, the residue (0.19 g) was characterised by GC-MS, showing the presence of 10 compounds. This mixture was applied onto a silica gel column (Merck Kieselgel 60; 0.063–0.2 mm particle size; 300 × 15 mm ID). The column was eluted with CH_2Cl_2/EtOAc (15:1 v/v). Eluates were collected as 26 × 20 ml subfractions, analysed by TLC, and those containing daucol were combined (fractions 10–17). The obtained compound was recrystallised from hexane at 4 °C for 48 h and analysed by GC-MS and ^{1}H NMR spectroscopy.

Structural studies

The analysis of the essential oil was performed using a Hewlett Packard 6890 gas chromatograph equipped with a FID detector. The diethyl ether solution (1 μl) was injected into a HP-1 capillary column (30 m × 0.32 mm bonded-phase fused silica). The initial oven temperature was maintained at 60 °C for 2 min and then raised at 10 °C min^{-1} to 280 °C. Helium was used as carrier gas. MS analyses were performed on a quadrupole Hewlett Packard 6897 instrument with ionisation at 70 eV. The structure of the active compound was found using a peak matching library search to published standard mass spectra and by comparison with literature data.

The NMR experiments were carried out using a Bruker DRX 300 MHz spectrometer. Chemical shifts were referred to TMS (tetramethylsilane). The proton and carbon assignments were performed by means of COSY, TOCSY, HMQC, DEPT-135 and HMQC-TOCSY experiments using a spin lock time in the range 80–120 ms. The TOCSY spectra were acquired with total spin-locking time of 80 ms using the MLEV-17 mixing sequence. In order to assign the carbon signal of the daucol molecule the HMBC spectra were additionally performed.

Antifungal activity

Fungal bioassays in 9-cm Petri dishes were designed to evaluate the influence of tested substances on mycelial growth of *Alternaria alternata.* The mixtures of: carotol, caryophyllene and daucol 8:2:1 w/w/w (at the same weight ratio as it was found in carrot seed oil and in carrot seeds); carotol and daucol 8:1 w/w; carotol and caryophyllene 4:1 w/w; caryophyllene and daucol 2:1 w/w as well as carrot seed oil were tested for antifungal activity. Appropriate amounts of above terpenoids were mixed (before sterilization) with Czapek medium to obtain the final concentration of 150 mg/l. The commercially available fungicide Funaben T was used as a control. The agar was allowed to solidify and experiments were initiated by placing 6-mm fungal plugs taken from the growing margins of 9-day-old cultures, mycelial side down, on Czapek medium. Plates were incubated at 24–25 °C. Radial growth of the strain was recorded daily, by taking the mean diameter of colonies from each plate. The same experiments were carried out for individual compounds: carotol, daucol and caryophyllene. All the experiments were performed four times, with four replications with four pure Czapek medium controls.

Data analysis

The data were subjected to analysis of variance to test the significance of all factors examined in each experiment. F test was done to determine the homogeneity of error variances among runs. Treatment means were separated using Tukey's HSD test at a 5% significance level (Statgraphics® *Plus* 5, 2000).

Results and Discussion

Carrot seed oil composition

The chemical composition of the carrot seed oil was determined by GC-MS analysis. The results are presented in Table I as percent of the total MS ion current. The compounds are listed according to their elution order. Monoterpenes and sesquiterpenes represent the major components of carrot seed oil. Out of 40 compounds detected in the chromatogram of the carrot seed oil 33 were identified. In our studies, only those components which were present in the oil in amounts higher than 0.1% have been taken into consideration.

Table I. Levels (peak area percent) of major components of carrot seed oil purchased from Augustus Oils Ltd.

Component	Carrot seed oil	
	RT[a]	RA[b] (%)
α-Thujene	3.06	1.90
α-Pinene	3.20	3.94
Camphene	3.43	0.92
β-Pinene	3.46	1.90
β-Myrcene	3.65	1.44
α-Terpinene	3.97	1.43
o-Cymene	4.03	1.34
Limonene	4.12	1.75
γ-Terpinene	4.49	1.43
Terpinolene	5.54	0.63
Linalool	6.08	0.51
Pinen-4-ol	7.32	0.42
Terpinen-4-ol	8.16	0.22
3-Carene	8.60	0.83
Neryl acetate	8.69	1.06
Calarene	8.80	3.23
Zingibren	8.99	2.13
α-Farnesene	9.10	3.35
β-Caryophyllene	9.18	10.66
α-Cedrene	9.35	2.74
α-Himachalene	9.44	0.55
β-Cubebene	9.57	0.53
α-Longipinene	9.73	0.76
Aromadendrene	9.93	1.92
β-Farnesene	10.08	4.03
Levomenol	10.18	0.34
Vitamin A aldehyde	10.33	0.66
Isolimonene	10.56	3.24
Caryophyllene oxide	10.97	4.34
Carotol	11.22	38.85
χ-Cadinene	11.49	0.25
Daucol	11.57	2.00
Total		*99.30*

[a] Retention time on HP-1 column in minutes.
[b] Relative area (peak area relative to total peak area).

The main components of the oil were carotol (38.8%) and β-caryophyllene (10.7%), accompanied by caryophyllene oxide (4.3%) and a second daucane sesquiterpene alcohol namely daucol which is present in significant amounts (2.0%). The other identified volatile components have been reported previously as constituents of another organs of carrot (Seifert and Buttery, 1978; Buttery *et al.*, 1979; Kjeldsen *et al.*, 2001). All of them, except the sesquiterpenic alcohols carotol and daucol are well-known compounds isolated from many other plant sources. Especially, β-caryophyllene is a widespread sesquiterpene. Carotol, daucol and β-caryophyllene comprised 51.5% of the oil.

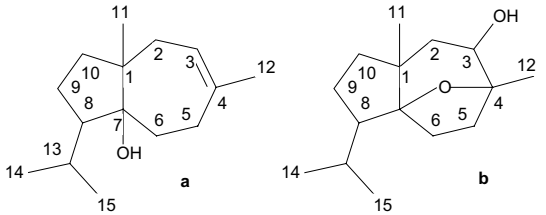

Fig. 1. Structures of carotol (a) and daucol (b).

Isolation of carotol and daucol

The previously described methods for isolation of carotol and daucol are based on fractional distillation (Sykora *et al.*, 1961; Hashidoko *et al.*, 1992). In our opinion column chromatography is a faster and simpler method since carotol was obtained from carrot seed oil by only one step by means of silica gel column chromatography with high efficiency (99%). Fractions with daucol were provided by the same column, however, they required further purification by means of additional silica gel column chromatography. These three chromatographic steps resulted in the isolation of daucol of 99% purity.

^{1}H NMR and ^{13}C NMR spectra of carotol and daucol (Fig. 1) assigned using the set of NMR experiments (see Materials and Methods) are summarized in Tables II and III.(The chemical shifts of proton and carbon peaks for the carotol molecule are in good agreement with those reported in the literature (Bülow and König, 2000; Platzer *et al.*, 1987; Hashidoko *et al.*, 1992). The proton chemical shifts of daucol are also in good consistence with only one up to now published data (Bülow and König, 2000), however, additional assignments for carbon atoms were performed (Table III).

Table II. ^{1}H and ^{13}C chemical shifts of carotol in chloroform ($CDCl_3$).

Number of carbon with its proton	^{1}H[a]	^{13}C[a]	^{1}H	^{13}C
1C		49.09		49.08
2CH_2	1.70/2.26	38.62	1.70/2.26	38.62
3CH	5.32	122.13	5.32	122.13
4C		138.60		138.59
5CH_2	2.08	29.45	2.08	29.45
6CH_2	1.94	34.45	1.63/1.94	34.41
7C		84.55		84.54
8CH	1.80	52.54	1.79	52.53
9CH_2		24.39	1.52/1.68	24.39
10CH_2	1.3	39.45	1.29/1.57	39.45
11CH_3	0.95	21.47	0.95	21.47
12CH_3	1.67	25.23	1.67	25.24
13CH	1.80	27.58	1.81	27.59
14CH_3	1.00	24.04	1.00	24.05
15CH_3	0.95	21.38	0.94	21.38
7C-OH	1.14		1.14	

[a] Bülow and König, 2000.

Table III. ^{1}H and ^{13}C chemical shifts of daucol in chloroform ($CDCl_3$).

Number of carbon with its proton	^{1}H[a]	^{1}H	^{13}C
1C			45.15
2CH_2	1.28/1.68	1.28/1.68	40.09
3CH	3.72	3.74	71.70
4C			85.22
5CH_2	1.36/1.86	1.37/1.86	29.50
6CH_2	1.55/2.15	1.58/2.15	41.15
7C			91.63
8CH	1.50	1.50	52.40
9CH_2	1.70	1.71	26.20
10CH_2	1.25/1.30	1.24/1.28	32.90
11CH_3	1.06	1.06	22.42
12CH_3	1.36	1.36	23.45
13CH	1.77	1.78	31.51
14CH_3	0.81	0.82	21.79
15CH_3	1.06	1.06	22.93

[a] Platzer *et al.*, 1987.

Antifungal activity

The antifungal activity of carrot seed terpenoids was tested on strains isolated from non-disinfected and untreated seeds. Seven strains of fungi belonging to the *Alternaria* family and one strain of *Acremonium* were isolated from the surface of carrot seeds. Thus, *Alternaria* predominated among all the identified genera of fungi. Because the phytopathogenic activity of *Alternaria* towards carrot plants is well documented they were chosen for further experiments.

At the start, we tested the effect of crude carrot seed oil and appropriate mixtures of carotol, caryophyllene and daucol in different weight ratios (see Materials and Methods) on the growth of *Alternaria alternata.* The composition of the mixture of the three terpenoids was set at the same ratio as it was found in crude oil. For comparison the additional tests with the commercially available fungicide Funaben T were also performed. The obtained results are shown in Fig. 2a. Carrot

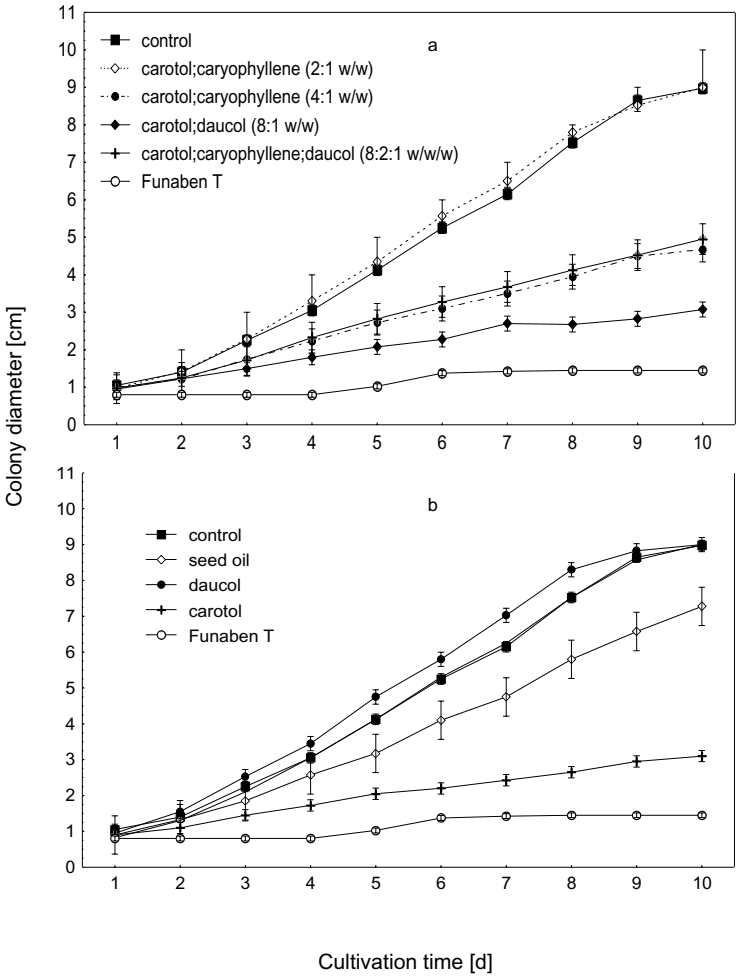

Fig. 2. The effect of tested mixtures (a) and poor substances (b) at 150 mg/l on mycelial growth of *Alternaria alternata* on solid media.

seed oil exhibited moderate inhibitory effects on mycelium radial growth of *Alternaria alternata* (20% of inhibition). Mixtures containing only carotol exhibited strong inhibitory activity. This activity increased with an increasing content of carotol in individual mixtures. The highest inhibition of the growth of pathogenic fungi was determined for a carotol and daucol mixture (66%) in which the content of carotol was the highest (90%).

The experiments with individual substances, namely carotol, caryophyllene and daucol were carried out to find out, whether the observed activity derives from the action of carotol only or from a synergetic nature. The kinetics of inhibition of *Alternaria alternata* by these terpenoids used at the concentration 150 mgl/l in Czapek medium is shown in Fig. 2b. It is clearly seen that these compounds started to influence the fungal growth after the third day of incubation (see the standard deviation bars) and the differences in their action had significantly grown with time. Carotol significantly inhibited the growth of the fungi and reduced the colony radial size by 65% at the 9th day of the experiment. Quite different effects were observed for daucol (second specific sesquiterpene alcohol of the oil) where slight stimulation of the development of *Alternaria alternata* was observed. Widespread in various plants the sesquiterpene β-caryophyllene failed to have any effect. Therefore, it can be concluded that carotol is the main agent attributed for antifungal activity of carrot seeds. The activity of carotol is nearly as strong as of the commercially abailable fungicide Funaben T (85%).

Acknowledgements

This research was supported by Polish State Committee for Scientific Research (Komitet Badań Naukowych) grants PBZ KBN – 060/T09/2001/37. Piotr Młynarz wishes to thank The Foundations for Polish Science for scholarship.

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