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Research Article

Gene Expression of Subtilisin Genes of UV-irradiated *Trichophyton indotineae* Isolated from Iraqi Patients

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Abstract:

Background: Virulence genes in dermatophytes have been a major focus of research in recent years. These fungi have a unique ability to degrade keratin, an essential structural protein in human skin, using protease enzymes. These enzymes play a crucial role in the development and spread of infections.

Objectives: To assess the impact of UV radiation on the expression of subtilisin virulence genes in *Trichophyton indotineae* isolates obtained from patients in Baghdad, Iraq.

Materials: Seven *T. indotineae* isolates were analyzed in this study, and their proteolytic activity was qualitatively assessed using skim milk agar. The isolate displaying the largest clearance zone in response to UV radiation (254–365 nm) was selected for further experiments with varying exposure durations. The expression of Subtilisin (SUB) genes was measured using the one-step RT-qPCR technique. These specimens were obtained from patients diagnosed with Tinea at Al-Yarmouk Teaching Hospital and Al-Zahra Consultative Center for Allergy and Asthma in Baghdad, and the laboratory procedures were conducted in the Department of Biology, College of Science, University of Baghdad, between October 2022 and February 2023.

Results: Distinct trends in SUB7 gene expression were observed following UV exposure. A significant increase was noted after 6 minutes of exposure to 365 nm UV, while a rapid rise was seen after 3 minutes of exposure to 254 nm UV. All the studied genes (SUB1, SUB3, SUB4, SUB6, and SUB7) exhibited higher expression levels following UV exposure for 3 or 6 minutes. Most genes were upregulated, except SUB3, which was repressed after 12 minutes of exposure to 254 nm UV.

Conclusion: The findings suggest that increased virulence gene expression may be a stress response to UV radiation, potentially influenced by rising global temperatures and increased solar irradiance during Iraqi summers.

Keywords: Dermatophytes; Gene expression; Subtilisin genes; *Trichophyton indotineae*; UV radiation.

Introduction:

Dermatophytes are a specialized group of pathogenic fungi capable of invading and reproducing in keratinized layers of the skin, hair, and nails in humans (1). These infections, commonly known as tineas, are more prevalent in tropical and subtropical regions, including India, China, Lebanon, Iraq, and Egypt, due to high temperatures and humidity (2)(3). It is estimated that 20–25% of the global population is affected by dermatophytosis (4). *Trichophyton indotineae*, a newly classified anthropophilic species, has been associated with recurrent infections and increasing resistance to terbinafine treatment (5) (2). In 2020, *T. indotineae* was identified as a distinct species, separate from *Trichophyton interdigitale* and *Trichophyton mentagrophytes*, based on internal transcribed spacer (ITS) region sequencing of terbinafine-resistant strains from Indian and Nepalese patients. These strains contain three distinct single nucleotides polymorphisms (SNPs) (6). Various infection models and virulence genes have been instrumental in understanding host-pathogen interactions in dermatophytes (7). Among these, subtilisin genes (SUB1–7), encoding serine proteases, play a vital role in dermatophyte virulence (8) (9). *Trichophyton* spp. has major endoproteases, subtilisins (SUB3 and SUB4), which are expressed during in vitro growth (10).

SUB6 is a robust marker of in vivo trichophytosis and onychomycosis caused by *Trichophyton* spp. It is the predominant virulence factor of *T. mentagrophytes* (11).

SUB3 and SUB4 are required for *T. rubrum* to invade the skin (12). The expression of SUB1, SUB6, and SUB7 was upregulated significantly after culturing in nail chips medium, suggesting they could contribute to *T. rubrum* pathogenicity (13). Some of these genes, such as SUB3 and SUB4, are essential for skin invasion, while others, like SUB6 and SUB7, have been identified as significant allergens (14).

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This study investigated the expression of subtilisin virulence genes in *T. indotineae* following UV exposure using RT-qPCR analysis.

Materials and Methods:

Fungal specimens: Seven *T. indotineae* isolates, previously characterized through morphological and molecular analysis (ITS region) and recorded in gene bank with code (OQ789642.1, OQ789635.1, OQ789644.1, OQ787108.1, OQ789643.1, OQ787436.1, OQ789636.1), were obtained from patients diagnosed with tinea at Al-Yarmouk Teaching Hospital and Al-Zahra Consultative Center for Allergy and Asthma in Baghdad, and the laboratory procedures were conducted in the Department of Biology, College of Science, University of Baghdad, between October 2022 and February 2023.

Proteolytic Activity Assay by Skim Milk Agar: The proteolytic activity of *T. indotineae* isolates was assessed using skim milk agar. Fifteen grams of agar was dissolved in 900 ml of distilled water by heating and stirring for 30 minutes, then sterilized in an autoclave. After cooling to 45°C, 100 ml of skim milk was added, mixed well, and distributed into Petri dishes. Skim milk agar plates were inoculated with 5 mm from actively growing cultures, and they were then incubated at 28 ± 2°C. The diameter of the clearance zones was measured over incubation periods of 5, 7, and 10 days. The isolate with the highest protease activity was selected for UV exposure experiments. (15) (16).

UV Radiation Treatment: Fungal spores (1×10^4 spores/mL) were suspended in sterile distilled water and exposed to UV radiation at 254 nm (UV-C) and 365 nm (UV-A) for durations of 3, 6, 9, and 12 minutes. Control samples were maintained without UV exposure. Following irradiation, the spores were cultured on Sabouraud dextrose agar (Hi-Media Company/ India) (SDA) and incubated at 28 ± 1°C (17) (18).

RT-qPCR Analysis of Subtilisin Gene Expression: Total RNA was extracted from the selected *T. indotineae* isolate using the TRIzol™ reagent protocol (19). Primers of genes and housekeeping Gene used in this study were provided by Macrogen (Korea) as shown in Table (1), (20) (21). Gene expression levels of SUB1, SUB3, SUB4, SUB6, and SUB7 were measured using the one-step RT-qPCR technique, reaction volume (10 µl) as shown in Table (2). Fold change in gene expression was calculated using the Livak (2^−ΔΔCT) method equation (20) (22):

Folding (amount of target) = 2 -ΔΔCT.

ΔCT = CT gene - CT House- Keeping gene.

ΔΔCT = ΔCT Treated or Control - ΔCT Control.

Statistical Analysis

Statistical analysis shows how different concentrations affected the research parameters. In this study, means were compared using the least significant difference (LSD) test.

Table (1): Sequences of primers for use with genes encoding secreted SUB Genes

Gene	Forward (5′ ➔ 3′)	Reverse (5′ ➔ 3′)	Annealing Temp. (°C)
SUB1	TGGGTGTTTTCAGATTCATTTC	ACGGCGGGTGATGTTATGG	54
SUB3	TCAAGGTTATCTCCGTCTTC	AAAGAGGACTTCTGGTCATC	
SUB4	TCGCTGCTGGTAATGACAACG	GGAGCATAGATGTCAACTGAAG	
SUB6	GCTCATACAACTTGGCTTAG	TCAGAGGCAGGAGAAGAGT	
SUB7	CGGCATCTGTCATCAACG	AGTGACCAGAGTATCCCTT	
18S rRNA	TCGACCCCGGAGAAGGA	GCCTGCTGCCTACCTTGGA	60

Table (2): RT-qPCR reaction components for detection of SUB gene expressions

Master mix components	Stock	Unit	Final	Unit	Volume
					1 sample
qPCR Master Mix	2	X	1	X	5
RT mix	50	X	1	X	0.25
MgCl2					0.25
Forward primer	10	µM	0.5	µM	0.5
Reverse primer	10	µM	0.5	µM	0.5
Nuclease Free Water					2.5
DNA		ng/µl		ng/µl	1
Total volume					10
Aliquot per single rxn	9µl of Master mix per tube and add 1µl of Template				

Results:

Proteolytic Activity: According to Table 3, all *T. indotineae* isolates exhibited extracellular protease activity, with the highest enzymatic efficiency observed after a 10-day incubation period, amounting to 20.7± 0.33 mm, and the lowest value enzyme. recorded at a 5-day incubation period, amounting to 12.0 ± 1.0 mm. Thus, the most virulent isolate was selected based on the highest percentage of the diameter of clearance zone (mm) produced by protease

Table (3): Activity of protease enzyme of *T. indotineae* grown on Skim Milk Agar medium at 28±2°C for 5, 7 and 10 days

No. of isolation	Lytic enzyme activity in diameter (clear zone) (mm) / days		
	5	7	10
1.	13.3 ± 0.33	15.3 ± 0.33	18.3 ± 0.33
2.	12.0 ± 1.0	15.0 ± 1.0	17.3 ± 0.33
3.	13.0 ± 1.0	14.3 ± 0.33	17.7 ± 0.33
4.	12.0 ± 1.0	15.7 ± 1.33	18.7 ± 0.33
5.	12.7 ± 0.33	14.7 ± 2.33	17.7 ± 0.33
6.	15.3 ± 2.33	17.0 ± 1.0	20.7 ± 0.33
7.	13.3 ± 0.33	15.0 ± 1.0	17.0 ± 1.0
*L.S.D.	P= 0.05		
between isolates	0.93		
between days	0.34		
Between Interaction	1.62		

**L.S.D: least Significant Difference*

Gene Expression Analysis: RT-qPCR analysis revealed that UV exposure (365 and 254 nm) significantly influenced SUB gene expressions as shown in Table (4).

SUB7 exhibited the highest fold change (284.05) following exposure to 365 nm UV for 6 minutes. SUB1 showed a (179.90-fold) increase after 3 minutes of exposure to 254 nm UV and was decreasing in expression (3.40) (254nm for 12 min.). SUB3 was the only gene that showed repression (0.85-fold change) following 12 minutes of exposure to 254 nm UV, while showing the highest folding change (134.26) when the sample was treated with 365nm for 6 minutes.

SUB 4 showed a high folding change (206.66) when treated with 254nm for 3 minutes and the lowest result when treated with 254nm for 12 minutes.

SUB6 and SUB7 showed the highest folding change (223.59) (284.05) respectively, when treated with 365nm for 6 minutes, Figure (1).

These findings suggest that UV exposure induces the expression of subtilisin virulence genes, potentially as a stress response mechanism.

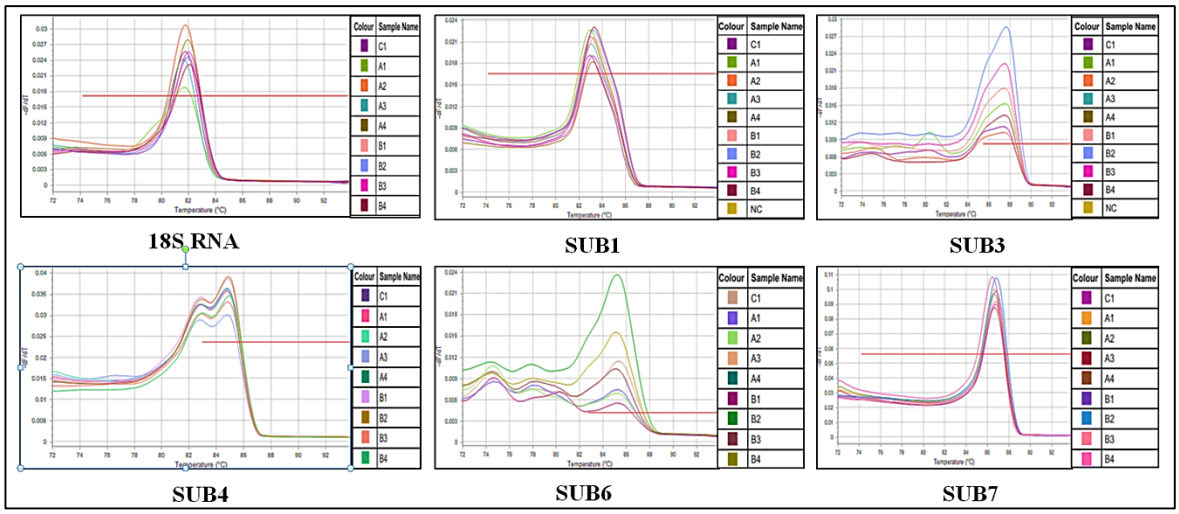

Figure (1): Melting peak of real-time PCR (qPCR) to detect of SUB genes. Extensive with fluorescence cut off of 5%

Table (4): Fold change of SUB genes expression for *T. indotineae* isolate treated with 365 and 254 nm UV irradiation, data analysis using the Livak method

SUB1						
Sample	**18S**	**SUB 1**	**ΔCT**	**ΔΔCT**	**Folding**	**L.S.D (0.05)**
C1	17.37	20.65	3.28	0.00	1.00	1.02
A1	26.23	22.01	-4.21	-7.49	179.90	
A2	20.93	22.02	1.09	-2.19	4.56	
A3	22.83	22.60	-0.23	-3.51	11.41	
A4	23.16	24.67	1.51	-1.77	3.40	
B1	23.52	21.35	-2.17	-5.45	43.69	
B2	24.89	20.89	-3.99	-7.27	154.45	
B3	24.14	22.43	-1.71	-4.99	31.68	
B4	24.25	20.94	-3.32	-6.60	96.70	
SUB3						
Sample	**18S**	**SUB 3**	**ΔCT**	**ΔΔCT**	**Folding**	**L.S.D (0.05)**
C1	17.37	26.55	9.18	0.00	1.00	2.31
A1	26.23	29.07	2.84	-6.34	81.02	
A2	20.93	28.37	7.44	-1.73	3.33	
A3	22.83	30.99	8.15	-1.02	2.03	
A4	23.16	32.57	9.41	0.23	0.85	
B1	23.52	30.24	6.72	-2.46	5.49	
B2	24.89	27.00	2.11	-7.07	134.26	
B3	24.14	28.32	4.18	-4.99	31.87	
B4	24.25	27.40	3.14	-6.03	65.55	
SUB4						
Sample	**18S**	**SUB4**	**ΔCT**	**ΔΔCT**	**Folding**	**L.S.D (0.05)**
C1	17.37	24.99	7.61	0.00	1.00	2.57
A1	26.23	26.15	-0.08	-7.69	206.66	
A2	20.93	26.04	5.11	-2.50	5.67	
A3	22.83	27.02	4.19	-3.42	10.73	
A4	23.16	29.02	5.86	-1.75	3.37	
B1	23.52	25.79	2.27	-5.34	40.56	
B2	24.89	24.83	-0.06	-7.67	203.75	
B3	24.14	26.35	2.22	-5.40	42.20	
B4	24.25	25.17	0.91	-6.70	104.27	
SUB6						
Sample	**18S**	**SUB6**	**ΔCT**	**ΔΔCT**	**Folding**	**L.S.D (0.05)**
C1	17.37	24.15	6.77	0.00	1.00	8.86
A1	26.23	25.91	-0.32	-7.10	136.85	
A2	20.93	25.80	4.87	-1.90	3.73	
A3	22.83	27.10	4.26	-2.51	5.70	
A4	23.16	28.08	4.92	-1.85	3.61	
B1	23.52	26.61	3.09	-3.68	12.84	
B2	24.89	23.86	-1.03	-7.80	223.59	
B3	24.14	25.23	1.09	-5.68	51.42	
B4	24.25	24.12	-0.13	-6.90	119.74	
SUB7						
Sample	**18S**	**SUB7**	**ΔCT**	**ΔΔCT**	**Folding**	**L.S.D (0.05)**
C1	17.37	27.29	9.92	0.00	1.00	3.24
A1	26.23	28.01	1.78	-8.14	281.89	
A2	20.93	28.10	7.17	-2.74	6.69	

A3	22.83	28.95	6.11	-3.80	13.95
A4	23.16	31.13	7.97	-1.94	3.85
B1	23.52	27.80	4.28	-5.63	49.63
B2	24.89	26.66	1.77	-8.15	284.05
B3	24.14	28.51	4.37	-5.54	46.66
B4	24.25	26.66	2.40	-7.51	182.80

Discussion:

A greater number of virulence factors such as proteases are important for infection to occur (23). The results of the present study confirm that *T. indotineae* secretes proteolytic enzymes, with protease activity increasing over time which may lead to increased virulence of *Trichophyton Indotineae* in Iraq (24) (25).

The expression of SUB genes was significantly upregulated following UV exposure, indicating that these genes are involved in stress adaptation. We observed distinct gene expression trends in SUB7 with UV365 nm exposure during 6-minutes recovering period was fast, following UV254 nm exposure during 3-minutes. All genes under study (SUB1, SUB3, SUB4, SUB6, and SUB7) show highly folding expression in UV365 nm exposure during 6-min and UV254 nm exposure during 3-minutes. All genes were more induced than repressed, except SUB3 gene which was the only gene inhibited when exposed to UV254 nm during 12-minutes.

Previous studies have shown that dermatophytes modulate virulence gene expression in response to environmental factors, including oxidative stress and keratin degradation. The current research systematically focused on studying and comparing the genomic responses of subtilisin genes from *T. indotineae* to two UV wavelengths (254-365nm) with different period of time of exposure. It was found that genes encoding the virulence proteinase enzyme are induced by UV radiation. Hence, the main effect after UV exposure (254-365 nm) appears to be the stimulation of gene expression. Although some genes have a highly active detoxification system that includes novel and traditional UV defense mechanisms, such as activation of heavy metal efflux pumps, multidrug-resistant enzymes, antioxidants, proteins, sequestration of transition metals, and activation of degradative pathways, they are still unable to protect against UV-induced oxidative stress (26). These results are in accordance with those developed by *Çavuşoğlu K et al* (27), who demonstrated that UV-induce many genes that coded many enzymes. *Bitencourt TA* (7) reported that UV radiation damages DNA by changing the pairing of nucleotides, resulting in the formation of a new bond between adjacent nucleotides on the same DNA strand. This increase in expression of virulence genes may be a response to stress conditions, as *T. indotinea* modulates the expression of its own virulence genes, as in many fungi spp. (7). This modification is essential for survival in vivo, as strains lack this ability due to a mutant gene, the product of which is involved in the expression of a signal-dependent virulence gene. Ionizing radiation, a physical agent that attacks DNA molecules either directly or by generating reactive oxygen species and free radicals, degrades DNA molecules to the point where they are no longer able to function properly (28).

UV radiation is known to induce genetic damage, altering nucleotide pairing and leading to stress-induced gene expression. The observed increase in SUB gene expression following UV exposure suggests that these genes play a role in fungal adaptation to environmental stressors, such as increased solar radiation due to global warming and the increase in the rate of radiation recorded in the summer in Iraq (29), which coincides with the increase in recorded cases of infection with tinea.

Limitation:

This work focused on the effects of short-term UV radiation under controlled laboratory conditions using a limited number of strains. The laboratory approach may not fully capture host-pathogen interactions or natural environmental variability. These factors warrant further investigation in more complex models.

Conclusion:

The findings of the current study indicate that UV exposure significantly influences subtilisin gene expression, which may contribute to the pathogen's virulence. Given the rising incidence of dermatophyte infections and increased solar radiation, further research is needed to explore the molecular mechanisms underlying this adaptive response.

Authors' declaration:

We confirm that all the Figures and Tables in the manuscript belong to the current study. Besides, the Figures and images, which do not belong to the current study, have been given permission for re-publication attached to the manuscript. Authors sign on ethical consideration's Approval-Ethical Clearance: The project was approved by the local ethical committee in the Department of Biology, College of Science, University of Baghdad Ref. No. CSEC/01223/00143 on December 20, 2023.

Conflicts of Interest: None.

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Authors' contributions:
Study conception & design: (Aseel H. Hendi, Alaa M. Al-Araji). Literature search: (Aseel H. Hendi). Data acquisition: (Aseel H. Hendi). Data analysis & interpretation: (Aseel H. Hendi, Alaa M. Al-Araji). Manuscript preparation: (Aseel H. Hendi). Manuscript editing & review: (Aseel H. Hendi, Alaa M. Al-Araji)

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التعبير الجيني لجينات السبتليزين بعد التعرض للأشعة فوق البنفسجية على فطريات الترايكوفيتون إندوتينيا المعزولة من المرضى العراقيين

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الخلاصة:
الخلفية: تعتبر جينات الضراوة للفطريات الجلدية من القضايا البحثية المهمة في السنوات الأخيرة. حيث تمتلك الفطريات الجلدية قابلية على تحليل الكيراتين، وهي بروتينات بنيوية مهمة موجودة في جلد الإنسان، بواسطة إنزيمات البروتييز التي تعد واحدة من أهم العوامل التي تؤثر على تطور وانتشار العدوى.
الأهداف: التحقيق في تأثير الأشعة فوق البنفسجية على التعبير الجيني عن جين الضراوة في السوبتيليزين على عزلات *Trichophyton indotineae* التي أجريت في بغداد، العراق.
المنهجية: تم استخدام سبع عزلات من *T.indotineae* في هذه الدراسة، وتم استخدام وسط أجارالحليب منزوع الدسم لتقييم النشاط البروتيني للعزلات نوعيًا. حيث تم اختيار عزلة واحدة من *T.indotineae* ذات أعلى قطر لهالة التحلل للبروتين في الوسط وتم تعريضها لطول موجة الأشعة فوق البنفسجية (254-365 نانومتر) لفترات تعرض مختلفة. تم قياس التعبير عن جين SUB باستخدام تقنية RT-qPCR بخطوة واحدة. تم الحصول على هذه العينات من المرضى الذين تم تشخيصهم بالإصابة في مستشفى اليرموك التعليمي ومركز الزهراء الاستشاري للحساسية والربو في بغداد، وأجريت الإجراءات المخبرية في قسم علوم الحياة، كلية العلوم، جامعة بغداد، بين تشرين الأول 2022 وشباط 2023.
النتائج: لاحظنا اتجاهات مميزة لتعبير جين SUB7 مع التعرض للأشعة فوق البنفسجية بطول موجة 365 نانومتر خلال فترة تعرض التي استمرت 6 دقائق وزادت بعد التعرض للأشعة فوق البنفسجية بطول موجة 254 نانومتر خلال 3 دقائق. أظهرت جميع الجينات المدروسة (SUB1 وSUB3 وSUB4 وSUB6 وSUB7) تعبيرًا عاليًا في التعرض للأشعة فوق البنفسجية لمدة 6 دقائق والتعرض للأشعة فوق البنفسجية لمدة 3 دقائق. كانت جميع الجينات أكثر تحريضًا من قمعها، باستثناء SUB3 الذي تم قمعه فقط عند تعرضه للأشعة فوق البنفسجية بطول موجة 254 نانومتر لمدة 12 دقيقة.
الإستنتاج: تُظهر النتائج أن الزيادة في التعبير عن جينات الضراوة قد تكون استجابة لظروف الإجهاد (تأثيرات الأشعة فوق البنفسجية) التي تعدل تعبير *T.indotinea* عن جينات الضراوة، خاصة بعد زيادة الانحباس الحراري العالمي وزيادة الإشعاع المسجل في الصيف في العراق.
الكلمات المفتاحية: الفطريات الجلدية، التعبير الجيني، جينات السوبتليزين، ترايكوفيتون إندوتينيا، الأشعة فوق البنفسجية.