Isolation and structure elucidation of xanthotoxin, a phototoxic furanocoumarin, from *Peucedanum galbanum*

N. Finkelstein,† C.F. Albrecht and P.P. van Jaarsveld*

Department of Pharmacology, Faculty of Medicine, University of Stellenbosch, P.O. Box 19063, Tygerberg, 7505, Republic of South Africa

† Present address: c/o Professional Services Division, S.A. Druggists Ltd., P.O. Box 45, Observatory, 7935, Republic of South Africa.

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The major vesicant principal of *Peucedanum galbanum* has been isolated for the first time and its structure confirmed as xanthotoxin on the basis of melting-point and spectroscopic evidence.

Die hoofkomponent wat vir die ontwikkeling van fitodermatitis na kontak met die blare van *Peucedanum galbanum* verantwoordelik is, is geïsoleer en die struktuur is bevestig as xantotoksien op grond van smeltpunt en spektroskopiese eienskappe.

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* To whom correspondence should be addressed.

Introduction

Peucedanum galbanum (L.) Benth. & Hook.f. (blister bush, Afr. 'bergseldery') is a robust, branching shrub up to 2.5 m tall with sessile, compound yellowish-green leaves, and bears diamond-shaped leaflets on waxy stems. The leaves are reminiscent of celery – hence the Afrikaans vernacular name. The inflorescence is a characteristic, compound umbel (Apiaceae was formerly known as Umbelliferae) with insignificant greenish-yellow flowers in summer. It occurs on rocky upper slopes, often in moist, sheltered places, and is distributed in mountain fynbos from Piketberg through the Cape Peninsula to Riversdale (Adamson 1950; Bond & Goldblatt 1984; Burman *et al.* 1985).

An infusion of the plant is used as an abortifacient, whilst a douche or local steaming is regarded as a remedy for miscarriages and an aid in the expulsion of retained placenta. In some parts of the country the infusion is co-administered with *Mentha longifolia* and *Pelargonium grossularioides* for suppression of the menses. When used in conjunction with *Diosma vulgaris*, it is claimed to act as a diuretic in dropsy and renal diseases (Watt & Breyer-Brandwijk 1962). In the Western Province, an infusion is used for vesical catarrh, all kidney and bladder ailments (including kidney stones), prostate problems (J.N. Eloff & D.T. Longland, 1987, pers. commun.), swelling of the glands and retention of urine (Watt & Breyer-Brandwijk 1962).

The infusion is a diaphoretic and several authors state that the plant *per se* produces dermatitis and blisters (vesicant effect) 40 – 50 h after contact with the bare skin (Watt & Breyer-Brandwijk 1962). Many authors (Adamson 1950; Levyns 1966; Moll & Scott 1981; Jackson 1980, 1982; Bond & Goldblatt 1984; Burman *et al.* 1985) who wrote on the Cape Flora, described a blistering cutaneous condition with erythema and vesicular/bullous lesions after exposed parts of the body (Quail 1983) come into contact with *P. galbanum*. Floral and hiking guides for the Cape Peninsula and its environs (Quail 1983; Lundy 1986) sound a similar cautionary note about this plant.

Levyns (1966) and Jackson (1982) were of the opinion that the unsightly blisters are only produced in susceptible people, thereby inferring that the condition is probably an allergic contact dermatitis. Jackson (1980) suggested that after exposure to the leaf, sunlight is required for vesiculation, hence the medical term to describe the condition as phytophotodermatitis. However, he qualified his postulate by stating that no definitive experiments had verified this. It has been observed that the manifestations of skin irritation are delayed for a day or two. The worst reaction occurs when the victim is perspiring (combination of heat and dampness) or when the leaves are bruised (Burman *et al.* 1985; Smith 1966).

Jackson (1982) asserted that the reaction is delayed as itching only occurs 2 – 3 days after exposure, followed by blisters which heal slowly and leave pigmented patches for several weeks. It seems uncertain whether everybody is susceptible, whether the bush is toxic at all seasons and whether sunlight is necessary for blistering to take place. The nature of the vesicant substance has also not been established (Jackson 1982; Watt & Breyer-Brandwijk 1962).

Early chemical investigations showed that the leaf yields a light-brown volatile oil with a characteristic aromatic odour. It was initially thought that the oil was responsible for the blistering action. Subsequent investigations have shown that the oil neither irritated the oral mucosa nor produced irritation on the skin, and it was suggested that the irritant properties are due to a non-volatile constituent. The leaf and stem of the plant were reported to contain an alkaloid hesperidine (sic) which has a vitamin P-like action (Watt & Breyer-Brandwijk 1962).

In this paper the isolation and structure elucidation of xanthotoxin as the major photosensitizer in *P. galbanun*, is described.

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Materials and Methods

UV spectra were recorded in ethanol with a Hewlett Packard 8451A diode array spectrophotometer, and IR spectra (KBr disc) on a Nicolet 5DXC FTIR spectrophotometer. ^{1}H-NMR spectra (90 MHz) were executed in $CDCl_3$ with a Brucker 90 instrument with TMS as internal standard. Mass spectra were measured at 70 eV on a VG F16 single focusing mass spectrometer. Melting points (uncorrected) were determined on a Kofler hot stage. Column chromatography was performed using Merck silica gel (Cat. No. 7754) and thin-layer chromatograms (TLC) were run on plates coated with silica gel (Kieselgel G Merck) and developed with toluene/ethyl acetate (8:2) at ambient temperature.

Plant material

The aerial parts of *P. galbanum* (L.) Benth. & Hook.f. were collected in March 1986 on Table Mountain (Newlands Ravine area) at approximately 700 m above sea-level and were air-dried under cover for 4 weeks. The plant was authenticated by Mrs Anne Bean, a botanist on the staff of the Bolus Herbarium, University of Cape Town, and a voucher specimen (No. 40531) was lodged there for reference.

In vitro phototoxicity test

Concentrated extracts of plant material and column fractions thereof were dissolved in chloroform (1% m/v) and small discs of filter paper (5 mm diameter) were dipped into the solution. The discs were dried in a vacuum desiccator and carefully placed on the surface of Sabouraud's Agar impregnated with a 24-h culture of *Candida albicans* (Weimarck & Nilsson 1980). After removal of the lids, the Petri dishes were irradiated with long-wave UV light (365 nm) 25 cm from the source for 12 h. Controls containing discs soaked in chloroform only and dried were similarly irradiated. One set of discs was kept in the dark. All plates were incubated for 24 h at 25°C and examined for zones of inhibition.

In vivo phototoxicity test

Any extract showing a positive *in vitro* test was applied with a cotton bud as a 0.1% m/v solution in ethanol to the flexor surface of the right forearm of a human volunteer. The exposed arm was subjected to bright sunlight for 4 h. Another application, which served as a control, was made to the left forearm, but covered to prevent exposure to sunlight. Observations for erythema and vesicles were recorded at 24, 36 and 48 h after exposure.

Comminution and extraction of plant material

The dried leaves were separated from the other aerial parts (stems, twigs and umbels) and 700 g of coarsely comminuted material was successively extracted for 36 h with 15 l each of light petroleum (b.p. 40 – 60°C), chloroform and methanol in a Soxhlet extractor (Quickfit large-scale extractor 11EX). The extracts were each concentrated under reduced pressure.

The light-petroleum extract on reduction in volume yielded 22 g of a waxy, white deposit which did not test positively for phototoxicity. The concentrated methanolic extract (63.4 g) was also not phototoxic *in vitro*. The chloroform extract (49.3 g), however, tested positive *in vitro* and was purified further as follows: 6 g of this extract was adsorbed on a silica gel column (120 g) and eluted with chloroform/light petroleum (1:1), pure chloroform, chloroform/ethyl acetate (1:1) and methanol. Like fractions (as established by TLC) were pooled and each pooled fraction was tested for phototoxicity *in vitro*. The positively phototoxic fractions 23 – 31 (3.91 g) were rechromatographed on another silica gel column (100 g) and eluted with benzene/ethyl acetate (9:1). 50-ml fractions were collected and like fractions pooled. After concentration *in vacuo*, fraction 8 yielded a solid deposit (290 mg), which after several recrystallizations from chloroform/light petroleum (b.p. 40 – 60°C), afforded colourless prisms of xanthotoxin (Structure I in Figure 1) with m.p. 145 – 147°C (60 mg). The compound exhibited a single, yellow fluorescent spot on TLC (*Rf* = 0.34) and produced a mauve to purple colour when sprayed with modified Dragendorff's reagent (Macheboeuf & Munier 1951).

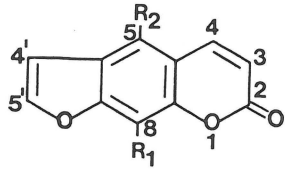
Results and Discussion

As bruised leaves were reported to be responsible for cutaneous blistering (Smith 1966), the dried leaves were carefully separated from other aerial parts and comminuted. Because phototoxic constituents had previously been isolated from the roots and fruits of other umbelliferous plants (Varga *et al.* 1979; Ceska *et al.* 1987), it was prudent to establish whether the phototoxicity in fact was associated with the leaves.

The successive extractions in solvents of increasing polarity affected crude separation. The methanolic extract showed no phototoxicity, thus suggesting the absence of polar photosensitizing compounds. The white crystalline deposit obtained from the light-petroleum extract was likewise not phototoxic. In most phytochemical investigations this deposit is commonplace and is generally attributed to a complex mixture of neutral plant fats and waxes.

TLC of the concentrated chloroform extract revealed several fluorescent spots which produced a positive colour reaction when the plates were sprayed with modified Dragendorff's reagent (Macheboeuf & Munier 1951). This evidence, coupled with a strongly positive phototoxicity test, suggested the presence of furanocoumarins (Murray *et al.* 1982a). This deduction is further strengthened by the fact that the plant family Apiaceae is known to accumulate various classes of secondary metabolites, *inter alia* coumarins, chromones, terpenoids, etc. (Varga *et al.* 1979).

Column chromatography on silica gel proved successful in separating these fluorescent compounds from other plant constituents and culminated in the isolation of xanthotoxin (structure I in Figure 1), the structure of which was



I Xanthotoxin: $R_1 = OCH_3$; $R_2 = H$
II Psoralen: $R_1 = R_2 = H$
III Bergapten: $R_1 = H$; $R_2 = OCH_3$

Figure 1 Structures of phototoxic furanocoumarins.

confirmed by comparison of its physical properties and spectral data with that reported in the literature (Kutney *et al.* 1972). Another closely related furanocoumarin is 5-methoxypsolaren (bergapten, structure **III**, Figure 1). However, its m.p. is 188 – 191°C, which is distinctly higher than that of xanthotoxin.

Xanthotoxin crystallized as colourless prisms with m.p. 145 – 147°C, and has the formula $C_{12}H_8O_4$ [M^+ 216). Like other coumarins and furanocoumarins (Murray *et al.* 1982a), it exhibited a yellow fluorescence under UV light (365 nm). It also gave a positive mauve to purple spot on TLC with modified Dragendorff's reagent (Macheboeuf & Munier 1951). This false positive reaction with an essentially alkaloidal reagent has been reported for non-nitrogenous compounds possessing ketone, aldehyde or lactone functions (Farnsworth *et al.* 1962).

The ultraviolet absorption bands at λ222, 250 and 302 nm are suggestive of a furanocoumarin (Murray *et al.* 1982b). The log ε values are also in close agreement with those reported (Kutney *et al.* 1972).

The presence of strong absorption bands at 1717 and 1713 cm^{-1} in the infrared spectrum are characteristic of a pyrone–carbonyl stretching frequency and coumarinyl lactone (α-pyrone), respectively, while the strong absorption at 1589 cm^{-1} is due to C=C stretching frequency of the pyrone ring. The two peaks at 1154 and 1102 cm^{-1} are considered to be characteristic C—O stretching vibrations for the furan group (Murray *et al.* 1982b). The peaks recorded accord well with those recorded in the literature (Scheel *et al.* 1963; Kutney *et al.* 1972).

The ^1H NMR spectrum of xanthotoxin defined all 8 protons. The two doublets at δ 6.33 and 7.73 (J = 9.5 Hz) are typical of a coumarin nucleus unsubstituted in the pyrone ring, and are attributed to the *cis* protons H-3 and H-4 of this ring. In coumarins lacking an oxygen function at C-5, the H-4 doublet occurs at δ 7.5 – 7.9 (in $CDCl_3$), whereas the H-4 resonance is shifted downfield to δ 7.9 – 8.2 ($CDCl_3$) should C-5 bear an oxygen function (Murray *et al.* 1982b). This is further corroborated by the presence of a one-proton singlet at δ 7.32. The signal consisting of three protons at δ 4.26 is assigned to one aromatic methoxyl group which, from an earlier deduction, must be sited on C-8. The two doublets at δ 6.79 and 7.66 (J = 2.5 Hz) are typical of furanocoumarins with the benzene ring substituted *ortho* to the furan oxygen and are attributed to the *cis* protons H-4′ and H-5′ of this ring. The signals obtained were virtually identical to those obtained by other investigators (Kutney *et al.* 1972).

The mass spectrum of xanthotoxin supported the structural evidence already presented. The molecular ion (M^+) at *m/e* 216 and the other fragments at 201 (loss of CH_3), 188 (loss of CO), 173 (loss of CH_3 + CO) and 145 (loss of CH_3 + 2 × CO) confirms the molecular structure. This is consistent with the literature values (Kutney *et al.* 1972). The expected loss of 15 atomic mass units occurs as a result of ejection of a methyl (–CH_3) radical of the aromatic methoxyl substituent, while the successive eliminations of 28 atomic mass units (CO) is characteristic of coumarins (Murray *et al.* 1982b). The ^1H NMR spectrum suggested no substitution at C-5, hence the only other possible substitution must be at C-8. Furthermore, since the m.p. of 5-methoxypsoralen (bergapten) is 188 – 191°C, the spectral evidence and melting point confirm that the compound isolated from *P. galbanum* must be 8-methoxypsoralen or xanthotoxin.

Among the natural linear furanocoumarins, xanthotoxin follows psoralen in photosensitizing potency when these two compounds are subjected to solar or 365-nm UV radiation.

Xanthotoxin intercalates readily into DNA and forms photocycloadducts with the participation of the 3 and 4 positions of xanthotoxin and positions 5 and 6 (double bond) of the pyrimidine bases. Such photosensitizing effects are not confined to skin DNA, but have also been reported for bacterial fungi, DNA viruses and bacteriophages (Murray *et al.* 1982c). This effect therefore makes *Candida albicans* a suitable and rapid *in vitro* model for the establishment of phototoxicity. As no toxic effects occurred in the control series of dishes kept in the dark, it follows that any inhibition of *Candida* growth in UV light is due to phototoxicity.

When sunlight was excluded from the skin area in the *in vivo* phototoxicity test, no erythema or vesiculation was observed, thus confirming the indispensability of light plus a photosensitizer (xanthotoxin) for the induction of phytophotodermatitis.

Conclusions

Although xanthotoxin is not a novel furanocoumarin, this is the first report of its isolation from the leaves of *P. galbanum*. The phytophotodermatitis reported after contact with the bruised leaves of this plant in the presence of sunlight can now be conclusively explained. The severe reaction on sweaty (humid) skin is probably due to the relative ease of adherence of the plant sap under those conditions. The observation that the phototoxicity is greater at certain times of the year can possibly be attributed to the duration and intensity of solar radiation or seasonal variation in the concentration of xanthotoxin. The lack of reaction by certain individuals is undoubtedly due to incomplete or superficial contact with the leaf and/or insufficient exposure to solar radiation.

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