ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2024;10(3):237-243 10.52547/iem.10.3.237

Detection of the *In-Vitro* Inhibitory Effects of Nitroglycerin on *Candida albicans*, *Trichophyton rubrum*, and *Aspergillus flavus*

ARTICLE INFO

Article Type
Original Article

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How to cite this article
Mohamadi A., Hashemi S.J., Daie-Ghazvini R., Rezaie S., Rafat Z., Ardi P. Detection of the In-Vitro Inhibitory Effects of Nitroglycerin on *Candida albicans*, *Trichophyton rubrum*, and *Aspergillus flavus*.Infection Epidemiology and Microbiology. 2024;10(3): 237-243.

Article History
Received: March 10, 2024
Accepted: June 19, 2024
Published: August 20, 2024

ABSTRACT

Background: The present investigation aimed to survey the *in-vitro* inhibitory effects of nitroglycerin against *Candida albicans*, *Trichophyton rubrum,* and *Aspergillus flavus*. .
Materials & Methods: In the current investigation, 99 fungal isolates were gathered from patients referred to the Medical Mycology Laboratory of Tehran University of Medical Sciences**.** The disk diffusion method was done based on Clinical and Laboratory Standards Institute (CLSI) M44-S2 guidelines. Also, the microdilution method was performed base on CLSI guidelines for filamentous fungi (document M38-A2) and yeasts (document M27-A3).
Findings: In the disk diffusion method, all isolates of *C. albicans* (n=33, 100%) and *A. flavus* (n=33, 100%) showed sensitivity to nitroglycerin, whereas all isolates of *T. rubrum* (n=33, 100%) showed resistance to nitroglycerin. On the other hand, in the microdilution method, the minimum inhibitory concentration (MIC) of nitroglycerin against *C. albicans* and *A. flavus* isolates was 0.5 mg/mL, whereas the MIC of nitroglycerin against *T. rubrum* was 0.12 mg/mL.
The results showed that the MIC of nitroglycerin against dermatophytes was about one-quarter of its MIC against *C. albicans* and *A. flavus*, and this difference was statistically significant ($p < .05$).
Conclusion: Considering the potential and efficacy of nitroglycerin against yeasts and filamentous fungi (saprophytes and dermatophytes), complementary *in-vivo* and *in-vitro* studies should be done.

Keywords: Nitroglycerin, Minimum inhibitory concentrations, *Candida albicans*, *Aspergillus flavus*, *Trichophyton rubrum*

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Introduction

Until the 1980s, most common fungal infections were not life-threatening, and dangerous systemic fungal infections had a very low prevalence; thus, researchers showed little enthusiasm to discover new antifungal drugs [1-3]. On the other hand, the preparation of antifungal drugs was more difficult and time-consuming compared to antibacterial drugs [1, 4]. Since the beginning of the 80s, due to the evolution of medical science and therapeutic advances, as well as the global spread of AIDS (acquired immune deficiency syndrome) and the increase in the frequency of patients with immune deficiency, the prevalence and significance of opportunistic fungal infections has increased more than before, despite the constant prevalence of pathogenic fungal infections [4, 5]. Therefore, the need for effective antifungal drugs with few side effects and long-term usability has also been felt more than ever.

Although many effective antifungal drugs have been discovered so far, their solubility, stability, absorption, and toxicity have limited their clinical use [6-8]. Besides, since the number of accessible systemic antifungals is low, antifungal resistance has become a global problem for clinicians in the treatment of invasive mycoses (fungal infections). In addition, toxicities and drug interactions may be limiting factors for long-term use or dose escalation of currently accessible antifungals [9, 10]. Amphotericin B is a complex with sodium cholate bile salts. Although fungal resistance to Amphotericin B is a rare phenomenon, it has been reported many times in recent years [9-11].

Therefore, antifungal treatment failure is a major clinical concern affecting patients with fungal diseases. The poor bioavailability and high toxicity of antifungals, the inability of topical agents to penetrate nonviable tissues, the emergence of antifungal-resistant isolates, and drug-interactions are all attributed to this failure. Therefore, it is important to find novel drugs or compounds with effective antifungal properties.

Nitroglycerin, also known as 1,2,3-trinitroxypropane, is a colorless, dense, oily liquid frequently used to treat or prevent chest pain, heart disease, high blood pressure, and anal fissures [6, 7]. Until now, the antifungal activity of nitroglycerin has been investigated in only one study [8]. Palmeira-de-Oliveira et al. (2012) found that nitroglycerin had a significant anti-*Candida* effect [8].

Objectives: Since there is limited information regarding the antifungal activity of nitroglycerin, the current investigation aimed to survey the inhibitory effect of nitroglycerin against clinical isolates of *Candida albicans*, *Trichophyton rubrum*, and *Aspergillus flavus* under *in-vitro* conditions to provide a new drug with fewer side effects.

Materials and Methods

Fungal isolates: In the current investigation, the antifungal activity of nitroglycerin was evaluated against clinical isolates of *C. albicans* (n=33), *A. flavus* (n=33), and *T. rubrum* (n=33). All the strains were previously gathered from clinical samples of patients with fungal infections, referred to the Medical Mycology Laboratory of Tehran University of Medical Sciences, Tehran, Iran.

Direct examination and culture: For direct microscopic examination, the specimens were mixed with 10% potassium hydroxide solution and observed under a microscope (Zeiss, Germany). Then all specimens were cultured on Sabouraud dextrose agar medium (SDA, Merck, Germany). Identification properties such as growth rate, colony morphology, and colony pigmentation were evaluated for any fungal growth observed. Microscopic examination of fungal reproductive structures and mycelia was performed as required. Based on the formation of

chlamydoconidia on cornmeal agar (Becton, France) and colony color on chromogenic CHROMagar *Candida* medium (CHROMagar, Paris, France), yeast isolates were identified.

PCR-sequencing: All the isolates were subjected to PCR and sequencing techniques in order to confirm the diagnosis. The High Pure PCR Template Preparation Kit (GeneAll Bldg, 303-7 105 Dongnam-ro, Songpa-gu, Seoul, South Korea) was used to isolate the genomic DNA of colonies grown on SDA according to the manufacturer's recommended guidelines. As previously mentioned, PCR amplification was performed on each isolate [12]. In order to discriminate *Aspergillus* isolates at the species level, the beta tubulin gene of *Aspergillus* species was amplified with specific forward (Bt2a:5 '-GGTAACCAAATCGGTGCTGCTTTC-3') and reverse (Bt2b:5'-ACCCTCAGTGTAGTGACCCTTGGC-3') primers. Besides, in order to identify other fungal isolates at the species level, universal primers for fungal amplification were used, including ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) that hybridizes at the end of 18S rDNA and ITS4 (5′TCC TCC GCT TAT TGA TAT GC 3) that hybridizes at the beginning of 28S rDNA (Life Technologies, Barcelona, Spain). At the Bioneers Advanced Nucleic Acid Core Facility, positive PCR products were sequenced. Then using the BLASTn algorithm on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi), sequences were used separately to perform individual searches for nucleotides. With this method, fungal identification was done with a maximum identity of 99% and query coverage of 98%.

Screening for antifungal activity: NITROJECT® 5 mg/mL (nitroglycerin for injection, USP) containing 50 mg of nitroglycerin was used to screen for its antifungal activity against clinical isolates of *A. flavus*, *C. albicans*, and *T. rubrum*. The disk diffusion method was done according to the international standard CLSI (Clinical and Laboratory standards institute) M44-S2 protocol [20]. In this method, fungal cells with a concentration of 0.5 x 10^8 CFU/mL (cells per milliliter) were prepared from clinical isolates in sterile distilled water and cultured on SDA plates with a sterile swab. After 15 min, a well was created in the center of each plate using a sterile tube. Then 200 µL of the prepared nitroglycerin solution was poured into each well. *C. albicans* isolates were incubated for 24 hours, *A. flavus* isolates were incubated for 48 hours, and *T. rubrum* isolates were incubated for 21 days at 35 °C. Finally, the growth inhibition zone diameter in the plates was measured and examined according to the CLSI standard protocol.

In vitro antifungal susceptibility testing of the isolated fungal strains was performed in accordance with the procedures set out in CLSI recommendations: document M38A2 for filamentous fungi [13] and document M27A3 for yeasts [14].

In short, 24-hr cultures of yeast isolates on SDA were prepared. Spectrophotometric measurements of homogenous yeast conidial solutions were done at 530 nm with a percent transmission between 75 and 77%. The final inoculum suspension was adjusted to 10^5 conidia/mL in RPMI 1640 medium (GIBCO, UK) buffered at pH 7.0. The microdilution plates were incubated at 35 °C for 24 hours after adding 100 µL of the inoculum suspension. The plates were visually read in accordance with the recommendations provided in CLSI M27A3 document. Furthermore, for filamentous fungi, sterile cotton swabs soaked in sterile saline solution with Tween 40 (0.05%) were used to gently scrape the surface of mature colonies on potato dextrose agar (Merck, Germany) to create inoculum suspensions. The inoculum suspension was adjusted to 0.5-2.5 × 10^3 conidia/mL in RPMI 1640 medium (GIBCO, UK) buffered at pH 7.0 with

0.165 M morpholino propanesulfonic acid (MOPS, Sigma-Aldrich, St. Louis, MO, USA). The microdilution plates were incubated at 35 °C for 48 hours following the addition of 100 µL of the inoculum suspension. Visual reading of the plates was performed according to CLSI M38A2 instructions. For quality control, standard strains of *C. parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) were employed. The lowest concentration of the drug, in which the fungi did not have visible growth, was considered as the minimum inhibitory concentration (MIC) and recorded. The results were read with the help of a mirror.

Statistical analysis: Independent t-test was carried out to analyze the statistical data. A *p*-value of less than 0.05 was considered as statistically significant. All variables were analyzed using SPSS 20 and Excel software.

Findings

In the disk diffusion method, all *C. albicans* (n= 33) and *A. flavus* (n= 33) isolates created an inhibition zone of more than 17 mm after 24 hours and 48 hours of incubation with nitroglycerin (0.5 mg/mL), respectively, which means that all the isolates were sensitive to nitroglycerin. Also, all 33 isolates of *T. rubrum* created a zone of less than 13 mm after 21 days of incubation with nitroglycerin (0.5 mg/mL), which means that all the isolates were resistant to nitroglycerin (Table 1).

Figures 1 and 2 compare the growth inhibition zone diameter of nitroglycerin against *C. albicans, A. flavus,* and *T. rubrum* in the disk diffusion method.

On the other hand, in the microdilution method, the growth of all isolates of *C. albicans* and *A. flavus* was stopped after 48 hours of incubation with nitroglycerin at a concentration of 0.5 mg/mL. In addition, the results showed that the growth of all isolates of *T. rubrum* was stopped after 21 days of incubation with nitroglycerin at a concentration of 0.12 mg/mL (Table 2).

Figure 3 compares the MICs of nitroglycerin against *C. albicans, A. flavus*, and *T. rubrum* (mg/mL) in the microdilution method.

According to the obtained results, the effective concentration of nitroglycerin against dermatophytes was about one-quarter of its effective concentrations against *C. albicans* and *A. flavus*, and this difference was statistically significant ($p< .05$).

Discussion

Despite the evidence regarding prevention, treatment, and therapeutic interventions, invasive fungal infections cause significant mortality in patients with immune system defects, and fungal drug resistance in these patients has become a major concern [15, 16]. Therefore, we need to find new drugs with more effects and fewer side effects. This experimental study aimed to investigate the inhibitory effects of nitroglycerin against clinical isolates of *C. albicans*, *T. rubrum*, and *A. flavus* in *in-vitro* conditions. In most studies, *C. albicans, A. flavus*, and *T. rubrum* have been determined as the most common

Table 1) The mean inhibition zone diameter of nitroglycerin against *C. albicans* after 24 hours, *A. flavus* after 48 hours, and *T. rubrum* after 21 days

Isolate	Mean Zone Diameter of Nitroglycerin	Sensitive (17 mm)	Intermediate (14-16 mm)	Resistant (13 mm)
Candida albicans isolates	20.27	Sensitive	-	-
Aspergillus flavus isolates	19.63	Sensitive	-	-
Trichophyton rubrum isolates	11.18	-	-	Resistant

Sensitivity and resistance to nitroglycerin based on inhibition zone diameters: ≥ 17 mm as susceptible, 14-16 mm as intermediate, and ≤ 13 mm as resistant

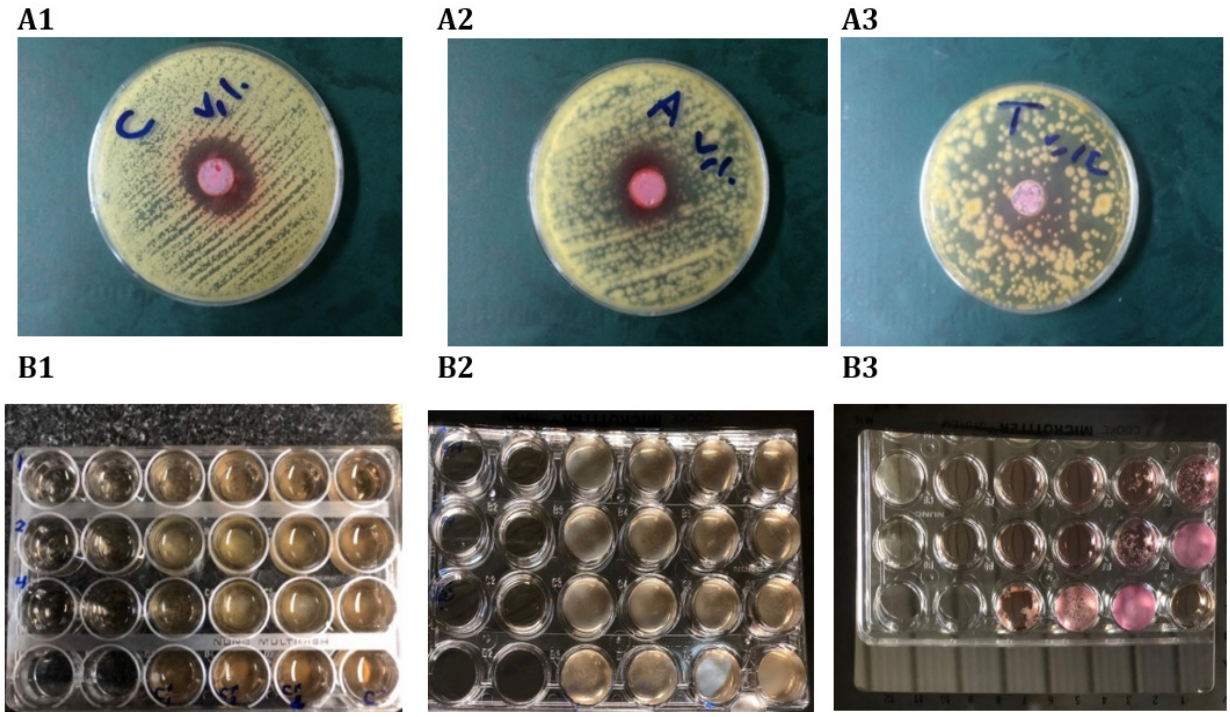

Figure 1) A1) Inhibition zone diameter of nitroglycerin against *C. albicans* after 24 hours; **A2)** zone diameter of nitroglycerin against *A. flavus* after 48 hours; **A3)** mean zone diameter of nitroglycerin against *T. rubrum* after 21 days; **B1)** minimum inhibitory concentration of nitroglycerin against *C. albicans* after 24 hours; **B2**) minimum inhibitory concentration of nitroglycerin against *A. flavus* after 48 hours; **B3)** minimum inhibitory concentration of nitroglycerin against *T. rubrum* after 21 days.

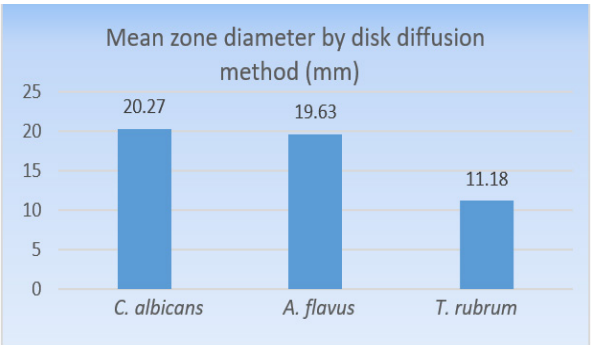

Figure 2) Comparison of the mean zone diameter of nitroglycerin against *C. albicans*, *A. flavus*, and *T. rubrum* (mm)in the disk diffusion method

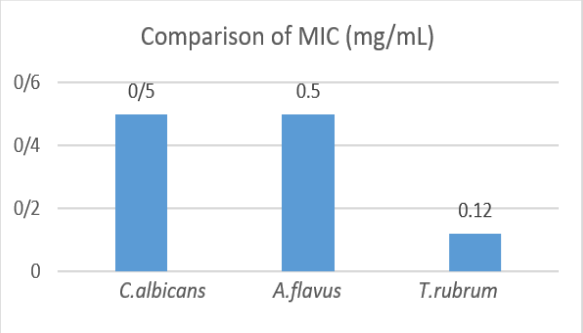

Figure 3) Comparison of the MICs of nitroglycerin against *C. albicans*, *A. flavus*, and *T. rubrum* (mg/mL) in microdilution method

Table 2) Minimum inhibitory concentrations (MICs) of nitroglycerin against *C. albicans* after 24 hours, *A. flavus* after 48 hours, and *T. rubrum* after 21 days

Isolate	Mean Nitroglycerin MIC (mg/mL)
Candida albicans isolates	0.5
Aspergillus flavus isolates	0.5
Trichophyton rubrum isolates	0.12

etiological agents of fungal infections caused by yeasts, saprophytes, and dermatophytes, respectively. Therefore, these species were selected for susceptibility testing in the present study [2, 17, 18].

Nitroglycerin is a vasodilator drug for the relief of an attack or anginal chest pain or acute prophylaxis of angina pectoris, which was approved by the FDA (Food and Drug Administration) in 2000 [6]. It was originally

synthesized in 1847 by Ascanio Sobrero in Turin, Italy [7].

The only study in this field was conducted by Palmeira-de-Oliveira et al. (2012) to investigate the anti-*Candida* effects of lidocaine and nitroglycerin alone and in combination with each other *in vitro* [8]. The finding indicated that both drugs had anti-*Candida* effects [8]. Their study was conducted only on *C. albicans* isolates, but the present study was conducted not only on *C. albicans* isolates but also on filamentous fungi, and the results showed that this drug affected filamentous fungi as well.

Novel nitroglycerin derivatives have also been synthesized, and their cytotoxic and antifungal effects against fluconazole-susceptible and resistant clinical isolates of *C. albicans* were evaluated in our previous study [19].

The findings indicated the potent antifungal activity of nitroglycerin derivatives against *C. albicans* isolates [19].

On the other hand, although *T. rubrum* isolates were resistant to nitroglycerin in the disc diffusion method, they were sensitive to this drug in the microdilution method. Thus, it seems that the disc diffusion method is not reliable for dermatophytes.

In the mentioned study, the MIC value obtained for *C. albicans* isolates was 0.15 mg/mL, which is different from the value obtained in the present study (0.5 mg/mL). The reason may be related to the differences in the native isolates of each country and the nitroglycerin drug used, or the fact that nitroglycerin loses its antifungal properties due to evaporation at the end of its effective half-life.

This study had some limitations as follows: 1) it was an *in-vitro* study, and studies on animal models and clinical trials are suggested, 2) drug susceptibility testing on other fungal species 3) and nitroglycerin effectiveness screening against drug-resistant yeasts, saprophytes, and dermatophytes were not performed in this study, which are suggested to be done in future studies.

Conclusion

Considering the emergence of drug resistance in fungal microorganisms and the increasing prevalence of patients with defects or weakness in the immune system, it seems necessary to find new antifungal drugs with fewer side effects. Considering the potential and efficacy of nitroglycerin against yeasts and filamentous fungi (saprophytes and dermatophytes), complementary *in vivo* and clinical studies should be done.

Acknowledgments

The authors would like to thank all the staff of the Medical Mycology Laboratory of Tehran University of Medical Sciences, Tehran, Iran.

Ethical permissions: Tehran University of Medical Sciences Research Ethics Committee (the number of ethics committee protocol: IR.TUMS.REC.1398.185) accepted the project.

Authors` contributions: Seyed Jamal Hashemi: conceptualization, methodology design, and project administration. Roshanak Daie-Ghazvini: conceptualization and methodology design. Zahra Rafat: writing the original draft, resources, visualization, data curation, writing, reviewing, and editing. Afsaneh Mohamadi: methodology design, investigation, and statistical analysis. Sassan Rezaie: methodology design and investigation. Pegah Ardi: investigation. All authors contributed to the article and approved the submitted version.

Conflicts of interest: The authors have no conflict of interest to declare.

Fundings: This study was part of an MSc thesis supported by Tehran University of Medical Sciences.

Consent to participate: None.

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