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Infrequent Production of Xanthomegnin by Fungal Strains Recovered from Patients with Ocular Mycoses

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Abstract Mycotoxins are putative virulence factors of fungi that play an important role in the pathogenesis of fungal infections. Mycotoxin production has been used as a diagnostic marker for the early diagnosis of fungal diseases. Using high-performance liquid chromatography, we investigated whether the fungal strains recovered from eye tissue samples obtained from patients with ocular mycoses produced the mycotoxin xanthomegnin. We tested 62 well-characterized strains of fungi, including *Aspergillus* spp. ($n = 14$), *Exophiala* spp. ($n = 9$), *Fusarium* spp. ($n = 15$), and several molds ($n = 24$). All isolates were identified to the species level using PCR and DNA sequencing of rRNA genes. We detected xanthomegnin activity (0.02 μg/ml) in one of the three *Aspergillus flavus* strains. However, we were unable to detect xanthomegnin in any of the other 61 fungal strains. Our result suggests that xanthomegnin production was infrequent in fungal strains recovered from patients with ocular mycoses.

Keywords Early diagnosis · Ophthalmic fungal strains · Pathogenicity · Xanthomegnin

Introduction

Mycotoxins, which are secondary metabolites produced by fungi, are involved in the pathogenesis of fungal infections; these metabolites either cause or exacerbate the disease [1]. Some well-known mycotoxins such as aflatoxins, gliotoxin, and ochratoxin A [2] are putative virulence factors that suppress the host immune system, thereby enhancing the infectivity of the fungus [3–5]. Recently, the secondary fungal

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metabolite xanthomegnin has attracted interest as a local virulence factor because of its potent toxicity and mutagenic, cytocidal, and immunosuppressive properties within the lesion [6].

Ocular mycoses is a serious fungal infection of the cornea, orbit, and other ocular structures that may result in blindness or reduced vision [7, 8]. Given the many fungal genera and species implicated in ocular infections, we hypothesized that xanthomegnin production might be a potential diagnostic marker that can be readily detected in patients with ocular mycoses. We studied xanthomegnin production by 62 fungal strains isolated from patients with non-outbreak ocular mycoses, representing three clinical presentations and 20 countries.

Materials and Methods

Fungal Strains

We used 62 fungal strains of human origin, recovered from eye tissue samples obtained from patients with eye infections [i.e., keratitis ($n = 59$), endophthalmitis ($n = 2$), and orbital infections ($n = 1$)]. All isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS)-KNAW Fungal Biodiversity Centre at Utrecht, the Netherlands (http://www.cbs.knaw.nl). Table 1 lists the clinical sources, geographical origins, and reference numbers of the strains used in this study. We included *Aspergillus* spp. ($n = 14$), *Exophiala* spp. ($n = 9$), *Fusarium* spp. ($n = 15$), and several molds ($n = 24$). All isolates were identified down to the species level by using PCR and DNA sequencing of rRNA genes. Before testing, all strains were inoculated on malt extract agar (Merck, Darmstadt, Germany); the plates were incubated at 28 °C for 5 days, and the purity and identity were controlled.

Extraction of Fungal Strains and High-Performance Liquid Chromatography (HPLC)

The extraction process and HPLC (Agilent Technologies, Tokyo, Japan) technique were performed as described previously [6, 9]. Briefly, approximately 2.5 cm^2 of the each fungal strain was transferred from the agar plate to a clean microcentrifuge tube, crushed, and extracted with 1 ml of methanol–chloroform–acetic acid (50:50:1 vol/vol/vol) for 10 min. The extract was filtered through a layer of glass wool, and the solvent was eliminated by evaporation at 40 °C. The residue was extracted with 500 µl of acetonitrile for 5 min and filtered through glass wool, after which the solvent was eliminated by evaporation. Fifty microliters of acetonitrile was added to the tube on a vortex mixer, and the tube was centrifuged at 13,000 rpm for 10 min. The upper layer was transferred to a clean vial wrapped in aluminum foil. The total preparation time was approximately 3 h.

The second portion of the same fungal strain was transferred to a clean microcentrifuge tube and crushed with 500 µl of methanol–chloroform–acetic acid (50:50:1, vol/vol/vol). The extract was filtered through glass wool, after which the solvent was eliminated by evaporation at 40 °C. Then, 100 µl of acetonitrile was added to the tube on a vortex mixer, and the tube was centrifuged at 13,000 rpm for 10 min. The top layer was transferred to a clean vial wrapped in aluminum foil. The entire procedure was completed within 2 h. The samples were protected from exposure to light during both extraction periods.

HPLC was performed on an quaternary solvent delivery system with a manual injector (G1328B), quat pump (G1311A), degasser (G1379A), and an ACE 4.6 mm × 25 cm C_8 (Serial Number: A137853) reverse-phase column. Detection was performed with a variable wavelength detector (VWD, G1314A) with a ChemStation integrator. The sample was monitored at 405 nm. The column was equilibrated at 1 ml/min with a mobile phase of acetonitrile–water–potassium dihydrogen phosphate KH_2PO_4 (55:45:0.14, vol/vol/wt) adjusted to pH 3.0 with phosphoric acid. All separations were performed isocratically.

Xanthomegnin (BioViotica, Goettingen, Germany) standard solution was prepared by dissolving a dried film of an authentic sample in acetonitrile for approximately 30 min prior to HPLC. Xanthomegnin standard solution (5 pg/µl) was used as an internal control at the start of each run. After injection of each sample, the amounts and peak areas of xanthomegnin were automatically calculated and ordered by the ChemStation program on the basis of a standard curve. A standard curve was created using 1/10 and 1/100 dilutions of an authentic xanthomegnin standard. *Trichophyton rubrum* CBS 132250 and *T. erinacei* CBS 344.79 were used as the positive and negative control strains, respectively [6, 9].

Table 1 Ophthalmic fungal isolates used in this study

Species	CBS no.	Clinical source	Geography
Aspergillus brasiliensis	122723	Keratitis, human	India
A. brasiliensis	122724	Keratitis, human	India
A. calidoustus	121609	Post-cataract surgery endophthalmitis	Ankara, Turkey
A. calidoustus	121610	Post-cataract surgery endophthalmitis	Ankara, Turkey
A. flavus	126857	Keratitis, human	Sao Paulo, Brazil
A. flavus	126858	Keratitis, human	Sao Paulo, Brazil
A. flavus var. *flavus*	616.94	Orbita tumor, human	Aachen, Germany
A. niger	122720	Keratitis	India
A. niger	122721	Keratitis	India
A. niger	122722	Keratitis	India
A. nomius	123901	Corneal scraping	Coimbatore, India
A. tamarii	121598	Keratitis	India
A. tubingensis	122719	Corneal ulcer, keratitis	India
A. tubingensis	122725	Corneal ulcer, keratitis	India
Exophiala dermatitidis	120470	Cyst from eye	USA
E. dermatitidis	120562	Eye	USA
E. dermatitidis	120567	Eye scraping	USA
E. dermatitidis	639.96	Keratitis after cornea transplantation	France
E. oligosperma	463.80	Eye, prosthetic lens, human	Basel, Switzerland
E. phaeomuriformis	121744	Eye duct	Aurora, USA
E. xenobiotica	117648	Sclera	USA
E. xenobiotica	117661	Vitreous fluid	USA
E. xenobiotica	117671	Eye vitreous	USA
Fusarium delphinoides	116510	Corneal ulcer	Argentina
F. delphinoides	110140	Eye, human	Florida, USA
F. delphinoides	109831	Corneal ulcer, human	South Africa
F. dimerum	116637	Left eye vitreous	Texas, USA
F. dimerum	116524	Anterior chamber	Texas, USA
F. dimerum var. *dimerum*	110314	Eye	Ohio, USA
F. dimerum var. *dimerum*	116523	Vitreous fluid	San Antonio, Texas, USA
F. lichenicola	109048	Cornea, human	Formosa, Argentina
F. penzigii	116508	Eye infection	Sri Lanka
F. polyphialidicum	111770	Keratomycosis	Zaragosa, Spain
F. proliferatum	116324	Eye, human	Allicante, Spain
F. sacchari	121683	Endophthalmitis	India
F. solani	124625	Cornea	Lille, France
F. solani	113934	Keratitis	Sao Paulo, Brazil
F. solani	109696	Eye infection	Ontario, Canada
Acremonium recifei	188.82	Ulcus corneae, human	The Netherlands
Acrophialophora fusispora	120409	Corneal scraping	Sevagram, India
Alternaria infectoria	124072	Keratitis, human	Athens, Greece
Botryosphaeria subglobosa	448.91	Keratomycosis, human	United Kingdom
Chaetomium globosum	120346	Infected eye, human	China
Curvularia senegalensis	102171	Keratitis	Rio de Janeiro, Brazil

Table 1 continued

Species	CBS no.	Clinical source	Geography
Dicotomophtoropsis nymphaearum	446.91	Keratitis	Chittagong, Bangladesh
Lecythophora hoffmannii	140.96	Keratitis after cornea transplantation	Berlin, Germany
Neosartorya fennelliae	598.74	Eye ball	USA
Neocosmospora vasinfecta	121152	Corneal ulcer	Coimbatore, India
Paecilomyces variotii	124.97	Vitreous tumor	Hong Kong
Periconia minutissima	562.83	Cornea pus	The Netherlands
Phaeoacremonium alvesii	408.78	Keratitis	California, USA
Phaeoacremonium cf. rubrigenum	112046	Infected eye, vitreous fluid, human	Illinois, USA
Phaeoisaria clematidis	102276	Keratomycosis	Sao Paulo, Brazil
Phialemonium curvatum	365.61	Cornea, human	Germany
Phialemonium curvatum	489.82	Mycotic keratitis	Ohio, USA
Phialophora bubakii	254.81	Human, eye	Indiana, USA
Pleurostomophora richardsiae	723.74	Lacrimal stone	Unknown
Pseudallesheria boydii	119708	Corneal scraping	Japan
Pseudallesheria boydii	119698	Corneal scraping	Japan
Pyrenochaeta keratinophyla	121759	Corneal scraping, keratitis	Alicante, Spain
Sarcopodium oculorium	110031	Keratomycosis	Brazil
Stagonosporopsis oculo-hominis	634.92	Corneal ulcer	Nashville, Tennessee, USA

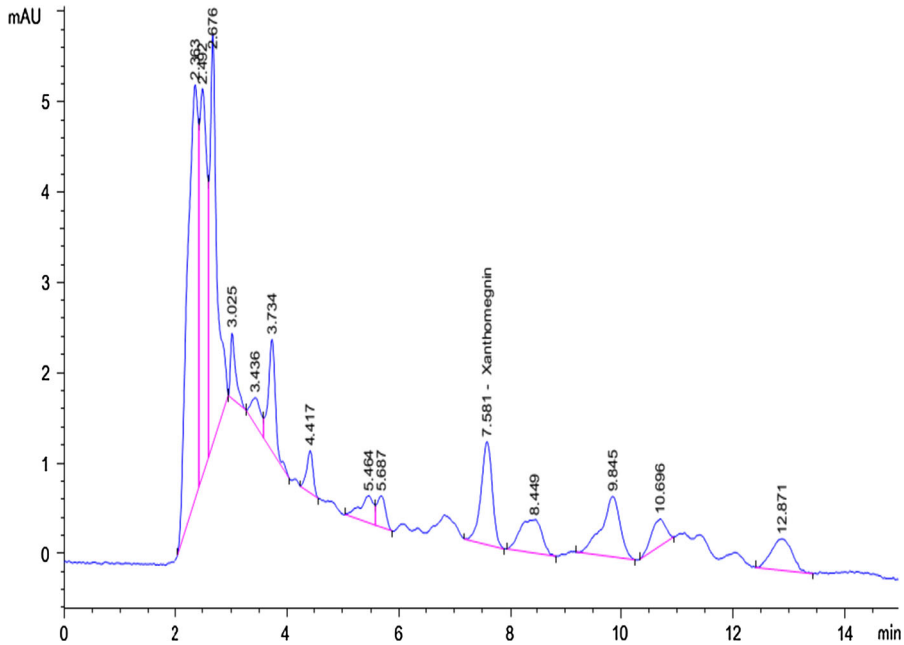


Fig. 1 HPLC chromatogram of *A. flavus* CBS 126857. Retention time: 7.581 min; amount of xanthomegnin: 0.02 µg/ml; area: 16.00 mAU/s

Results

For each assay, the chromatograms of standard xanthomegnin, the positive control *T. rubrum* CBS 132250, and the negative control *T. erinacei* CBS 344.79 were available. We identified xanthomegnin activity (0.02 µg/ml) in one of the three *A. flavus* strains (Fig. 1). However, we were unable to measure

xanthomegnin in any of the other 61 well-characterized fungal strains recovered from patients with ocular mycoses.

Discussion

Collectively, our data suggest that xanthomegnin production was infrequent in fungal strains recovered from patients with ocular mycoses. In total, 61 out of 62 fungal strains tested did not produce detectable amount of xanthomegnin in culture, to be observed by HPLC.

Notably, in this study, we tested several fungal genera that have not been reported to possess xanthomegnin activity (i.e., *Exophiala* spp. and *Fusarium* spp.). In addition, we found that xanthomegnin activity might not be related to the anatomical site of the disease, but might, instead, be related to the genus of the causative agent. To understand this hypothesis, further studies on ocular tissues are also warranted to explore whether these tissue offers a favorable setting for xanthomegnin production.

Historically, the nephrotoxic and hepatotoxic effects of xanthomegnin were identified in 1976 [10]. It is potentially genotoxic and mutagenic and is mainly produced by *Aspergillus* spp. and *Penicillium* spp. [11], and less frequently by dermatophytes [6, 9]. There is limited literature on the role of fungal secondary metabolites as virulence factors in mycotic eye infections. Leema et al. [12] reported that aflatoxin B_1 was detected in a significantly higher percentage of *A. flavus* keratitis-causing strains (80 %), compared to environmental strains (40 %). Leema et al. [13] investigated oxidative stress in experimental rodent corneas infected with aflatoxigenic and non-aflatoxigenic *A. flavus* strains and showed that the former is associated with more intense oxidative stress than the latter. Importantly, in tropical and subtropical countries, *A. flavus* has most frequently been reported the predominant fungal pathogen causing mainly ocular infections and/or cerebral or sino-orbital aspergillosis [14, 15]. Experimental data also suggest that *A. flavus* produces some of the most significant mycotoxins known including aflatoxins, gliotoxin, and ochratoxin A, which are the putative virulence factors to suppress host immunity, thereby enhancing the infectivity of the fungus [16].

Leal et al. [17] also detected distinct roles for dectin-1 and toll-like receptor 4 (TLR4) in the pathogenesis of *Aspergillus fumigatus* keratitis. Nayak et al. [18] examined 200 filamentous mycotic keratitis isolates (*Aspergillus* spp. and *Fusarium* spp.) and suggested that proteinases might be important virulence factors that are associated with amphotericin B resistance, as their production significantly correlates with amphotericin B resistance.

Presence of fumonisin B_1 was demonstrated in patients with *Fusarium* keratitis [19, 20]. Analysis of mycotoxin-producing *Fusarium* spp. recovered from 29 patients with mycotic keratitis revealed that 72.4 % of these *Fusarium* spp. were members of the *Fusarium solani* species complex. Members of this complex produce mycotoxins including fusaric acid, moniliformin, and fumonisin B1, which are known to be highly toxic to experimental animals and at the cellular levels [21]. The ability to form biofilms is another key determinant of the pathogenicity of *F. solani* and *F. oxysporum* species complexes, irrespective of the thickness of these biofilms [22].

Previous studies have shown that the members of the *T. rubrum* complex exhibit xanthomegnin activity [6, 9]. Moreover, we recently showed that strains belonging to the *Trichophyton mentagrophytes* complex and the teleomorph *Arthroderma vanbreuseghemii* might show xanthomegnin activity [9].

As a limitation of the current study, it should be noted that we only tested clinical strains, and not clinical samples, which limits the value of our study. The novelty of this study is that we investigated xanthomegnin production as a potential diagnostic marker for various fungi causing ocular mycoses. Although the presence of mycotoxins, i.e., aflatoxins, is well described in *Aspergillus*, we found that only one strain among the 14 *Aspergillus* strains tested in this study had xanthomegnin activity. Based on these results, we believe that mycotoxin activity in ocular samples should be investigated according to the fungal genus detected. Future studies investigating genus-related mycotoxins such as aflatoxin B_1 and fumonisin B_1 will contribute to the existing knowledge regarding the production of fungal mycotoxins. Further research is warranted to validate the presence of xanthomegnin in eye tissue samples obtained from patients with ocular mycoses.

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Compliance with Ethical Standard

Conflict of interest Seyedmojtaba Seyedmousavi received research from Gilead Sciences from Astellas Pharma B.V. and Gilead Sciences. All the other authors have no competing interests.

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