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THIN-LAYER CHROMATOGRAPHIC ASSAY OF PHOTOACTIVE COMPOUNDS (FUROCOUMARINS) USING THE FUNGUS *PENICILLIUM EXPANSUM* AS A TEST ORGANISM

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SUMMARY

A simple method using silica gel thin layers for the detection and determination of phototoxic compounds has been developed, using the fungus *Penicillium expansum* as a test organism. The method is suitable for the determination of phototoxic components in mixtures or in impure compounds, as the chromatographic separation and the growth-inhibition test based on photoactivity are carried out on the same thin-layer plate. The highly photoactive linear furocoumarins psoralen, bergapten, xanthotoxin and trioxalen can be detected even more sensitively on the thin-layer plate by means of this photoactivity bioassay than by examination under UV light at 366 nm. The occurrence of xanthotoxin and bergapten as minor constituents in the extract of *Ammi visnaga* could be detected using this method. Sphondin was weakly photoactive. We could not find any photoactivity for the linear furocoumarins imperatorin and isopimpinellin, which have been described by other authors to be slightly photoactive. Samples of these compounds, however, were found to be contaminated with minor amounts of the highly photoactive furocoumarins xanthotoxin and bergapten, respectively.

INTRODUCTION

Some fungi[1,2], green algae[3] and bacteria[4,5] have been shown to be excellent test organisms for assaying the phototoxic properties of furocoumarins and related compounds. The method applied for the detection of photoactivity in all of these studies is similar to that used for testing the antimicrobial properties of compounds. In the photoactivity test, however, the sample to be tested is irradiated with UV light at 366 nm in the presence of the test organism on agar dishes. A limitation of the method on

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agar dishes is that with mixtures (or impure compounds) it is not possible to distinguish between active and inactive components.

In this respect, improvement is achieved by combining a chromatographic procedure with detection of the antimicrobial properties of the separated components of the sample under investigation. Several studies using the same thin-layer plates for a chromatographic separation and the subsequent antimicrobial test have been described[6–9]. In particular, sporulating fungi have been shown to be excellent test organisms for this purpose[6].

In a previous study we used the innoxious fungus *Penicillium expansum* for the visualization of the fungitoxicity of polyene antibiotics and saponins[10] on silica gel thin-layer chromatographic (TLC) plates. To study samples containing photoactive compounds we have developed a similar bioassay directly on TLC plates using *P. expansum* as a test organism. This paper describes this method and the results obtained particularly with furocoumarins and furochromones.

EXPERIMENTAL

UV apparatus

A Desaga UVIS UV–visible spectrophotometer, supplied with two Sylvania F T4/BLB UV lamps (366 nm), was used.

Samples tested

Furocoumarins. The following were used: xanthotoxin (Carl Roth, Karlsruhe, G.F.R.; batch No. 4580277), imperatorin (Carl Roth; batch No. 3006462), trioxsalen (TMP) (Paul B. Elder Co., Bryan, OH, U.S.A.; rec. 23148), psoralen (synthesized by N. J. de Mol, University of Leiden, Leiden, The Netherlands), bergapten and isopimpinellin (isolated by N. J. de Mol from *Heracleum mantegazzianum* Somm. et Lév.), pimpinellin and isobergapten (F. C. Fischer; isolated from fruits of *H. mantegazzianum* Somm. et Lév.) and angelicin (synthesized by N. J. de Mol).

Furochromones. Khellin was obtained from O. P. G. (Utrecht, The Netherlands) and visnagin from Carl Roth (batch No. 1930347; donated by Dr. O. Schimmer).

Plant extracts. The extract of *Heracleum sphondylium* L. ssp. *orsinii* Guss. [1 g of fresh roots per 0.9 ml of 70% (v/v) aqueous ethanol] contained per ml about 0.5 mg of isobergapten, 0.5 mg of sphondin, 0.5 mg of angelicin, 0.2 mg of bergapten, 0.2 mg of xanthotoxin, 0.9 mg of isopimpinellin and 1.6 mg of pimpinellin per millilitre, determined by means of gas–liquid chromatography and high-performance liquid chromatography[11].

Ammi visnaga $D_1 = \varnothing$ [1 g of dried fruits per 10 ml of 70% (v/v) aqueous ethanol] was a commercial plant extract (VSM, Zaandam, The Netherlands).

Sorbent layers. The following pre-coated TLC plates were obtained from Merck (Darmstadt, G.F.R.): silica gel 60 F-254 (5 × 20 and 20 × 20 cm), silica gel silanized F-254 (5 × 20 cm) and aluminium oxide (type T) (5 × 20 cm).

Test organism. Penicillium expansum was used.

Conidial spray suspension[6]

Conidio-spores of the test organism culture, suspended in a solution of inorganic salts and glucose (30%) in water, were used. The salt solution contained 7 g of

potassium dihydrogen orthophosphate, 3 g of disodium hydrogen orthophosphate, 4 g of potassium nitrate, 1 g of magnesium sulphate and 1 g of sodium chloride per litre of water. A conidial suspension of 35 ml (30 ml of salt solution and 5 ml of glucose solution) is sufficient for spraying four TLC plates (20 × 20 cm).

Optimization

Sample solutions. A 1-mg amount of xanthotoxin (8-MOP) was dissolved in 1 ml of methanol, and from this solution 1/10, 1/100, 1/250, 1/500, 1/1000 and 1/2500 dilutions were prepared. Volumes of 2 μl of the sample solutions were applied as spots on 5 × 20 cm silica gel, silanized silica gel and aluminium oxide TLC plates.

Irradiation. Identically prepared TLC plates with the xanthotoxin samples were irradiated with UV light at 366 nm (distance to the UV lamp, 10 cm) either immediately or 24 h after spraying the plates with the conidal spray suspension and for 5, 10, 20, 40, 60, 120 or 240 min.

The influence of the usual laboratory illumination (fluorescent light) on the phototoxic activity was tested only on silica gel TLC plates. The different durations of exposure to laboratory illumination were 0, 10, 20, 40, 60, 120 and 240 min. The distance to the TL tubes (Philips TL 40W/34 De Luxe) was about 4 m.

Incubation. The TLC plates were kept for 2–3 days in a moist atmosphere at about 25°C in the dark.

Evaluation. Photoactivity was observed as white zones of inhibition of growth on the TLC plates. Optimal conditions were judged by comparing the presence and areas of inhibition zones.

Photoactivity bioassays

Sample solutions. Amounts of 1 mg each of TMP, psoralen, bergapten and xanthotoxin were dissolved in 1 ml of methanol and from each of the solutions dilutions of 1/10, 1/100, 1/250, 1/500, 1/1000 and 1/2500 were prepared. Amounts of 1 mg each of angelicin, imperatorin, pimpinellin, isopimpinellin, khellin and visnagin were dissolved in 1 ml of methanol and from each of the solutions dilutions of 1/10, 1/25, 1/50, 1/100 and 1/250 were prepared. From the extract of *Heracleum sphondylium* dilutions of 1/2, 1/5, 1/10, 1/25, 1/50 and 1/100 were prepared.

From each sample solution 2 μl were spotted on the 20 × 20 cm silica gel TLC plates.

Solvent systems. The following solvent systems were used: (a) toluene–ethyl acetate (9:1)[2]; (b) hexane–ethyl acetate (7:3 and 7.5:2.5)[12]; (c) dichloromethane–ethanol (99:1); (d) dichloromethane–ethanol (98.5:1.5); (e) dichloromethane[3]; (f) chloroform[3]; (g) chloroform–ethanol (98.5:1.5)[13]; and (h) ethyl acetate[14].

Development. An unsaturated chamber was used, with a solvent migration distance of 15 cm.

Detection. Detection was effected by using UV light of wavelength 366 and 254 nm, the spots being marked with a pencil, and by photoactivity bioassay.

Photoactivity bioassay. The well dried thin-layer chromatograms were sprayed with the conidial spray suspension and irradiated for 1 h with UV light at 366 nm from a distance of 10 cm to the UV lamp. Incubation was performed by keeping the thin-layer chromatograms for 2–3 days in a moist atmosphere at about 25°C in the dark.

The results were evaluated by observing the presence or absence of inhibition zones (white spots on a green background). Photoactivity of a compound was detected by comparing its effects on two identical chromatograms, one of which had been exposed to UV irradiation (366 nm) and the other kept in the dark.

RESULTS AND DISCUSSION

Optimization

Xanthotoxin (8-MOP, methoxsalen), a compound known to be highly phototoxic to many organisms[2–5], was used as a test compound throughout the pilot experiments and the optimization of the assay. Amounts ranging between 1 and 2000 ng, dissolved in 2 μl of methanol, were applied as spots on the TLC plates. Variables tested included the nature of the sorbent on the TLC plate, the timing of irradiation and the duration of UV treatment. The plates were always incubated in the dark.

On the non-irradiated TLC plates xanthotoxin was found to be not fungitoxic in any of the amounts tested. On the irradiated TLC plates the photo-fungitoxic activity could be optimally demonstrated by using silica gel layers, irradiated with UV light at 366 nm for about 60 min immediately after spraying with the conidial suspension. Amounts down to 2 ng of xanthotoxin were found to be photoactive under these test conditions; amounts as small as this remain undetected as far as their fluorescence under UV light at 366 nm is concerned before spraying with the conidial

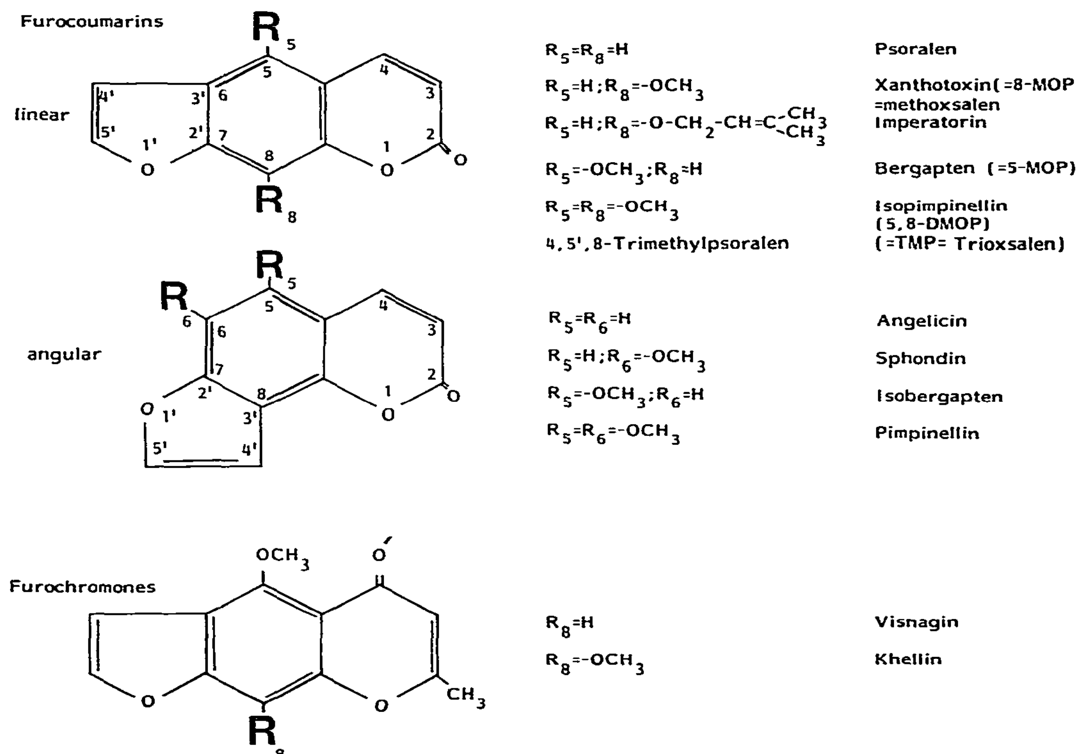

Fig. 1. Structural formulae of the furocoumarins and furochromones investigated.

suspension (the fluorescence detection limit is about 20 ng). A shorter period of irradiation and also a much longer period (*e.g.*, 4 h) reduce the sensitivity of the detection of photoactivity. Irradiation 24 h after spraying with the conidial suspension also results in a decrease in sensitivity of detection. The inhibition zones are larger, however, probably owing to increased diffusion of xanthotoxin.

On aluminium oxide plates the bioassay is also much less sensitive and the inhibition zones are much larger. Moreover, the mycelium covers aluminium oxide plates less smoothly than it covers silica gel plates.

On silanized silica gel plates photo-fungitoxic activity cannot be detected for any of the amounts of xanthotoxin tested. The phenomenon that fungitoxic compounds like polyene antibiotics and saponins fail to show their activity in a bioassay on silanized silica gel layers has been described earlier[10].

Although less effective than long-wavelength UV light (366 nm), irradiation with daylight and fluorescent lighting (TL tubes) also induce xanthotoxin to exhibit photofungitoxic activity on silica gel TLC plates. Xanthotoxin applied to a silica gel plate and exposed for 1 h to ordinary room illumination with fluorescent light (TL tubes) on the laboratory bench exhibits comparable photoactivity to that when it is

(a)

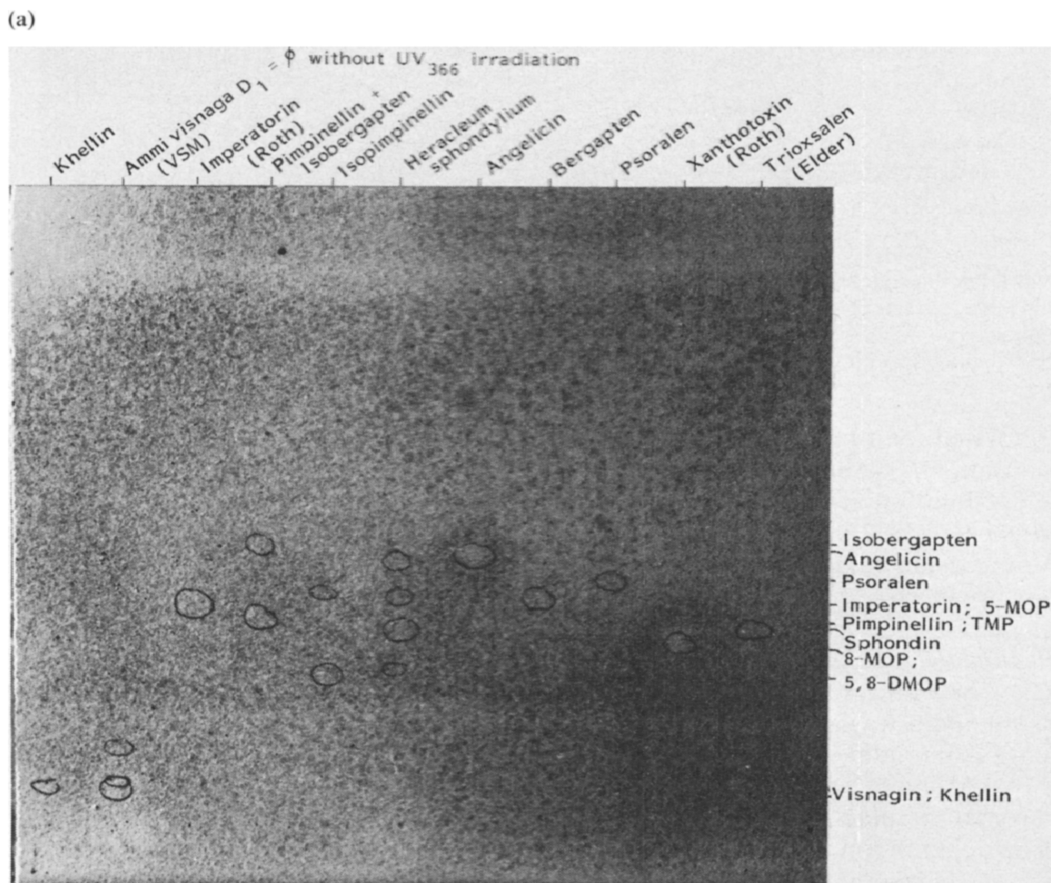

Fig. 2.

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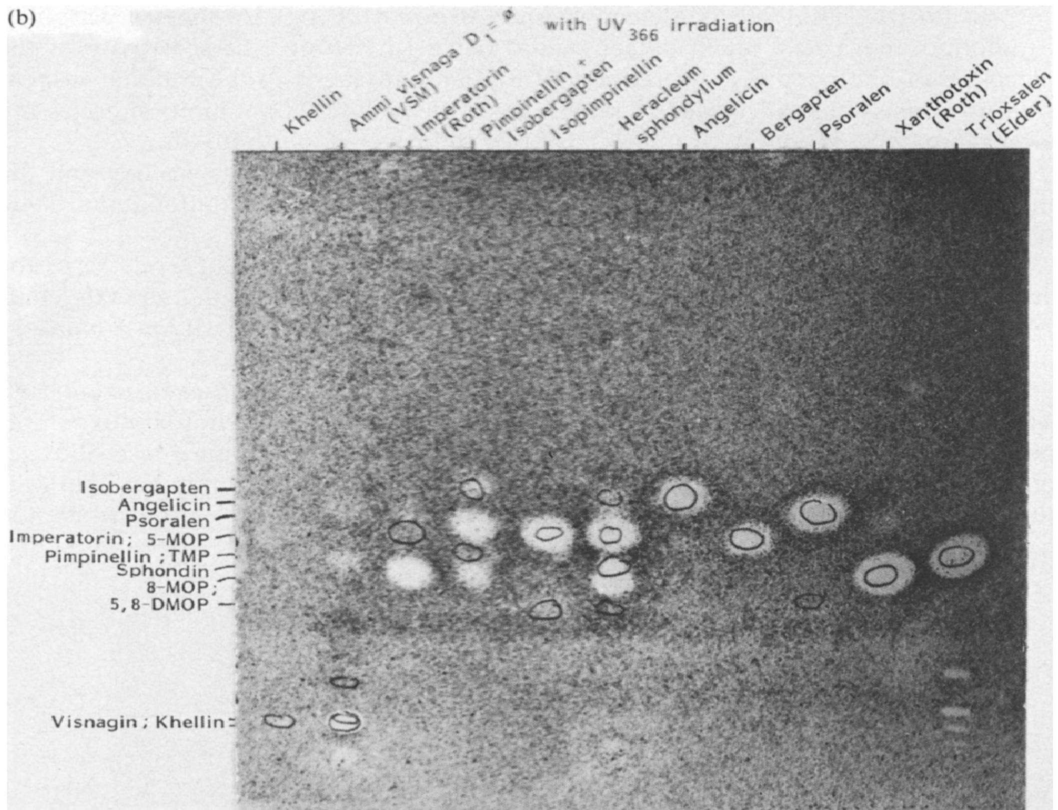

Fig. 2. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and of the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system c. Ringed areas show areas of compound spots detected under UV light (366 nm), before spraying with the conidial suspension. (a) Without UV irradiation; (b) with UV irradiation (366 nm).

irradiated for 10 min with UV light at 366 nm; under these test conditions 20 ng of xanthotoxin is about the smallest amount that shows photoactivity.

Johnson *et al.*[9] found xanthotoxin to be strongly fungitoxic against *Helminthosporium carbonum*. Our experiments indicate that incomplete exclusion of light during the biological tests caused the fairly high fungitoxicity of xanthotoxin for this test organism.

Photoactivity bioassays of the samples after thin-layer chromatographic separation

Several furocoumarins and furochromones (Fig. 1) were tested. As no sample of sphondin was available, an extract of *Heracleum sphondylium* with a known content of sphondin was used for the bioassay of this compound.

A limitation of the bioassay is set by the solvents which can be used in the chromatographic separation procedure on thin layers prior to irradiation and the growth inhibition test. In general, only solvents that can be evaporated quantitatively and easily from the thin layer and solvents that are non-fungitoxic themselves can be used[6].

Different solvent systems were tested for this purpose and the results obtained with three different solvent systems are shown in Figs. 2–4. None of the solvent systems tested separated all compounds. Hence, different solvent systems have to be used in order to characterize mixtures of furocoumarins and furochromones.

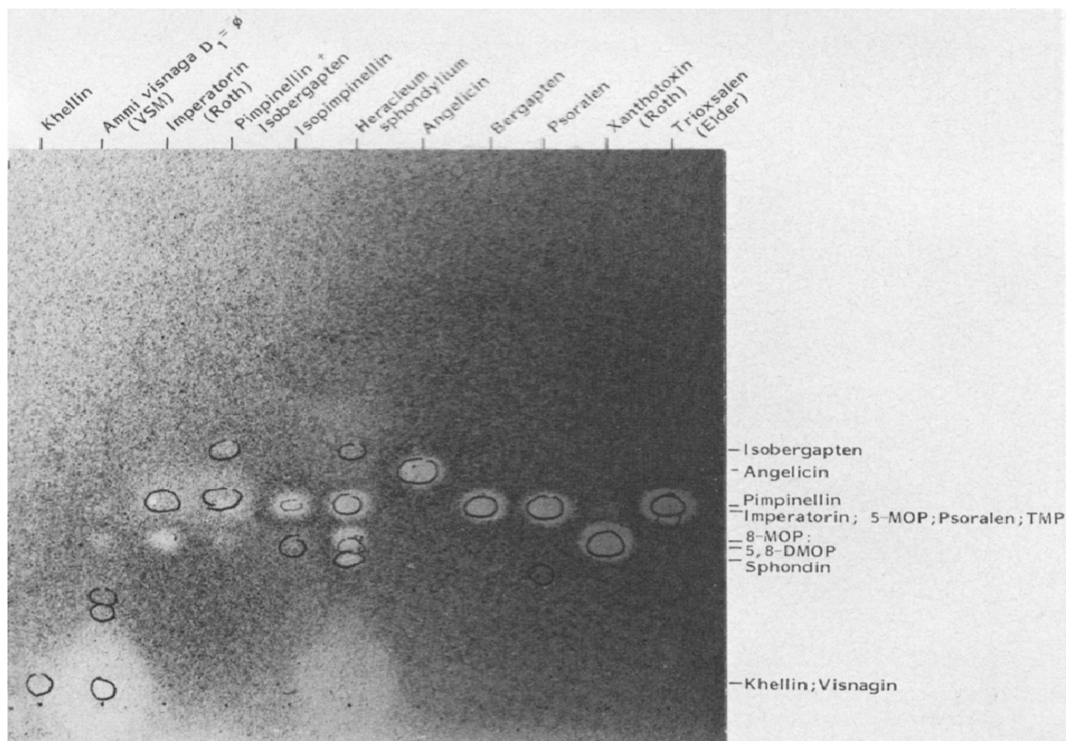

Fig. 3. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and of the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system a. Ringed areas as in Fig. 2.

With the solvent systems tested only psoralen and bergapten could be separated and even then hardly with solvent systems containing chloroform or dichloromethane. Dichloromethane–1% ethanol gave the best results (Fig. 2).

With the solvent systems tested only ethyl acetate[14] is able to separate visnagin and khellin from each other; however, under these conditions most other compounds are not separated from each other.

In our experiments khellin, pimpinellin, isopimpinellin and imperatorin were found not to be photo-fungitoxic in the amounts tested. The samples of the other compounds were tested over a wide range of concentrations after chromatographic development with solvent systems a and c.

The minimal amounts for which photo-activity is detectable were determined. The detection limits of a compound based on its photo-fungitoxicity were compared with those based on its fluorescence under UV light at 366 nm on the chromatogram before spraying with the conidial suspension. The results are summarized in Table I

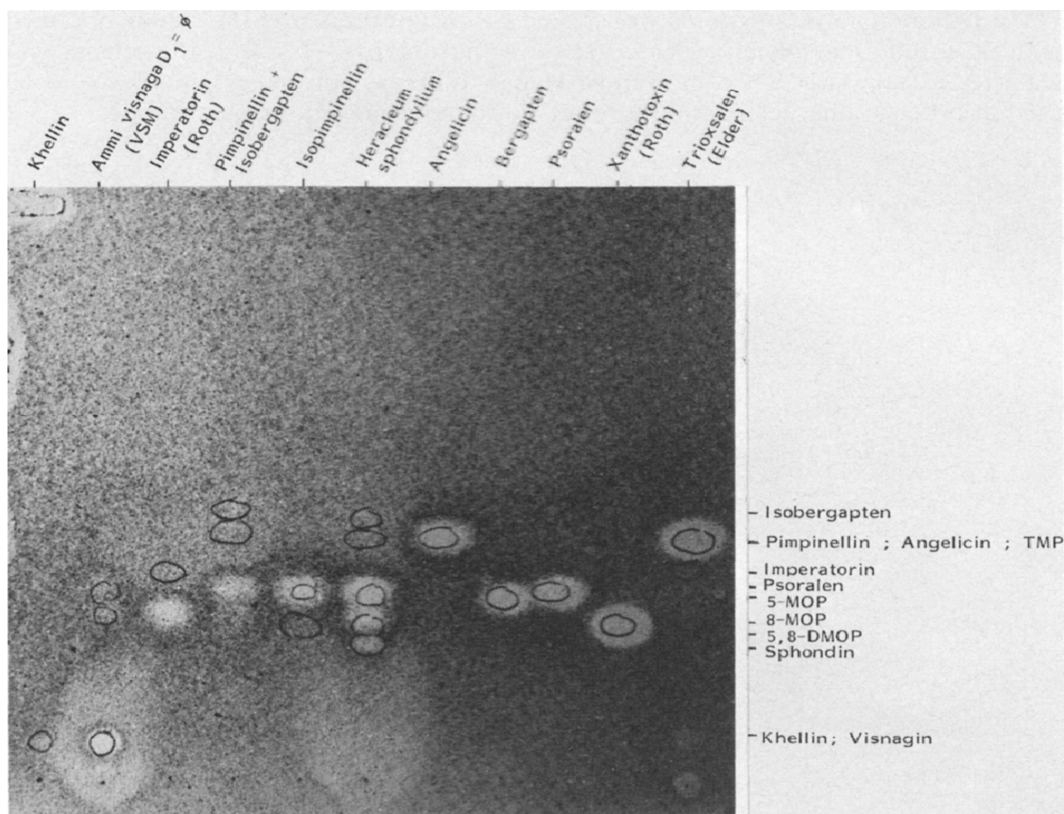

Fig. 4. Photoactivity bioassay of the sample solutions of the furocoumarins and furochromones (1 mg/ml) and the extracts of *Ammi visnaga* and *Heracleum sphondylium* on silica gel TLC plates after development with solvent system b. Ringed areas as in Fig. 2.

and an example of these experiments with TMP is shown in Fig. 5. It can be seen that the sample of TMP contains at least three other photoactive compounds. Although the detection limits of the compounds are in principle dependent on the extent of diffusion on the TLC plate, as a consequence of chromatographic development there is no great difference in the results obtained with the two solvent systems tested.

As shown in Table I, xanthotoxin, bergapten, psoralen and TMP are strongly phototoxic towards the test organism. These compounds can be detected even more sensitively by means of the photoactivity bioassay than by examination under UV light at 366 nm. This demonstrates that the photoactivity bioassay is suitable as a very specific and for some compounds also very sensitive detection method for photoactive compounds on thin-layer chromatograms.

With this method the occurrence of xanthotoxin and bergapten as minor constituents in the extract of *Ammi visnaga* (Figs. 2–4) could be detected. The results of an investigation of several commercial plant extracts and also extracts of plants belonging to the Umbelliferae and Rutaceae will be published elsewhere[11].

Most of the data given in Table I are in good agreement with literature data

TABLE I

DETECTION LIMITS OF FUROCOUMARINS AND FUROCHROMONES ON SILICA GEL TLC-PLATES AFTER DEVELOPMENT WITH SOLVENT SYSTEM a OR c, DETERMINED UNDER UV LIGHT AT 366 nm AND WITH THE BIOASSAY

Compounds	*UV (366 nm)*		*Bioassay detection limit (ng)**	
	Colour	*Detection limit (ng)**	*With UV irradiation (366 nm)***	*Without irradiation***
Furocoumarins				
(a) Linear				
Psoralen	Blue	40–80	4–8	–***
Bergapten (5-MOP)	Yellow	20–40	4–8	–***
Xanthotoxin (8-MOP)	Yellow	20–40	2–4	–***
Isopimpinellin (5,8-DMOP)	Brown	80–200	–***	–***
Imperatorin	Yellow	40–80	–***	–***
TMP	Blue	4–8	1–2	–***
(b) Angular				
Angelicin	Blue	80–200	80–200	–***
Isobergapten	Yellow	20–40	±(2000)	–***
Pimpinellin	Brown	80–200 §	–***	–***
Sphondin	Blue	10–20	40–100	–***
Furochromones				
Khellin	Brown	10–20	–***	–***
Visnagin	Yellow	10–20	10–20	–***

* The higher figure represents an easy detectable amount; the lower figure represents a non-detectable amount.

** –, Inactive; ±, very weakly active.

*** Maximum amount tested = 2000 ng.

§ More sensitively detectable in UV light at 254 nm: detection limit = 8–20 ng.

concerning photoactivity against microorganisms[1–5,15] and skin photo-sensitizing activity[16,17]. However, our results did not agree with data on photoactivity described for imperatorin[2,3] and for isopimpinellin[4]. Imperatorin itself showed no photoactivity in our bioassay, but the sample we used was found to be contaminated with xanthotoxin (Figs. 2–4). This casts some doubts on reports of a low photoactivity for imperatorin[2,3]; in at least one instance the sample used[3] was from the same source as ours. Our sample of isopimpinellin was slightly contaminated with bergapten (Figs. 2–4); the same sample was used by De Mol[4], who found very weak photoactivity for this sample.

Sphondin was weakly photo-active. To our knowledge, no data have been published previously concerning the photoactivity of this compound.

Although furocoumarins have been described as stable in long-wavelength UV light, in contrast to the also highly photoactive phenylheptatriyne[18], a negative result in the photoactivity bioassay could be due to instability of the compound in UV light at 366 nm. To test this, UV spectra of the compound adsorbed on silica gel plates were measured before and after 2 h of UV irradiation at 366 nm. The spectra of all compounds except imperatorin coincided before and after irradiation. Although

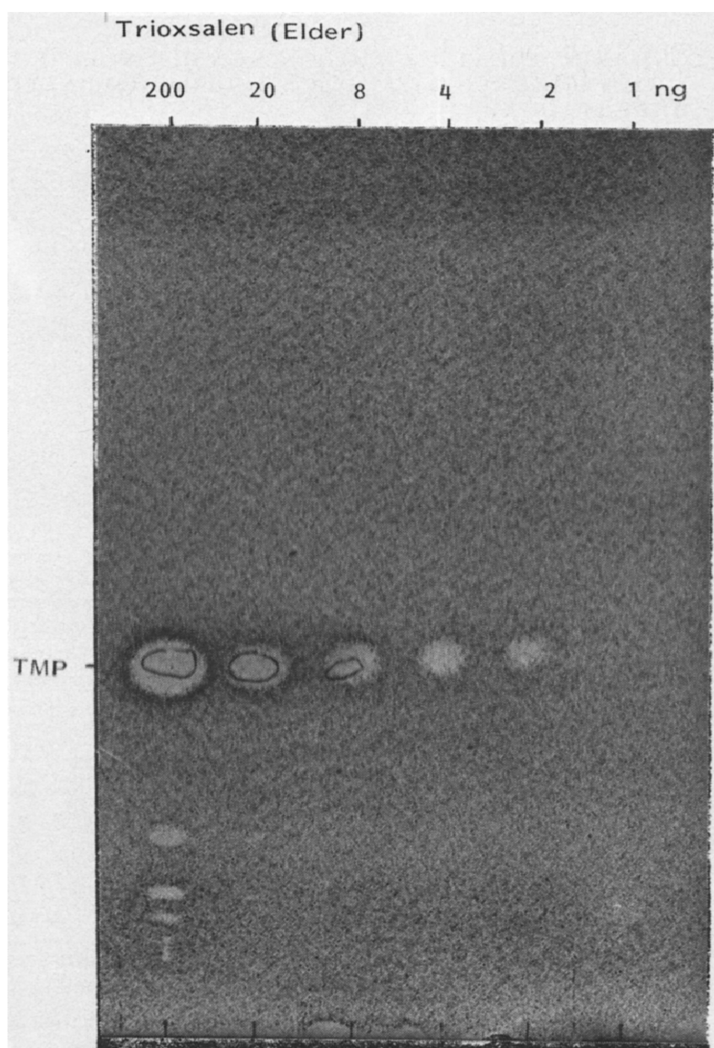

Fig. 5. Photoactivity bioassay of different amounts of trioxsalen (Elder) on silica gel TLC plates after development with solvent system c. Ringed areas as in Fig. 2.

the colour of the spot of imperatorin turned yellow, only a small part of the imperatorin decomposed; on the thin-layer chromatogram, developed two-dimensionally with the same solvent system before and after irradiation, the amount of imperatorin had not diminished appreciably and the decomposition product was observed as a non-migrating yellow spot.

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