APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1978, p. 819-823
0099-2240/78/0036-0819$02.00/0

Vol. 36, No. 6
Printed in U.S.A.

Production of Xanthomegnin and Viomellein by Species of *Aspergillus* Correlated with Mycotoxicosis Produced in Mice

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Received for publication 2 October 1978

By using thin-layer chromatography and infrared spectroscopy, xanthomegnin and viomellein have been isolated and identified from species of the *Aspergillus ochraceus* group. A correlation was established between the occurrence of these fungal quinones in the fungal cultural products and the ability of these products to induce mycotoxicosis in mice. In addition, a method was employed to estimate the amount of xanthomegnin and viomellein produced by the fungi.

Xanthomegnin and viomellein have been isolated from a toxigenic strain of *Penicillium viridicatum*. These fungal metabolites have been shown by Carlton et al. (4) to produce hepatic lesions in mice which were identical to those obtained from crude extracts of *P. viridicatum* cultures (6).

Zimmermann et al. (14) have demonstrated that the cultural products of an isolate of *Aspergillus ochraceus* causes the development of hepatic lesions identical to those produced in mice and rats by products of *P. viridicatum*; consequently, these workers suggest that *A. ochraceus* produces either xanthomegnin, viomellein, or chemically similar metabolites. Although xanthomegnin was first isolated from species of *Trichophyton* (1, 10, 13) and is found with the chemically related viomellein in cultures of *Aspergillus melleus* and *Aspergillus sulphureus* (7), these compounds have not been reported to occur in *A. ochraceus*. It was the objective of this study to investigate strains of *A. ochraceus*, as well as other species of the *A. ochraceus* group, for the production of xanthomegnin and viomellein and for the occurrence of characteristic hepatic lesions associated with the toxic manifestations produced when these fungal quinones are fed to mice.

MATERIALS AND METHODS

Culture procedures. The strains of organisms used in this study are listed in Tables 1 and 2. *P. viridicatum* Purdue 66-68-2 is a known toxigenic strain which was isolated at Purdue University from infected corn (6). *A. ochraceus* M-298-SC and *Aspergillus ostianus* M-298-PM were obtained from A. F. Schindler and P. B. Mislivec, respectively, both from the Food and Drug Administration, Washington, D.C.

In the preparation of rice cultures, 2-liter flasks containing 280 g of rice and 128 ml of water were autoclaved at 121°C for 45 min. The sterilized substrate was inoculated with spores of the fungus and incubated at 23°C for 17 days. After treating the cultures with 1% propionic acid to kill the fungus, the cultures were dried at 40°C for 5 days.

Fungal mats were obtained from cultures grown in 500-ml flasks containing 100 ml of liquid medium consisting of Czapek medium 57 (12) containing 1% corn steep solids and 30 g of cerelose per liter and with the pH adjusted to 7.0 with 1 N NaOH. The medium was autoclaved at 121°C for 15 min, inoculated with spores of the fungus, and incubated at 23°C for 10 days. After removal of the fungal mats from the broth, they were covered with 125 ml of $CHCl_3$ for 12 h to kill the spores and then removed for drying at 40°C for 72 h.

For toxicity testing a portion of the yield of both the rice culture and the fungal mats was ground in a mortar and mixed with a purified diet (9). For extraction of quinones, the remaining portion of the $CHCl_3$-treated fungal mats was ground in a Wiley laboratory mill to a 0.97-mm (20-mesh) powder, and a 500-g portion of the remaining mortar-ground material of the original rice culture was used.

Toxicity testing in mice. Rice culture was fed at a dietary concentration of 50% to groups of 10 male and female Swiss albino mice. Ground fungal mats were fed at a dietary concentration of 3% to similar groups of 10 mice. The mice were provided with diet and water ad libitum and were weighed at the beginning and at the end of the experiment. Necropsy was performed on the mice that died and on those killed when moribund or when the experiments were terminated after 10 days of feeding. The liver and kidneys were fixed in 10% buffered Formalin, processed for paraffin sections, and stained with hematoxylin and eosin for histopathological examination.

Isolation of quinones. A 100-ml portion of *n*-hexane was used to defat the ground mycelium by shaking in an Erlenmeyer flask on a rotary shaker for 5 h. The

mycelium was recovered by vacuum filtration, air dried, and then extracted with three successive 75-ml portions of $CHCl_3$ with 2 h of shaking each time. The combined $CHCl_3$ extracts were dried with anhydrous sodium sulfate and evaporated to dryness with a rotary evaporator. The ground rice cultures were extracted in the same manner as the fungal mats; however, with the larger amount of material to be extracted, the volumes of *n*-hexane and $CHCl_3$ were increased proportionately.

TABLE 1. *TLC analysis of fungal mats of species of Aspergillus for xanthomegnin and viomellein, correlated with toxicity studies*

Organism	Strain	Amt (μg/g of mycelium) of:		Presence of cholangiohepatitis and hepatic lesions
		Xanthomegnin	Viomellein	
A. alliaceus	315[a]	—[b]	—	–
A. alliaceus	1237	—	—	–
A. alliaceus	4181	—	—	–
A. auricomus	387	—	<1	–
A. auricomus	388	—	—	–
A. elegans	4850	—	—	–
A. melleus	386	—	<1	+
A. melleus	5103	215	1,290	+
A. petrakii	404	—	—	–
A. petrakii	416	—	—	–
A. petrakii	4369	—	—	–
A. ochraceus	398	172	989	+
A. ochraceus	399	—	—	–
A. ochraceus	400	43	258	+
A. ochraceus	402	9	65	–
A. ochraceus	410	<1	<1	–
A. ostianus	420	—	—	–
A. ostianus	3524	—	13	–
A. sclerotiorum	415	—	—	–
A. sclerotiorum	1598	—	—	–
A. sclerotiorum	4901	—	—	–
A. sclerotiorum	5166	—	—	–
A. sclerotiorum	5170	—	—	–
P. viridicatum	Purdue 66-68-2	>250	>500	+

[a] Northern Regional Research Laboratory (NRRL) strain number.
[b] —, Not detected.

To determine the presence of xanthomegnin or viomellein in the extracts, thin-layer chromatography (TLC) with reference compounds on Bakerflex silica gel plates was employed, using benzene–methanol–acetic acid (18:1:1) (BMA) and toluene–ethyl acetate–formic acid (6:3:1) (TEF) as developing solvents. With both solvent systems after the solvent was evaporated from the developed plate, xanthomegnin was yellow, turning orange after standing for 6 h, and viomellein was yellowish-green, turning yellowish-brown after 6 h.

The identity of the compounds in the extract was further confirmed by spraying the chromatograms with a 1% aqueous solution of fast blue BB salt (Sigma Chemical Co.), allowing them to dry, and then spraying with a half-saturated Na_2CO_3 aqueous solution. Xanthomegnin turned a violet color, and viomellein turned red-brown.

The fungal quinones were isolated from the extracts by preparative TLC on silica gel plates (500 μm thick), using BMA as the developing solvent. The xanthomegnin- and viomellein-containing bands were scraped from the plates and extracted by shaking for 20 min on a laboratory mixer three successive times with $CHCl_3$ to which had been added a few drops of acetic acid. Both the xanthomegnin- and the viomellein-containing extracts were rechromatographed on preparative silica gel plates and developed in TEF. This eliminated minor contaminating compounds from the quinones.

Xanthomegnin and viomellein were crystallized from $CHCl_3$-benzene (1:2). Melting points were determined with a Mel-Temp Apparatus (Laboratory Devices) and were uncorrected. Infrared (IR) spectra of the compounds in $CHCl_3$ were obtained with a double-beam Beckman IR-33 spectrophotometer.

Quantitative assay procedure. A chromatographic assay for xanthomegnin and viomellein was developed based on the serial dilution procedure employed by Brown et al. (2). The technique involved

TABLE 2. *TLC analysis of rice cultures of species of Aspergillus for xanthomegnin and viomellein, correlated with toxicity studies*

Organism	Strain	Amt (μg/g of rice culture) of:		Presence of cholangiohepatitis and hepatic lesions	Mortality
		Xanthomegnin	Viomellein		
A. auricomus	NRRL 387	<1	6	–	0/10
A. ochraceus	NRRL 399	—[a]	8	–	0/10
A. ochraceus	NRRL 3174	<1	141	–	5/10[b]
A. ochraceus	M-298-SC	72	926	+	8/10
A. ostianus	M-298-PM	2	5	–	4/10
A. ostianus	NRRL A 19242	—	—	–	6/10[b]
A. ostianus	NRRL 5223	—	—	–	0/10
A. ostianus	NRRL 420	<1	75	–	0/10
P. viridicatum	Purdue 66-88-2	24	36	+	0/10

[a] —, Not detected.
[b] Focal tubular degeneration and necrosis in renal tubules.

visual observation of the minimum detectable quantity of the quinones after TLC on Bakerflex silica gel plates. It was determined that 0.3 μg of viomellein and 0.1 μg of xanthomegnin could be observed with certainty on chromatograms developed in BMA. For viomellein 0.2 μg was equivocal, and for xanthomegnin 0.05 μg was equivocal; therefore, the quantity of quinone producing a just-detectable spot was considered equivalent to 0.3 μg for viomellein and 0.1 μg for xanthomegnin. Weights of the dried $CHCl_3$ extracts from the rice cultures and the fungal mats were determined, and a known amount of extract was diluted with a known amount of $CHCl_3$ until the dilutions no longer contained detectable quantities of quinones as determined with TLC.

RESULTS AND DISCUSSION

As can be seen from Tables 1 and 2, xanthomegnin and/or viomellein can be detected in the fungal mats and rice cultures of strains of *Aspergillus auricomus*, *A. melleus*, *A. ochraceus*, *A. ostianus*, as well as a known toxigenic strain of *P. viridicatum* which served as an experimental control. Although these data represent a TLC analysis, their reliability is increased because in each case the chloroform extracts were purified by using preparative TLC. Because cultural products of *A. ochraceus* M-298-SC have been reported by Zimmermann et al. (14) to produce the characteristic toxigenic effects that have been reported for *P. viridicatum* (6) and for xanthomegnin and viomellein (4), it was decided to reconfirm the occurrence of the quinones in extracts of this strain by the use of IR spectroscopy.

The IR spectra comparing xanthomegnin and viomellein isolated from *A. ochraceus* M-298-SC with reference compounds are illustrated in Fig. 1. In both cases the IR spectrum is identical to

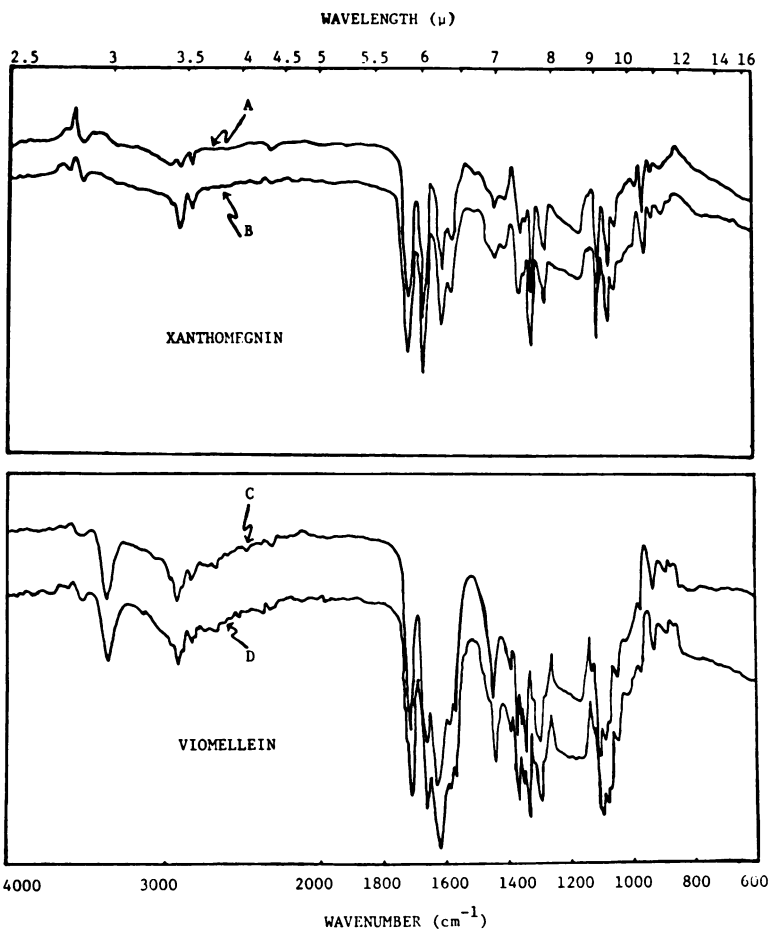

FIG. 1. *Comparison of IR spectra of xanthomegnin and viomellein isolated from A. ochraceus M-298-SC with reference compounds. A, Reference xanthomegnin; B, xanthomegnin from A. ochraceus; C, reference viomellein; D, viomellein from A. ochraceus.*

that of the authentic sample. The IR absorption maxima for xanthomegnin are 3,510 (broad), 1,715, 1,665, 1,610, and 1,580 (shoulder) cm^{-1}. For viomellein the maxima are 3,360 (broad), 2,920, 2,815, 1,710, 1,660 (shoulder), and 1,625 cm^{-1}. Upon crystallization viomellein was obtained as reddish-brown small beads which sintered above 275°C without melting. Orange plates of xanthomegnin sintered above 235°C.

Our results indicate that there is some correlation between the production of xanthomegnin and/or viomellein by *Aspergillus* strains and the occurrence of a characteristic acute toxic syndrome which has been produced in laboratory animals by strains of *P. viridicatum* (3, 5, 6). In no case were cholangiohepatitis and hepatic lesions produced in mice when xanthomegnin or viomellein could not be detected by TLC analysis. On the other hand, in several instances xanthomegnin and viomellein were detected in strains of *Aspergillus*, but the characteristic lesions were not produced in mice. A logical explanation for these observations would be that there is quantitative variation in the amount of the quinones produced by different strains of fungi. For this reason we employed an assay procedure that would provide a general idea as to the amount of quinone produced. Because of the subjective nature of the assay, the values reported in Tables 1 and 2 should be examined on a comparative basis and should not be considered absolute values. Carlton et al. (4) determined that xanthomegnin and viomellein produced hepatic alterations in mice at dietary concentrations of 448 and 456 mg/kg of feed, respectively. If the quantitative values for quinones used in Tables 1 and 2 are extrapolated to milligrams per kilogram of feed, in the case of the fungal mats 20 μg/g of mycelium would translate to 0.6 mg/kg of feed; with the rice cultures 20 μg/g of rice culture would represent 10 mg/kg of feed. Consequently, even though the minimum dose to produce lesions has not been determined for xanthomegnin and viomellein, the possibility exists that the level of quinones in some feed samples would be too low to elicit the toxic effect.

This investigation did not analyze for rubrosulphin and viopurpurin, other fungal quinones which have been isolated from *P. viridicatum* (11). These compounds have not been tested for their toxigenic effect; however, they may also be responsible for the hepatic alterations which are produced when the cultural products of certain fungi are fed to mice. In this regard the apparent anomalous result seen in Table 1 with *A. melleus* NRRL 386, in which this strain produces hepatic alterations but only a small amount of viomellein, might be explained by the production of rubrosulphin or viopurpurin by this strain. In fact Durley et al. (7) have isolated viopurpurin from a strain of *A. melleus*.

The question can also be raised that some strains of organism belonging to the *A. ochraceus* group are known producers of ochratoxin and penicillic acid, mycotoxins which might contribute to the toxic effect observed in mice. Isolates of the strains shown in Tables 1 and 2 which were positive for the production of cholangiohepatitis and hepatic lesions have been examined in our laboratories for the production of ochratoxin A and penicillic acid (15). These previously reported results show that our isolates did not produce ochratoxin A or penicillic acid, with the exception of *A. melleus* NRRL 386 and NRRL 5103, which produced a minimal amount of penicillic acid (34 μg/g or less).

In the case of the rice cultures in Table 2 which produced focal tubular degeneration and necrosis in the renal tubules, namely, *A. ochraceus* NRRL 3174 and *A. ostianus* NRRL A 19242, the possibility exists that these toxic effects are due to mycotoxins other than xanthomegnin and viomellein. We have not examined our isolates of these strains for the production of ochratoxin A or penicillic acid, and *A. ochraceus* NRRL 3174 is a known producer of ochratoxins A and B (8).

ACKNOWLEDGMENTS

We thank R. M. Eppley, Food and Drug Administration, Washington, D.C. for authentic samples of xanthomegnin and viomellein.

This work was supported in part by a grant from the Indiana Elks through the Purdue University Cancer Research Committee.

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