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# Xanthomegnin detection does not discriminate between *Trichophyton rubrum* and *T. mentagrophytes* complexes

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ABSTRACT

Here, we aimed to discriminate the *Trichophyton rubrum* and *Trichophyton mentagrophytes* complexes, via detection of xanthomegnin using HPLC. We concluded that it is not a reliable technique for discriminating the two complexes, because strains belonging to both *T. rubrum* and *T. mentagrophytes* complexes displayed xanthomegnin activity.

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## 1. Introduction

Xanthomegnin, a naphthoquinone, is a well-known animal mycotoxin produced by fungi in the mold genera *Aspergillus* and *Penicillium* in poorly stored feed materials (Carlton et al., 1973; Stack and Mislivec, 1978). Xanthomegnin is derived from the same polyketide chemical pathway that produces aflatoxins. Oral intake of xanthomegnin in animals results in kidney and liver abnormalities that often first present as jaundice (Carlton et al., 1976). It should also be noted that no association has been reported between dermatophytosis and either jaundice or kidney damage. Blank et al. (1963) first detected xanthomegnin from the anthropophilic dermatophyte *Trichophyton megninii*, from which the component derives its name, using high-performance liquid chromatography (HPLC). Wirth et al. (1965) demonstrated that the colony reverse color of *Trichophyton rubrum* sensu stricto is typically characterized as blood-red and occasionally characterized as yellow. This phenomenon is attributable to the metabolite xanthomegnin. One year later, xanthomegnin was described in *Trichophyton violaceum* strains (Blank et al., 1966). Conclusively, modern taxonomic studies have revealed that *T. megninii*, *T. rubrum* s. str., and *T. violaceum* belong to the *T. rubrum* species complex (Gräser et al., 2000; Ohst et al., 2004; Ilkit and Durdu, 2015), in which xanthomegnin was described.

HPLC is a technique used in clinical chemistry to separate the components of a mixture and to identify and quantify each component. HPLC relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material (Gerber et al., 2004). HPLC has been used for the following purposes: (i) medical [e.g., the identification of hemoglobin variants (Çürük et al., 1993) and the detection of vitamin D levels in blood serum (Ford, 2013) or vanillylmandelic acid (Lionetto et al., 2008)], (ii) legal [e.g., identification of performance enhancement drugs in urine (Boccard et al., 2011)], (iii) research [e.g., separating the components of a complex biological sample (Kutlar et al., 1986)], and (iv) industrial [e.g., production of pharmaceutical products (Pang et al., 2014)].

Using HPLC, Gupta et al. (2000) extracted xanthomegnin in 100% of skin scrapings and nail clippings known to be infected with *T. rubrum*, suggesting that this method might be a powerful diagnostic tool for this pathogen. Xanthomegnin was also extracted in milligram quantities from tissue infected by *T. rubrum* s. str. but not identified in non-infected controls. Therefore, the presence of xanthomegnin in a clinical sample is attributable to its production during growth on the human host. The authors also discussed the significance of the presence of xanthomegnin. According to these authors, xanthomegnin potentially acts as (i) a local virulence factor in the lesion, (ii) a factor that does not affect on the human host but aids in defending *T. rubrum* s. str. against bacterial or fungal competitors in the epidermis, and (iii) a factor that affects either other microbes or the human host. In addition, various dermatophytic components (such as xanthomegnin) and materials with antigenic activity are mobilized into the circulation and thus may be related to the id reaction (Gupta et al., 2000).

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Currently, the two most predominant dermatophyte species in medical mycology laboratories are members of the *T. rubrum* and *Trichophyton mentagrophytes* complexes (Ilkit and Durdu, 2015). *T. rubrum* s. str. accounts for 60% of all superficial fungal infections in humans (Shankar et al., 2002). Discrimination between the *T. rubrum* and *T. mentagrophytes* complexes in clinical samples or isolates is crucial for defining epidemiological and ecological characteristics, therapeutic management strategies, and control parameters of any dermatophytic fungus in question (Ilkit and Durdu, 2015). The objective of this study was to determine whether the detection of xanthomegnin activity via HPLC could be used to discriminate between the two major human dermatophytic pathogens, *T. rubrum* and *T. mentagrophytes* (as well as its teleomorphs of the *Arthroderma* spp.) complexes, by examining a set of clinical strains as well as reference strains taken from a variety of infections and countries.

## 2. Materials and methods

### 2.1. Reference strains

Table 1 lists the clinical sources, geographical origins, and reference numbers of the 32 dermatophyte strains used in this study: representatives of *Arthroderma* spp. ($n = 7$), *T. mentagrophytes* ($n = 7$), and *T. rubrum* ($n = 18$) complexes. The majority of these strains were obtained from the culture collection service at Centraalbureau voor Schimmelcultures (CBS)-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands. In addition, three *Trichophyton interdigitale* strains and one *T. violaceum* strain from the working collection of Macit Ilkit (MI) were included in the study (Table 1). These strains were previously identified using DNA sequencing. The fungal names were assigned based on the current taxonomy and/or the CBS list of cultures (http://www.cbs.knaw.nl/databases). Before testing, all of the strains were inoculated on Sabouraud glucose agar (SGA; Merck; Darmstadt, Germany) plates containing 100 μg/ml cycloheximide (Sigma; Steinheim, Germany), 100 μg/ml chloramphenicol (Sigma), and 50 μg/ml gentamicin (Sigma). The plates were incubated at 28 °C for 14 days, and the purity and identity were controlled.

### 2.2. Clinical samples and strains

The clinical samples investigated herein were collected from patients at the Department of Dermatology at Çukurova University 2 months prior to the analysis. The samples were kept in an incubator at 28 °C. Here, we tested 13 clinical samples, which compromised patients with suspected tinea pedis ($n = 8$), tinea unguium ($n = 4$), and tinea cruris ($n = 1$). Clinical strains were identified on the basis of macroscopic and microscopic characteristics in addition to growth and alkalinization patterns on bromocresol purple-milk solids-glucose agar; urease degradation tests (Ateş et al., 2008) and in vitro hair perforation tests were also performed (Gümral et al., 2013). The strains included in this study were also identified as *T. rubrum* ($n = 9$) and *T. interdigitale* ($n = 4$) via internal transcribed spacer (ITS)-sequencing using an ABI PRISM 3130XL genetic analyzer (Refgen Biotechnology; Ankara, Turkey). These strains were deposited in the laboratory facility of the Division of Mycology at Çukurova University. The Ethics Committee at the Faculty of Medicine at Çukurova University reviewed and approved the study protocol.

### 2.3. Extraction of reference and clinical strains and samples

The extraction was performed as described by Gupta et al. (2000) with minor modifications. For dermatophyte isolates, approximately 2.5 $cm^2$ of the sample was moved 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 removed 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 removed 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 a maximum of 3 h.

The second portion of clinical specimens 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 removed 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 and 10 min. The top layer was transferred to a clean vial wrapped in aluminum foil. The entire procedure was completed in 2 h. The samples were protected from exposure to light during both extraction periods.

### 2.4. High-performance liquid chromatography

HPLC (Agilent Technologies, Tokyo, Japan) was performed on a quaternary solvent delivery system with a manual injector (G1328B), Quat

**Table 1**
Dermatophyte strains of *Arthroderma* and *Trichophyton* species used in this study.

| Species | CBS no. | MI no. | Clinical source | Geography |
|---|---|---|---|---|
| *Arthroderma strains* | | | | |
| *A. simii* | 417.65 | | Chicken | India |
| *A. simii* | 132352 | | Tinea inguinalis | Mersin, Turkey |
| *A. simii* | 448.65 | | Poultry | India |
| *A. vanbreuseghemii* | 428.63 | | Skin, foot sole | Netherlands |
| *A. vanbreuseghemii* | 132252 | 19654 | Human, groin carrier | Adana, Turkey |
| *A. vanbreuseghemii* | 117724 | | Human | France |
| *A. vanbreuseghemii* | 138553 | 19761 | Human, folliculitis | Adana, Turkey |
| *T. mentagrophytes complex* | | | | |
| *T. erinacei* | 344.79 | | Human, skin, arm | Netherlands |
| *T. erinacei* | 511.73 | | *Erinaceus europaeus* (hedgehog) | New Zealand |
| *T. erinacei* | 677.86 | | Human, nail | Germany |
| *T. interdigitale* | – | 19670 | Tinea pedis | Adana, Turkey |
| *T. interdigitale* | – | 19671 | Tinea unguim | Adana, Turkey |
| *T. interdigitale* | – | 19672 | Tinea unguium | Adana, Turkey |
| *T. m.* var. *mentagrophytes* | 110.65 | | Human, pubic hair | Netherlands |
| *T. rubrum complex* | | | | |
| *T. kuryangei* | 518.63 | | – | – |
| *T. megninii* | 389.58 | | Human, nail | Utrecht, Netherlands |
| *T. raubitschekii* | 102856 | | Human, fingernail | Italy (patient from Cameroon) |
| *T. raubitschekii* | 287.86 | | Human, skin | Ontario,Toronto, Canada |
| *T. raubitschekii* | 125604 | | Human, skin | Adana, Turkey |
| *T. raubitschekii* | 125605 | | Human, skin | Adana, Turkey |
| *T. rubrum* | 392.58 | | Human, tinea pedis | Rotterdam, Netherlands |
| *T. rubrum* | 138551 | 19762 | Human, folliculitis | Adana, Turkey |
| *T. rubrum* | 132251 | 19651 | Human, groin carrier | Adana, Turkey |
| *T. rubrum* | 132249 | 19652 | Human, groin carrier | Adana, Turkey |
| *T. rubrum* | 132250 | 19653 | Human, groin carrier | Adana, Turkey |
| *T. rubrum* | 138550 | 19763 | Human, folliculitis | Adana, Turkey |
| *T. rubrum* | 138552 | 19764 | Human, folliculitis | Adana, Turkey |
| *T. soudanense* | 436.63 | | Skin, head | Africa (Country unknown) |
| *T. violaceum* | – | 20141 | Tinea capitis | Adana, Turkey |
| *T. violaceum* | 119446 | | Tinea capitis | Gabon |
| *T. violaceum* | 120322 | | Tinea capitis | Switzerland |
| *T. yaoundei* | 677.82 | | Scalp | Netherlands (patient from Morocco) |

CBS, Centraalbureau voor Schimmelcultures; MI, Macit Ilkit's working collection.

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 VWD (G1314A) variable wavelength detector with ChemStation integrator. The sample was monitored at 405 nm. The column was equilibrated at 1 ml per min with a mobile phase of acetonitrile–water–$KH_2PO_4$ (55:45:0.14 vol/vol/wt) adjusted to pH 3.0 with phosphoric acid. All of the separations were performed isocratically. Standard xanthomegnin (BioViotica, Goettingen, Germany) solutions were prepared by dissolving a dried film of an authentic sample in acetonitrile for approximately 30 min prior to HPLC analysis. A standard xanthomegnin solution (5 pg/μl) was used as an internal control at the start of each run. After injection of each sample, the amount and peak areas of xanthomegnin were automatically calculated and ordered by the ChemStation program based on a standard curve. A standard curve was created by using 1/10 and 1/100 dilutions of an authentic xanthomegnin standard. The identification of xanthomegnin in all of the samples was confirmed by agreement with the xanthomegnin retention time in the most recent standard chromatogram (Gupta et al., 2000).

## 3. Results

### 3.1. Reference strains

All of the strains were analyzed by the authors over the same time period at Çukurova University. Here, we observed that xanthomegnin purification, detection, and measurement could be performed via HPLC. In our study, the xanthomegnin measurements were conducted using a 20-μl injection, which accounted for only a small fraction of the 500 μl extract prepared for each strain. In this investigation, the xanthomegnin values of 12 reference *T. rubrum* complex strains yielded xanthomegnin values in the range of 0.03 to −106.19 μg/ml; however, we failed to obtain xanthomegnin values for the remaining 6 *T. rubrum* complex strains. In addition, as expected, 6 of the 7 *T. mentagrophytes* complex strains and 5 of the 7 *Arthroderma* strains did not yield any xanthomegnin values. We also measured xanthomegnin activity in 1 *T. interdigitale* (0.14 μg/ml) strain and 2 *A. vanbreuseghemii* (1.14 and 2.07 μg/ml) strains (Table 2).

The HPLC chromatogram of standard xanthomegnin, the positive control *T. rubrum* CBS 132250, and the negative control *Trichophyton erinacei* CBS 344.79 were tested. We also observed xanthomegnin activity for 2 *A. vanbreuseghemii* (CBS 132252 and CBS 138553) and 1 *T. interdigitale* (MI 19672) reference strains, which was compatible with the range of the *T. rubrum* complex.

### 3.2. Clinical samples and strains

Notably, we identified xanthomegnin in 11 of the 13 clinical samples tested (range 0.004–0.27 μg/ml), and xanthomegnin was detected in 11 of the 13 clinical strains (range 0.21–14.43 μg/ml). Nonetheless, the amount of xanthomegnin in the clinical samples was reduced compared to clinical isolates. We were unable to measure xanthomegnin in the patients with tinea pedis and tinea unguium, yet their clinical strains exhibited xanthomegnin activities of 0.31 and 8.90 μg/ml, respectively. Xanthomegnin activity was measured not only in the *T. rubrum* isolates (0.21–14.43 μg/ml) but also in the *T. interdigitale* isolates (0.55–1.23 μg/ml). Furthermore, xanthomegnin was measured in all 4 of the clinical samples containing *T. interdigitale* (0.05–0.21 μg/ml). Xanthomegnin activity did not differ according to the origin of the clinical samples, species diversity, or patient demographic information (Table 3).

## 4. Discussion

In the present study, we aimed to discriminate between the *T. rubrum* and *T. mentagrophytes* (as well as teleomorphs representing *Arthroderma* spp.) complexes. We examined a set of reference strains, clinical samples, and clinical strains using HPLC. Here, the HPLC method previously described by Gupta et al. (2000) was used with minor modifications, and this method allowed the determination of xanthomegnin at a microgram level. Although our results revealed that only two-thirds of the reference *T. rubrum* complex strains produce xanthomegnin in a short-turnaround time (max. 3 h), 2 *A. vanbreuseghemii* strains and 1

**Table 2**
Xanthomegnin activity in reference strains of *Arthroderma* and *Trichophyton* species.

| Species | CBS no. | MI no. | Xanthomegnin (μg/ml) | Peak area (mAU/s) |
|---|---|---|---|---|
| *Arthroderma strains* | | | | |
| *A. simii* | 417.65 | – | – | – |
| *A. simii* | 132352 | – | – | – |
| *A. simi* | 448.65 | – | – | – |
| *A. vanbreuseghemii* | 428.63 | – | – | – |
| *A. vanbreuseghemii* | 132252 | 19654 | 1.14 | 28.32 |
| *A. vanbreuseghemii* | 117.724 | – | – | – |
| *A. vanbreuseghemii* | 138553 | 19761 | 2.07 | 51.69 |
| *T. mentagrophytes complex* | | | | |
| *T. erinacei* | 344.79 | – | – | – |
| *T. erinacei* | 511.73 | – | – | – |
| *T. erinacei* | 677.86 | – | – | – |
| *T. interdigitale* | – | 19670 | – | – |
| *T. interdigitale* | – | 19671 | – | – |
| *T. interdigitale* | – | 19672 | 0.14 | 3.57 |
| *T. m.* var. *mentagrophytes* | 110.65 | – | – | – |
| *T. rubrum complex* | | | | |
| *T. kuryangei* | 518.63 | – | – | – |
| *T. megninii* | 389.58 | – | 0.03 | 0.73 |
| *T. raubitschekii* | 102856 | – | 82.36 | 2048.87 |
| *T. raubitschekii* | 287.86 | – | 13.01 | 323.55 |
| *T. raubitschekii* | 125604 | – | 10.07 | 250.64 |
| *T. raubitschekii* | 125605 | – | 42.79 | 1064.57 |
| *T. rubrum* | 392.58 | – | – | – |
| *T. rubrum* | 138551 | 19762 | 8.25 | 205.29 |
| *T. rubrum* | 132251 | 19651 | 16.07 | 399.86 |
| *T. rubrum* | 132249 | 19652 | 0.18 | 4.44 |
| *T. rubrum* | 132250 | 19653 | 106.19 | 2641.55 |
| *T. rubrum* | 138550 | 19763 | 15.85 | 394.20 |
| *T. rubrum* | 138552 | 19764 | 4.26 | 105.95 |
| *T. soudanense* | 436.63 | – | – | – |
| *T. violaceum* | – | 20141 | 9.72 | 241.86 |
| *T. violaceum* | 119446 | – | – | – |
| *T. violaceum* | 120322 | – | – | – |
| *T. yaoundei* | 677.82 | – | – | – |

CBS, Centraalbureau voor Schimmelcultures collection; MI, Macit Ilkit's working collection.

**Table 3**
Xanthomegnin activity in clinical samples and isolates of *T. rubrum* s. str. and *T. interdigitale*.

| Patient no. | Clinical source | Sex | Age | HPLC | | | |
|---|---|---|---|---|---|---|---|
| | | | | Clinical specimens | | Clinical isolates | |
| | | | | Amount (μg/ml) | Peak area (mAU per/s) | Amount (μg/ml) | Peak area (mAU per/s) |
| 1 | *T. unguium*§ | M | 48 | 0.21 | 5.25 | 1.09 | 27.09 |
| 2 | *T. pedis*§ | F | 44 | 0.05 | 1.16 | 0.55 | 13.68 |
| 3 | *T. pedis* | M | 63 | 0.004 | 0.11 | – | – |
| 4 | *T. cruris* | M | 17 | 0.02 | 0.48 | 14.43 | 359.00 |
| 5 | *T. pedis* | M | 67 | – | – | 0.31 | 7.76 |
| 6 | *T. pedis* | M | 55 | 0.16 | 4.08 | 0.21 | 5.25 |
| 7 | *T. pedis*§ | M | 40 | 0.20 | 4.89 | 1.23 | 30.59 |
| 8 | *T. unguium* | M | 58 | 0.23 | 5.74 | 1.02 | 25.44 |
| 9 | *T. unguium* | F | 36 | – | – | 8.90 | 221.41 |
| 10 | *T. pedis* | F | 63 | 0.27 | 6.66 | 5.45 | 135.59 |
| 11 | *T. unguium* | M | 53 | 0.16 | 4.08 | 2.05 | 51.01 |
| 12 | *T. pedis*§ | F | 44 | 0.07 | 1.70 | – | – |
| 13 | *T. pedis* | F | 47 | 0.26 | 6.59 | 4.02 | 100.12 |

T, tinea; M, male; F, female; §, *T. interdigitale* isolates.

*T. interdigitale* strain yielded a coincidental HPLC peak in the extraction. Most notably, we observed that either clinical samples or strains of *T. interdigitale* also exhibited xanthomegnin activity compatible with the *T. rubrum* complex, which consequently limits the value of this method. Therefore, this study indicates that xanthomegnin is not a reliable target for the differentiation of *T. rubrum* complex strains from *T. mentagrophytes* complex (and its teleomorph *Arthroderma* spp.) strains.

Gupta et al. (2000) suggested that *T. rubrum* infections might be exposed to xanthomegnin and may be used for diagnostic purposes. However, the authors noted that it was unknown whether xanthomegnin is found in every *T. rubrum* lesion. Here, we demonstrated that xanthomegnin activity was absent in clinical samples but present in clinical isolates in 2 patients diagnosed with a *T. rubrum* infection. Although we verified the earlier findings of these authors, we demonstrated that clinical samples exhibited lower xanthomegnin values compared with clinical isolates (13 of each produced xanthomegnin activity). *T. rubrum* can survive (without propagation) up to 18 months in its arthroconidial form (Baer et al., 1955).

Although xanthomegnin activity did not provide the correct taxonomic identification in this investigation, Gupta et al. (2000) noted that this target could provide useful information when examining clinical samples containing *T. rubrum* strains. Our findings suggest that this target does not provide new insights in medical mycology diagnostics and is not recommended for directly examining clinical samples in routine daily practice. We also conclude that this method can serve as a complementary test for *T. rubrum* complex identification. One of the limitations of the present study is the medical mycologists' lack of experience and familiarity with the HPLC method. Thus, we collaborated with an ad hoc biochemist to resolve this issue. This limitation can be overcome via collaboration with scientists in the field.

Over the past 6 decades, *T. rubrum* s. str. has been the most common dermatophyte isolated from glabrous skin and nail dermatophytic infections worldwide (Borman et al., 2007; Taylor and Gurr, 2014). This phenomenon is also likely due to urbanization, the use of sports and fitness facilities, the growing prevalence of obesity, and an aging population (Ilkit and Durdu, 2015). Similar to most other anthropophiles, *T. rubrum* s. str. is typically associated with chronic long-term, but limited, disease and tissue destruction (White et al., 2014). Therefore, medical mycologists have focused on developing rapid and reliable methods to identify this human fungal pathogen. However, phenotypic parameters are often insufficient to differentiate between the *T. rubrum* and *T. mentagrophytes* complexes (Ateş et al., 2008). In addition to poorly sporulating clinical isolates [except *T. rubrum* with the "*raubitshekii*" morphotype (Ilkit et al., 2011)], these two species complexes may exhibit either pleomorphic or atypical morphological characteristics (Ateş et al., 2008).

Considerable work has focused on this aim; molecular and phenotypic diagnostics such as MALDI-TOF MS technology have been applied worldwide to resolve this issue directly using either clinical samples or a colony (Erhard et al., 2008). In addition, molecular and genotypic techniques such as several PCR-based techniques, real-time PCR (Arabatzis et al., 2007), nested-PCR (Verrier et al., 2013), PCR-ELISA (Beifuss et al., 2011), and multiplex-based PCR (Mehlig et al., 2014) also offer highly sensitive and specific results. Recently, we successfully differentiated these two complexes using FT-IR spectroscopy (Ergin et al., 2013); their ability to produce profuse macroconidia on several media was also different (Döğen and Ilkit, 2013). In contrast to the *T. mentagrophytes* complex, species in the *T. rubrum* complex are genetically indistinguishable via ITS-sequencing but exhibit different morphological and physiological characteristics (Gräser et al., 2000; Ilkit et al., 2011; Ilkit and Durdu, 2015).

The novel contributions of this study include the finding that xanthomegnin activity is not restricted to *T. rubrum* complex strains, nor do all of these strains produce mycotoxin. In addition to the 3 reference strains of the *T. mentagrophytes* complex that exhibited xanthomegnin activity, the majority of the clinical samples and/or strains of *T. interdigitale* had a detectable amount of xanthomegnin. Thus, we concluded that xanthomegnin detection is a non-discriminatory tool. Further studies will highlight whether non-dermatophytic keratinophilic fungi possess xanthomegnin activity, particularly those strains recovered from patients with onychomycosis.

## Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the manuscript.

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