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ANTIFUNGAL ACTIVITY OF OXIDATIVE DAMAGING AGENTS IN COMBINATION WITH METAL CATIONS AGAINST *TRICHOPHYTON RUBRUM* ISOLATED FROM A DERMATOLOGICAL PATIENT

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SUMMARY

Trichophyton rubrum is a causative agent responsible for human chronic dermatophytosis worlwide. It is a common causative agent of the dermatophyte infections and resistant to many antifungal drugs. Oxidative damaging agents belong to a strong antimicrobial group and have been intensively investigated. The synergistic oxidative damage can occur if the agents react with metal cations. Therefore, in this study the antifungal activity of formulas containing the two oxidative damaging agents, including hydrogen peroxide (H_2O_2) and 8-hydroxyquinoline (8HQ), in combination with metal cations has been determined to examine the synergistic damage in *T. rubrum* isolated from the skin of a dermatological patient. The isolate was identified by morphology observations, biochemical tests and sequence analysis of ITS1-4 gene, in comparison with the similar ITS nucleotide sequences of others. The obtained results showed that the isolated fungal strain had physiological and biochemical characteristics of *T. rubrum* with more than 99% identity of the *T. rubrum* CBS 392.58 ITS sequence with OL347577 accession numbers. The antifungal tests indicated that the combinations of 2 mM Cu^{2+} + 0.05% H_2O_2; 2 mM Cu^{2+}+ 0.005% 8HQ; 2 mM Cu^{2+} + 0.005% 8HQ + 0.05% H_2O_2 exerted the best antifungal activity against the isolated *T. rubrum* with the diameter of inhibition zones of 22, 25 and 28 mm, respectively. Among the combinations containing Zn^{2+}, the only formula of 2 mM Zn^{2+} + 0.05% H_2O_2 exhibited the desired effect, while the combinations containing Fe^{2+} did not show any activity. Thus, our study suggested a potential use of Cu^{2+} + 0.05% H_2O_2; 2 mM Cu^{2+}+ 0.005% 8HQ and 2 mM Cu^{2+} + 0.005% 8HQ + 0.05% H_2O_2 formulas as new potential antifungal agents against *T. rubrum* due to their synergistic oxidative damage.

Keywords: 8-hydroxyquinolin, antifungal activity, hydrogen peroxide, oxidative damage, *Trichophyton rubrum*

INTRODUCTON

Trichophyton rubrum belongs to the filamentous fungi group that is a common causative agent of dermatophyte infections in the skin, nails, and hair (Sentamilselvi, 1998; Smith *et al.*, 2001). *T. rubrum* is found all over the world and its antibiotic resistance has been reported in South America, North America, Northern Europe, and Central Europe (Squeo *et al.*, 1998; Pereira *et al.*, 2013). In addition, *T. rubrum* often causes chronic infections with the high recurrence rate of 25–40% (Fachin *et al.*, 2006; Pereira *et al.*, 2013). Antifungal agents of

the azole group, such as terbinafin, are considered the first choice for treatment of the dermatophyte disease. However, these agents could cause some unwanted effects, as well as antifungal resistance, that resulted in a reduction in the treatment efficacy (Pereira *et al.*, 2013). In recent years, the studies on the development and application of new antifungal agents for the effective treatment of *T. rubrum* infections have been intensively carried out. Deavall *et al*. (2012) indicated that reactive oxygen species (ROS), highly reactive chemicals formed from O_2, such as hydrogen peroxide (H_2O_2) were formed during cellular metabolism in response to irritating or harmful agents. The high concentrations of ROS can cause oxidative damage to inhibit the growth or kill the pathogens (Deavall *et al.*, 2012). Therefore, oxidative damaging agents, that can produce ROS, are classified as one of the potentially antimicrobial groups for therapeutic applications. However, full assessments on their biological actions have not been intensively investigated, especially, the combination of these agents and metal cations to enhance their inhibitory effects on bacteria/fungi for practical applications.

The antibacterial role of H_2O_2 has broadly been confirmed. The 3% H_2O_2 solution is widely used as an antiseptic and antibacterial agent. H_2O_2 is a ROS, capable of causing oxidative damage (Imlay, 2003). The antibacterial effect of H_2O_2 is increased in the presence of metal cations such as Fe^{2+} and Cu^{2+}, even at very low concentrations. These substances promote the breakdown of H_2O_2 to hydroxyl radicals ($^{\bullet}OH$) through the Fenton reaction. The reaction of H_2O_2 and Fe^{2+} would result in an enhanced decomposition of H_2O_2, along with the formation of $^{\bullet}OH$, a ROS with very strong oxidative damages in cells (Henle, Linn, 1997; Almeida *et al.*, 1999; Cabiscol Català *et al.*, 2000; Symons *et al.*, 2001; Dalecki *et al.*, 2017).

Another substance, 8-hydroxyquinoline (8HQ) has long been used as a chelating agent with metal ions to generate potent products against bacteria and fungi (Shen *et al.*, 1999). It was also found to be a strong oxidative damaging agent by generating $^{\bullet}O_2$ (Nguyen Thi Mai Phuong *et al.*, 2004). Therefore, 8HQ exhibits strongly inhibitory effects on biochemical process and therefore, can be a potent chemical to microorganisms (Nguyen Thi Mai Phuong *et al.*, 2004). The previous studies indicated that 8HQ and its derivatives were effective the antifungal and antibacterial agents (Shah *et al.*, 2018; Li *et al.*, 2020; Saadeh *et al.*, 2020; Nguyen Thi Mai Phuong *et al.*, 2004).

However, the antibacterial and antifungal effects of the combinations of oxidative damaging agents, such as H_2O_2 and 8HQ with metal cations to evaluate their synergistic effects on oxidative damage in microorganisms for practical application have not been well investigated. This study was carried out to determine the antifungal activity of the combinations of H_2O_2 and 8HQ with metal cations against *T. rubrum* isolated from a dermatological patient for future application.

MATERIALS AND METHODS

Materials

T. rubrum was collected from the specimens of a dermatological patient at the National Hospital of Dermatology according to the ISO standard procedures. The isolated *Trichophyton* sp. strain was identified by sequencing method of ITS1-4 region. Sabouraud Dextrose Agar (SDA) medium for the growth of *T. rubrum* was purchased from Sigma (USA). The test formulas containing metal cations 2 mM $Fe^{2+}/Cu^{2+}/Zn^{2+}$+ 0.05% H_2O_2 and 2 mM $Fe^{2+}/Cu^{2+}/Zn^{2+}$+ 0.005% 8HQ and 2 mM $Fe^{2+}/Cu^{2+}/Zn^{2+}$+ 0.05% H_2O_2 + 0.005% 8HQ used in this study were selected based on our previous results (Phan Tuan Nghia *et al.*, 2003; Nguyen Thi Mai Phuong *et al.*, 2004) (Table 1).

Isolation of *T. rubrum* from a dermatological patient

Specimen collection

The patient's skin lesions were disinfected with 70% alcohol. A typical skin lesion margin

specimen was obtained with a blunt knife and placed on a sterile slide. Then, direct observation was performed under 10x and 40x objectives of a light microscope (Olympus, US) to determine the presence of mycelium, including branched, segmented, old and young hyphae. The specimens were then inoculated into SDA agar medium with a distance of 1.5 cm – 2.0 cm apart, and incubated at 25-27°C for 7-10 days.

Table1. The selected formulas for determination of antifunfal activity.

Fe^{2+}	Cu^{2+}	Zn^{2+}
1. H_2O_2 0.05%	1. H_2O_2 0.05%	1 H_2O_2 0.05%
2. 8HQ 0.005%	2. 8HQ 0.005%	2. 8HQ 0.005%
3. Fe^{2+} 2 mM + H_2O_2 0.05%	3. Cu^{2+} 2mM + H_2O_2 0.05%	3. Zn^{2+} 2mM + H_2O_2 0.05%
4. Fe^{2+} 2 mM + 8HQ 0,005%	4. Cu^{2+}2mM + 8HQ 0.005%	4. Zn^{2+} 2mM + 8HQ 0.005%
5. Fe^{2+}2 mM + 8HQ 0,01% + H_2O_2 0.05%	5. Cu^{2+} 2mM + 8HQ 0.005% + H_2O_2 0.05%	5. Zn^{2+} 2mM + 8HQ 0.005% + H_2O_2 0.05%

Identification by morphological observation

Macroscopic observation of *T. rubrum* colonies was performed to identify its characteristics, including: (i) The front surface of the inoculum has dark yellow, seeded or covered with fluff; (ii) The back of the culture plate is dark red or burgundy, sometimes brown, yellow-orange or colorless.

Microscopic observation of *T. rubrum* colonies was done by culturing the colony on 1% corn meat medium containing tween 80 to examine the following characteristics: (i) Hyphaeis transparent with the septum (from 1-4); (ii) Macroconidia may or may not be present with a size of 4-8 x 40-60 m, waist length, thin wall and two parallel pen-shaped walls lead. Microconidia have a single teardrop or pear shape attached along the hyphae side.

Identification by biochemical tests

- Urea generation: negative reaction (in the liquid SDA medium containing idol gives yellow-orange color).

- Acidogenic generation: The dermatophyte was culture on agar medium containing bromocresol purple (BCP), milk and glucose. The dermatophyte will have a limited growth rate and not produce acid, therefore pH and color of the medium will not change.

- Red pigment production: The dermatophyte will proliferate and produce red pigment on the back of the SDA agar plate containing glucose and cornstarch.

- Growth ability: The dermatophytecan grow in the medium without growth factors.

The potential colony was then re-inoculated on SDA agar plates and placed at 25-27°C for 7-10 days. The white mycelium with the *T. rubrum* morphology is grown on the top of the Petri dish.

Identification by analysis of ITS1-4 sequencing

The ITS region between the 18S rRNA and 28S rRNA genes (including ITS1; 5.8S rRNA; ITS4) in fungi has the highest degree of variability compared with the 18S rRNA and 28S rRNA genes. Therefore, this sequence region was commonly used in fungal taxonomy (Curran et al., 1994; Korabečná et al., 2003). Total DNA from the isolate was extracted using a yeast/mold DNA extraction kit from NorGene Biotek (Canada). The total DNA was then electrophorezed on 0.8% agarose gel and the DNA concentration was determined by using a NanoDrop spectrometer (Thermo Scientific, USA) at 260/280 nm. The qualified DNA sample was used to amplify the ITS1-4 region by PCR using a versatile primer pair ITS1/ITS4 specific for a broad spectrum of fungal species: (i)

Forward primer ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'; (ii) Reversed primer ITS4: 5'-TCCTCCGCTTATTGATATGC-3'.

PCR thermal cycling was as follows: 94°C/6 min for one cycle; 30 cycles at 94°C/30 seconds, 58°C/30 seconds, 72°C/40 seconds to 1.5 minutes; and last cycle at 72°C/10 min; then kept at 4°C for store. PCR products were electrophoresed on 1% agarose gel and vizualized under the ultraviolet lamp in the Gel Doc XR System (Bio-Rad, USA). The PCR product was purified using the Promega purification kit (USA) and was direct sequenced using an ABI 3500XL sequencer (Thrermo Fische Scientific, USA). The ITS nucleotide sequence obtained in this study was compared with the similar sequences in the GenBank database.

A phylogenetic tree was reconstructed using MEGA X software based on the analysis of 12 nucleotide sequences, including the Derma 001VN and other 11 fungal sequences of different genera retrieved from GenBank (*Trichophyton*, *Arthroderma*, *Penicillium*, *Aspergillus*, *Lophophyton*, *Microsporum*) and *Paraphyton* (as an outgroup). The sequences were aligned using Clustal W and phylogenetic inferences were collected using the maximum likelihood method in MEGA X with a bootstrap of 1000 replications (Kumar *et al*., 2018).

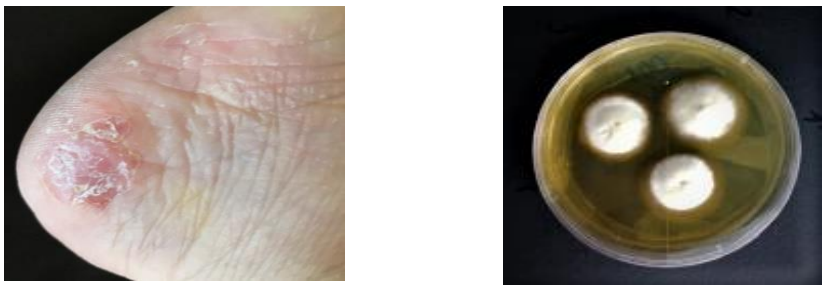
Determination of antimicrobial activity on agar plate

Agar plate diffusion method was used to determine the antifungal activity of the investigated combinations. The plate was inoculated with 100 µL of fungal suspension, evenly spreadedout on the agar surface and then the wells on we agar plate were punched. Different volumes of 100 µL of test sample were placed in the wells and the plates were refrigerated at 4^{o}C for 2 h for well substance diffusion before placing at 25-27°C for 7-10 days. The growth inhibition zone (mm) was measured after incubation. Compounds such as itraconazole, nystastin and cycloheximide were used as positive controls.

RESULTS AND DISCUSSION

Isolation of *T. rubrum* from a dermatological patient

Skin specimens collected from a dermatological patient's lesions (Fig. 1A) were cultured in a SDA medium and incubated at 25-27^{o}C for 7 days.



A **B**

Figure 1. Skin arear infected with Derma 001 VN (A); Fungus mycelinum on SDA agar plate after 7 days incubation (B).

The mycelium was formed on the surface of SDA agar plates with cottony morphology and white color while the underside of the colony was yellow (Figure 1B). On Littman Oxgall agar, colonies were green after 14 days of culture. Red colonies with no pH change were observed on agar medium supplemented with bromocresol. This result suggests that the *Trichophyton* fungus

was isolated and it was named Derma 001 VN (Figure 2A). The mycelium morphology was observed under the microscope at x40 magnification (Figure 2B).

Identification of isolated *Trichophyton sp* by sequencing the rDNA ITS1-4 region

Total DNA from the isolate was extracted using the Canadian NorGene Biotek yeast/mold DNA extraction kit. The DNA products were electrophoresed on a 0.8% agarose gel (Fig. 3). The absorption ratio of DNA concentration at A260/A280 nm was 1.8, thus, indicating the required level of purity.

The extracted DNA of the Derma 001 VN dermatophyte was used to amplify the ITS1-4 region by using PCR with the primer pairs ITS1/ITS4. PCR product after electrophoresis on agarose gel, showed a specific band with a molecular size of 700 bp as expected. The PCR product was then purified and directly used to analyse the nucleotide sequence of the ITS1-4 region.

Nucleotide sequence comparison of the ITS1-4 region of the isolate with those of the fungal strains available in the Genbank database showed that the Derma 001 VN strain with OL347577 accession numbers had the highest nucleotide similarity (99.03%) with the strain *T. rubrum* CBS 392.58. The phylogenetic tree of the Derma 001VN was drawn using MEGA X software (Figure 3) showed that the isolated dermatophyte was placed in a branch closely related to *T. rubrum*.

A **B**

Figure 2. Isolated Derma 001 VN in inclined agar tube (A); Fungus mycelinium of the isolated Derma 001 VN observation under microscopy at 40X magnification (B).

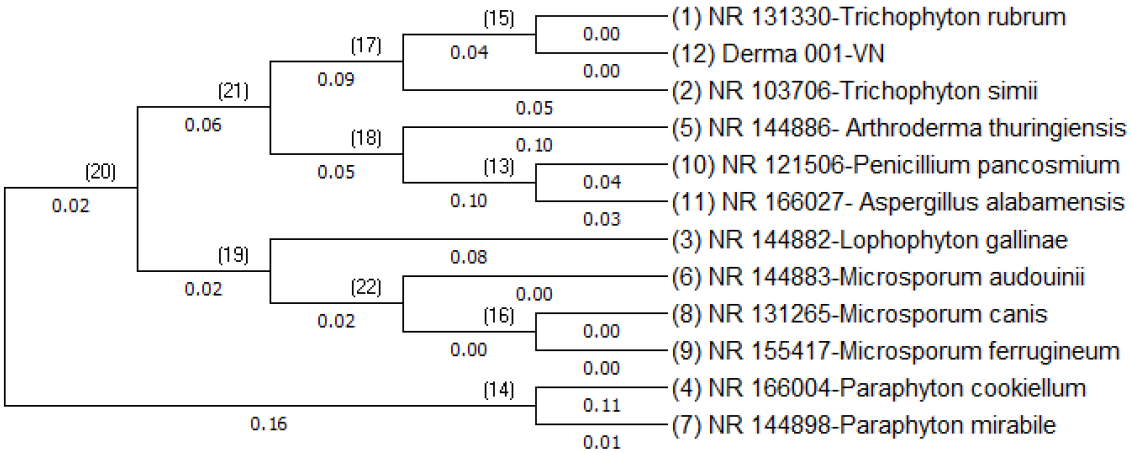
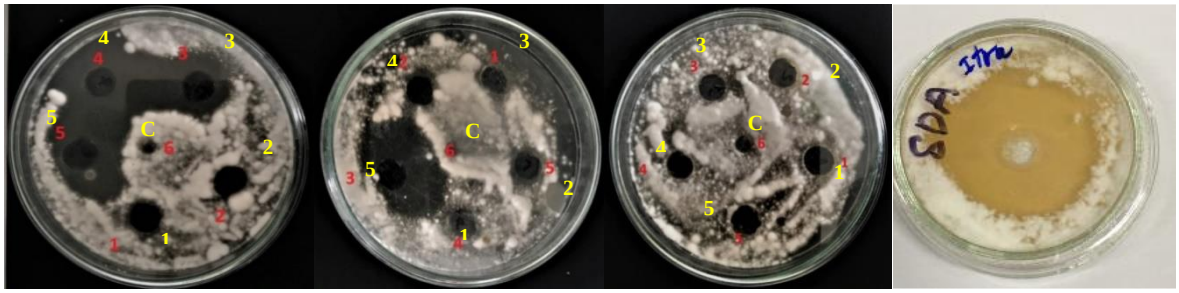


Figure 3. Phylogenetic tree of the isloated fungus *T. rubrum* Derma 001-VN.

Determination of the antifungal activity of *T. rubrum* using oxidative damaging agents in combination with metal cations

In order to find new antifungal agents for *T. rubrum* as well as, to evaluate the synergistic effects of oxidative damage in the isolated *T. rubrum*, the combinations of oxidative damaging agents H_2O_2 and 8HQ in combination with metal cations Fe^{2+}, Cu^{2+}, Zn^{2+} were tested. The formulations were selected with very low H_2O_2 and 8HQ concentrations to demonstrate that they had an enhanced antifungal effect on the *T. rubrum* in comparison with the single components H_2O_2, 8HQ and Fe^{2+}, Cu^{2+}, Zn^{2+}. The formulas in this study were selected based on our previous finding on synergistic oxidative damages occurred in *S. mutans* treated with the combinations of 2 mM Fe^{2+}, Cu^{2+}, Zn^{2+} metal cations with H_2O_2 and 8HQ, while the single forms had no effects (Phan Tuan Nghia *et al.*, 2003; Nguyen Thi Mai Phuong *et al.*, 2004). The data presented in figure 4 indicated that the formulas N°3, 4, 5 containing Cu^{2+} combined with H_2O_2 and 8HQ have enhanced activity compared to the single components (8HQ, H_2O_2 or metal ions alone) with diameters of inhibition zones of 22, 25, and 28 mm. The controls nystatin and cycloheximide had no antifungal activity against the Derma 001VN (data not shown) butitraconazole exhibited good activity with an antifungal ring diameter of 44 mm at the concentration of 5 µg/well (ca 7% solution). This concentration is significantly higher than those of H_2O_2, 8HQ, and metals in the combinations (0.05% H_2O_2, 0.005% 8HQ, and 2 mM metal cations). Interestingly, among the test samples containing Zn^{2+}, the only formula of 2 mM Zn^{2+}+ 0.005% 8HQ + 0.05%H_2O_2 showed the antifungal zone with a diameter of 26 mm. Surprisingly, no antifungal activity was found with the formulas containing Fe^{2+}. This result is quite different from that of previous studies, in which the combination of Fe^{2+} with 8HQ and Fe^{2+} with H_2O_2 had strong oxidative damage in *S. mutans* (Phan Tuan Nghia *et al.*, 2003; Nguyen Thi Mai Phuong *et al.*, 2004). The reasons for that may come from the difference in the test microorganisms and the form (solution or solid agar) of the culture medium. Moreover, the precipitation of Fe^{2+} found in the combinations of this experiment may cause a reduction of antifungal activity.



A B C D

Figure 4. Antifungal effect of the combinations of enhancing oxidative damaging agents with metal cations against *T. rubrum* isolated from a Vietnamese dermatological patient. A. Formula containing Cu^{2+}; B. Formula containing Zn^{2+}; C. Formula containing Fe^{2+}; D. Positive control Itraconazole. 1. 0.05% H_2O_2; 2. 0.005% 8HQ ; 3. 2 mM Cu^{2+} /Zn^{2+} / Fe^{2+} + 0.05% $H_2O_2$4; 2 mM Cu^{2+} /Zn^{2+} / Fe^{2+} + 0.005% 8HQ; 5.2 mM Cu^{2+}/Zn^{2+} / Fe^{2+} + 0,01% 8HQ + 0.05% H_2O_2; c. Control (H_2O).

Thus, the combinations, including 2 mM Cu^{2+} + 0.05% H_2O_2; 2 mM Cu^{2+}+ 0.005% 8HQ, and 2 mM Cu^{2+} + 0.005% 8HQ + 0.05% H_2O_2 are new formulas containing very low concentrations of oxidative damaging agents but exhibit good antifungal activity against *T. rubrum*. These new formulas can be used to effectively treat skin infection caused by *T. rubrum*. However, more works on different dermatophyte strains, as well as a full evaluation

of the skin irritation effects are needed to confirm the applicability of these test combinations.

CONCLUSION

A strain of *Trichophyton rubrum*, named Derma 001-VN (with OL347577 accession numbers on NCBI), was successfully isolated from the skin lesions of a dermatological patient and its ITS1-4 nucleotide sequence showed 99.03% identity to the strain *Trichophyton rubrum* CBS 392.58 in GenBank and closely related to the other *Trichophyton* and fungal strains.

Tested with different formulas of oxidative damaging agents combined with the metal cations, it was revealed that the combinations of 2 mM Cu^{2+} + 0.05% H_2O_2; 2 mM Cu^{2+} + 0.005% 8HQ, and 2 mM Cu^{2+} + 0.005% 8HQ + 0.05% H_2O_2 were effective in enhancing oxidative damage compared with the single components. These combinations, in this study, had a good antifungal activity against dermatophyte *T. rubrum* isolated from the dermatological patient.

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