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Amoebicidal Effect of Fluconazole and Verapamil Together Against Trophozoites and Cysts of *Acanthamoeba castellanii*

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Acanthamoeba castellanii, Fluconazole, Verapamil, Combination, Amoebicidal effect, Growth inhibition

Abstract: *Acanthamoeba* species are important pathogens that causes *Acanthamoeba* keratitis, which causes a visual loss, as well as central nervous system infection and death by causing encephalitis. Due to the limited available options to successfully treat *Acanthamoeba* infections, new therapeutic approaches must be developed, and especially combination drug therapy may be a successful and effective strategy. The aim of this study was to assess the combination efficacy of verapamil and fluconazole against *Acanthamoeba* trophozoites and cysts. The effects of drugs on growth inhibition against *Acanthamoeba* were tested using amoebicidal assays. The viability of *Acanthamoeba* was assessed using Trypan blue and hemocytometer counts. The effect of three different concentrations of "fluconazole", "verapamil" and "fluconazole + verapamil" combination on growth inhibition against *Acanthamoeba* trophozoites and cysts was significant compared to the control ($p < 0.05$). While *Acanthamoeba* reduced the viability of 250 µg/ml fluconazole and verapamil on trophozoites to 2.3 x 10^4 and 3 x 10^4 numbers, fluconazole + verapamil combination showed 100% growth inhibition on trophozoites. Moreover, the combination of 250 µg/ml fluconazole + verapamil showed up to 90% growth inhibition on cysts. As a result, it was revealed that the combination of fluconazole and verapamil was more effective against the trophozoites and cysts of *Acanthamoeba*.

Acanthamoeba castellanii'nin Trofozoitleri ve Kistlerine Karşı Flukonazole ve Verapamil'in Birlikte Amip Öldürücü Etkisi

Anahtar Kelimeler
Acanthamoeba castellanii, Flukonazol, Verapamil, Kombinasyonu, Amip öldürücü etkisi, Büyüme inhibisyonu

Öz: *Acanthamoeba* türleri, görme kaybına neden olan *Acanthamoeba* keratiti ve ayrıca merkezi sinir sistemi enfeksiyonuna ve ensefalite neden olarak ölüme yol açan önemli bir patojendir. *Acanthamoeba* enfeksiyonlarını başarılı bir şekilde tedavi etmek için mevcut seçeneklerin başarısı sınırlı olmasından dolayı yeni terapötik yaklaşımlar geliştirilmelidir ve özellikle kombinasyon ilaç tedavisi başarılı ve etkili bir strateji olabilir. Bu çalışmanın amacı, *Acanthamoeba* trofozoitleri ve kistlerine karşı verapamil ve flukonazol kombinasyonunun etkinliğini değerlendirmektir. İlaçların *Acanthamoeba'ya* karşı büyüme inhibisyonu üzerine etkileri amoebisidal yöntemler kullanılarak test edildi. *Acanthamoeba'nın* canlılığı, tripan mavisi ve hemasitometre sayımları kullanılarak değerlendirildi. Flukonazol, verapamil ve flukonazol + verapamil kombinasyonunun üç farklı konsantrasyonunun *Acanthamoeba* trofozoitleri ve kistlerine karşı büyüme inhibisyonu üzerindeki etkisi, kontrol ile karşılaştırıldığında anlamlı bulundu ($p < 0.05$). *Acanthamoeba*, 250 µg/ml flukonazol ve verapamilin trofozoitler üzerindeki canlılığını 2,3 x 10^4 ve 3 x 10^4'e düşürürken, flukonazol + verapamil kombinasyonu trofozoitler üzerinde % 100 büyüme inhibisyonu göstermiştir. Ayrıca, 250 µg/ml flukonazol + verapamil kombinasyonu, kistlerde % 90'a varan büyüme inhibisyonu göstermiştir. Sonuç olarak, *Acanthamoeba'nın* trofozoitlerine ve kistlerine karşı flukonazol ile verapamil'in kombinasyonunun daha etkili olduğu ortaya konuldu.

1. INTRODUCTION

The genus *Acanthamoeba* is widely found in the natural environment [1]. *Acanthamoeba* can also be found in many environmental specimens such as seawater, river water, lakes, swimming pools, soil, and contact lens and lens solutions [2]. Throughout its life cycle, *Acanthamoeba* has two forms: an active trophozoite form that may reproduce by binary fission and a latent cyst form under unfavorable environmental circumstances. The cyst stage is a defense mechanism for survival in adverse environmental conditions [1]. *Acanthamoeba* species are resistant to various antibiotics, chlorine and low temperatures in the cyst stage [3]. According to the sequence of study of the 18S rRNA gene, the molecular classification of *Acanthamoeba* species has been established, and 23 genotypes have been reported so far. [4, 5]. The *Acanthamoeba* genus can cause fatal brain infections known as granulomatous amebic encephalitis (GAE), as well as vision-threatening corneal ulcers called *Acanthamoeba* Keratitis (AK) [6].

The use of contact lenses is the biggest risk factor for AK in industrialized nations, whereas trauma (including contact with plant material) and corneal exposure to polluted soil and water are bigger risk factors in underdeveloped nations [7]. In addition to increasing the danger of corneal *Acanthamoeba* exposure, contact lens solutions also raise the possibility of being linked to ongoing AK outbreaks [8]. Even though there are an estimated 125 million contact lens wearers worldwide, that number is rising each year [9].

AK is not considered in the differential diagnosis since it is uncommon in comparison to other infections that cause keratitis [10]. For this reason, because the diagnosis cannot be made on time, empirical treatment with various drugs is started and long-term use is ensured [11]. Although there is no definitive treatment for the treatment of *Acanthamoeba* infection, long-term anti-amoebic treatment is required to entirely remove both the forms of the parasite. No selective drug has been developed for *Acanthamoeba* infections [4, 12, 13]. Polyhexamethylene biguanide (PHMB) and chlorhexidine digluconate are currently the most commonly used eye drops in the treatment of AK, and it is recommended to be used every 1-2 hours and then tapering off over time to achieve full recovery [14]. In addition, amphotericin B, voriconazole, itraconazole, azithromycin, rifampin, and miltefosine was used systemically to treat *Acanthamoeba* infections [15, 16]. Although some antifungals and antibiotics have been used in *Acanthamoeba* infections, the available anti-amoebic agents that will fully affect AK infection are still limited [10]. Hence, alternative strategies to the development of new anti-amebic drugs are urgently needed.

Fluconazole is important in preventing fungal keratitis infections in the azole group, with its low toxicity and cost even when used at high doses. However, new drugs available are limited due to increasing drug resistance [17]. The mechanism of action of fluconazole interacts with the cytochrome P-450 enzyme 14-demethylase, fluconazole, which catalyzes the conversion of lanosterol to ergosterol. Ergosterol is a crucial component of the cell membranes of fungi and *Acanthamoeba*, and fluconazole prevents its formation from making cells more permeable [18, 19]. Various calcium channel-blocking agents, such as amlodipine and loperamide have been reported to have anti-amoebic effects against *Acanthamoeba* and FLA [20]. Therefore, calcium channel blockers suggest that they are effective on FLA.

It has been demonstrated that calcium channel blockers, which are medications frequently used to treat cardiovascular diseases, have a variety of anti-fungal, antibiotic, and antiparasitic properties [21, 22]. It is known that calcium channels are involved in crucial cellular processes including the development of parasitic protozoan diseases [20]. Recent studies have provided evidence for the presence of voltage-sensitive calcium channels in *Acanthamoeba* [20, 23]. Various calcium channel blockers have been shown to have an anti-amoebic effect on *Acanthamoeba* [24]. It has been reported that some known antifungal agents are more effective with combinations of calcium channel blockers [25, 26]. In this study, we aimed to test the synergistic effects of fluconazole and verapamil together for the treatment of *Acanthamoeba* keratitis on the clinical strain *Acanthamoeba castellanii* using growth inhibition and viability assays.

2. MATERIAL AND METHOD

2.1. Chemicals

The chemicals including Trypan blue (Gibco), Page's saline solution, proteose peptone (Sigma Aldrich), gentamicin, yeast extract (Merck, Germany), glucose (Sigma Aldrich), agar (Oxoid no.1), phosphate-buffered saline (PBS) (Invitrogen Corp., Carlsbad, Calif.) was used in the present study. Fluconazole topical solution (Fluzamed, 0,3%, 3 mg/ml, World Medicine Pharmaceuticals Industry), verapamil hydrochloride (Isoptin, 80 mg, tablet, Abbott Laboratories) was purchased commercially. To prepare the verapamil hydrochloride, 80 mg was added into sterile 10 ml of distilled water to obtain a concentration of 8 mg/ml. Verapamil hydrochloride was prepared separately at three different concentrations (50 µg/ml, 100 µg/ml, and 250 µg/ml), and stored at +4°C until use.

2.2. *Acanthamoeba* Isolate and Culture

The clinical isolate of *Acanthamoeba castellanii* (GenBank No: ON600792.1) identified as genotype T4 used in this study was isolated from a patient with keratitis in previously study [27]. The clinical isolate was cultured axenically in PYG medium in 25 cm^2 culture flasks at 37 °C. Briefly, PYG medium was included proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), glucose 1.5% (w/v), Page's saline solution (PAS; 0.12 g NaCl, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4, 0.136 g KH_2PO_4 per liter of distilled water) one liter distilled water containing 40 µg/mL gentamicin. The *Acanthamoeba* culture flask was incubated at 30 °C, and

the media in flask was refreshed after every 24 h of incubation. *Acanthamoeba* trophozoite forms that adhered on the bottom of the flasks were collected by incubating on ice for 20 minutes and gently shaking.

2.3. Amoebicidal Activity Assay

To determine the anti-*acanthamoeba* activities of each drug, *Acanthamoeba* trophozoites (2 x 10^5 amoeba/ml/tube) were incubated in PBS with different concentrations of fluconazole (50 µg/ml to 250 µg/ml) and verapamil hydrochloride (50 µg/ml to 250 µg/ml) in capped microcentrifuge tubes (1.5 ml) at 37 °C for 24 hours. Following this incubation, the number of *Acanthamoeba* trophozoites was determined by hemocytometer counting. The number of *Acanthamoeba* incubated with PBS alone was used as control 100%. The viability of *Acanthamoeba* was assessed by adding 0.1% Trypan blue to live (unstained) and dark blue for dead amoebae using a hemocytometer under a light microscope. The results are representative as the mean ± standard error of three independent experiments performed in duplicate. The growth inhibition percentage was calculated according to the equation (A – B/A) x 100, where A = the mean number of viable *Acanthamoeba* in the control group and B = the mean number of viable *Acanthamoeba* in the treatment groups [24].

2.4. Cysticidal Activity Assays

For cysticidal assay, *Acanthamoeba* cyst form was prepared using 1.5 % non-nutrient agar plates as descript previously [24, 28]. Briefly, *Acanthamoeba* trophozoites (2 x 10^5 amoeba) were transferred from PYG medium to NNA plate and incubated at 37 °C for at least two weeks. Then, whether the trophozoites transformed into cysts was checked under an inverted microscope. After incubation, the cysts were removed from the NNA plates by adding PBS and using a cell scraper and transferred to 15 ml tubes. Tubes were centrifuged at 2000 x g for 10 minutes and cysts were counted using a hemocytometer. The *Acanthamoeba* cysts were transferred to microcentrifuge tubes as 2 x 10^5 cysts/ml and incubated in PBS with different drug concentrations for 24 hours at 37 °C. After incubation, centrifugation was done at 2000 x g for 10 minutes to remove the effects of the drugs and the supernatant was discarded. This process was performed with three-times washes with PBS to remove the effect of the drug. After incubation, whether the cysts transformed into trophozoite form was counted under the light microscope using a hemocytometer. Each drug was evaluated in triplicate, and each experiment was performed in duplicate.

2.5. Statistical Analysis

The data are presented as mean values with standard error and calculated the average of the groups on Microsoft Excel sheet. Two sample *t*-test; two-tailed distribution were used for analysis of the data. The statistical analysis was carried out using Graph Pad Prism v.8.0 software. The significant value $p < 0.05$ is used for all evaluations.

3. RESULTS AND DISCUSSION

3.1. Amoebicidal Effects Against *Acanthamoeba castellanii* Trophozoites

The anti-acanthamoebic effects of fluconazole and verapamil on *Acanthamoeba* trophozoites at various concentrations were evaluated. At 50 µg/ml fluconazole and verapamil caused a reduction in the number of *Acanthamoeba* trophozoites from 9 x 10^4 and 1 x 10^5 (from 2 x 10^5), respectively, resulting in 50 % inhibition on growth of *Acanthamoeba* ($p < 0.005$) (Figure 1). However, the combined effect of 50 µg/ml fluconazole + verapamil was significant compared to the control, in which it showed effects more than the effect of a single drug and showed 67 % growth inhibition on trophozoites of *Acanthamoeba castellanii* ($p < 0.005$) (Figure 2). The effects of 100 µg/ml fluconazole and verapamil on *Acanthamoeba* trophozoites were decreased 5 x 10^4 and 6.6 x 10^4 numbers, respectively (Figure 1). Although fluconazole and verapamil (100 µg/ml) showed growth inhibition of 70 % on the viability of *Acanthamoeba* trophozoites, the combination of these two drugs (fluconazole + verapamil) had a growth inhibition effect of 87% (Figure 2). At 250 µg/ml concentration of fluconazole and verapamil exhibited significant effects against *Acanthamoeba castellanii* trophozoites ($p < 0.001$) (Figure 1). At 250 µg/ml fluconazole + verapamil significantly reduced the number of viable *Acanthamoeba* trophozoites to 6.6×10^3 as compared to the control (2×10^5) ($p < 0.001$) (Figure 1). In particular, fluconazole + verapamil (250 µg/ml) showed approximately 100 % growth inhibition effects on trophozoites (Figure 2). The results showed that although the drugs had a dose-dependent lethal effect against the *Acanthamoeba* trophozoites, the combined effect of drug concentrations was much stronger on the trophozoites. *Acanthamoeba castellanii* trophozoites were found to be statistically significant with all drug concentrations as compared to the control ($p < 0.001$, $p < 0.005$, $p < 0.05$) (Figure 1). The previous two studies, the minimum amoebicidal concentration on trophozoites of *Acanthamoeba* of fluconazole was reported to be >320 µg/ml and > 1024 µg/ml [29, 30]. In another study was observed that the effects of fluconazole on clinical isolates of *Acanthamoeba castellanii* were more susceptible to environmental isolates such as *Acanthamoeba lenticulate* and *Acanthamoeba hatchetti* and also, the minimal inhibition concentration ranged from 64 to 256 µg/ml [31]. Baig et al. reported the presence of calcium channels in *Acanthamoeba castellanii*. It also revealed the effect of various calcium channel blockers on *Acanthamoeba* [20]. In addition, Baig et al. showed that the effects of voltage-sensitive calcium channel blockers such as verapamil and nifedipine on *Acanthamoeba castellanii* trophozoites range from 50 to 100 µg/ml [32]. In another study, the effect of fluconazole alone was more than two-fold as effective against *Candida albicans* when combined with calcium channel blockers such as amlodipine, nifedipine, benidipine, and flunarizine [17].

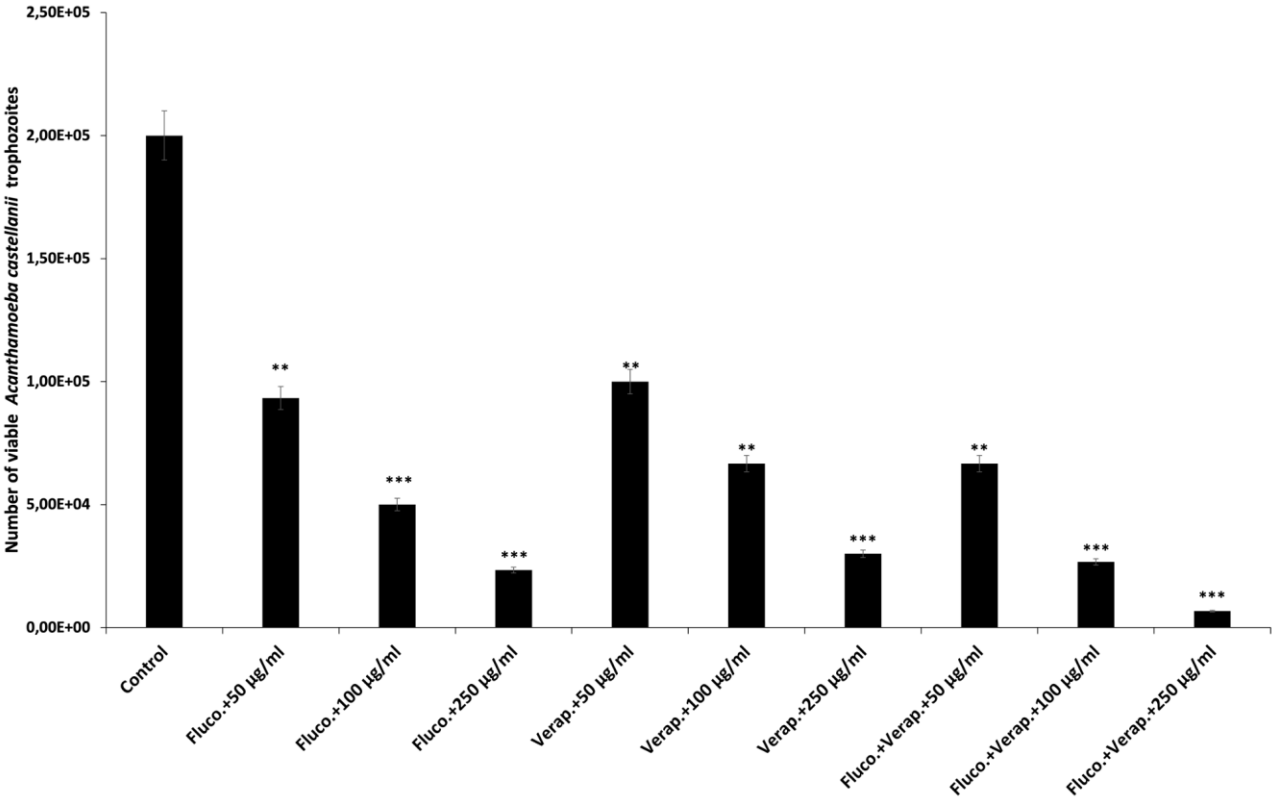

Figure 1. *Acanthamoeba castellanii* viability effects of various drugs were determined using Trypan blue staining. *Acanthamoeba castellanii* (2 x 10^5 trophozoites) was incubated with (50 µg/ml, 100 µg/ml and 250 µg/ml) fluconazole, verapamil and fluconazole + verapamil drugs at 37 °C for 24 h. The results show significant anti-*acanthamoebic* activity when compared to the control (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$)

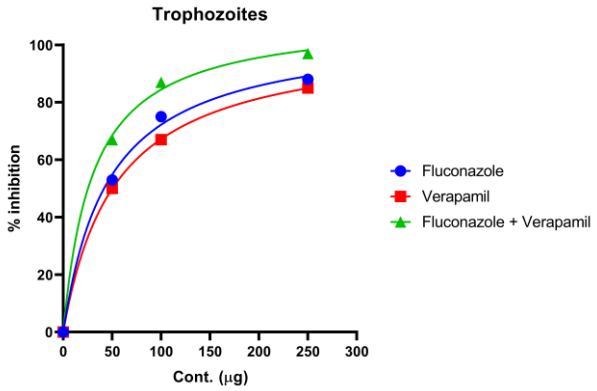

Figure 2. Growth inhibition curve of *Acanthamoeba castellanii* trophozoite at different concentrations. Growth inhibition curves of trophozoites treated with fluconazole, verapamil, and fluconazole + verapamil at 24 h.

3.2. Cysticidal Effects Against *Acanthamoeba castellanii* Cysts

Three different drug concentrations were evaluated by scraping *Acanthamoeba castellanii* cysts on non-nutrient agar plates after at least two weeks at 37 °C. It was observed that the drugs at 50, 100 and 250 µg/ml concentrations of fluconazole reduced the number of viable *Acanthamoeba castellanii* cysts by 1.48 x 10^5, 1.03 x 10^5, and 8 x 10^4 as compared to the control (2 x 10^5 cysts) (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) (Figure 3). At 50 µg/ml fluconazole showed 27 % growth inhibition on the viability of the cysts, while 250 µg/ml fluconazole had a growth inhibition of more than 50 % (Figure 4). Similar to the effect of fluconazole, 50, 100, and 250 µg/ml verapamil had a reduction effect of 1.53 x 10^5, 1.2 x 10^5, and 7 x 10^4 in the number of viable *Acanthamoeba castellanii* cysts (*$p < 0.05$, **$p < 0.005$, ***$p < 0.001$) (Figure 3). While the 250 µg/ml concentration of fluconazole and verapamil showed growth inhibition of 61 % and 66 %, respectively, on the viability of *Acanthamoeba* castellanii cysts, 100 µg/ml Fluconazole + verapamil showed 60 % growth inhibition (Figure 4). At 250 µg per ml of fluconazole + verapamil showed up to 90 % growth inhibition (Figure 4).

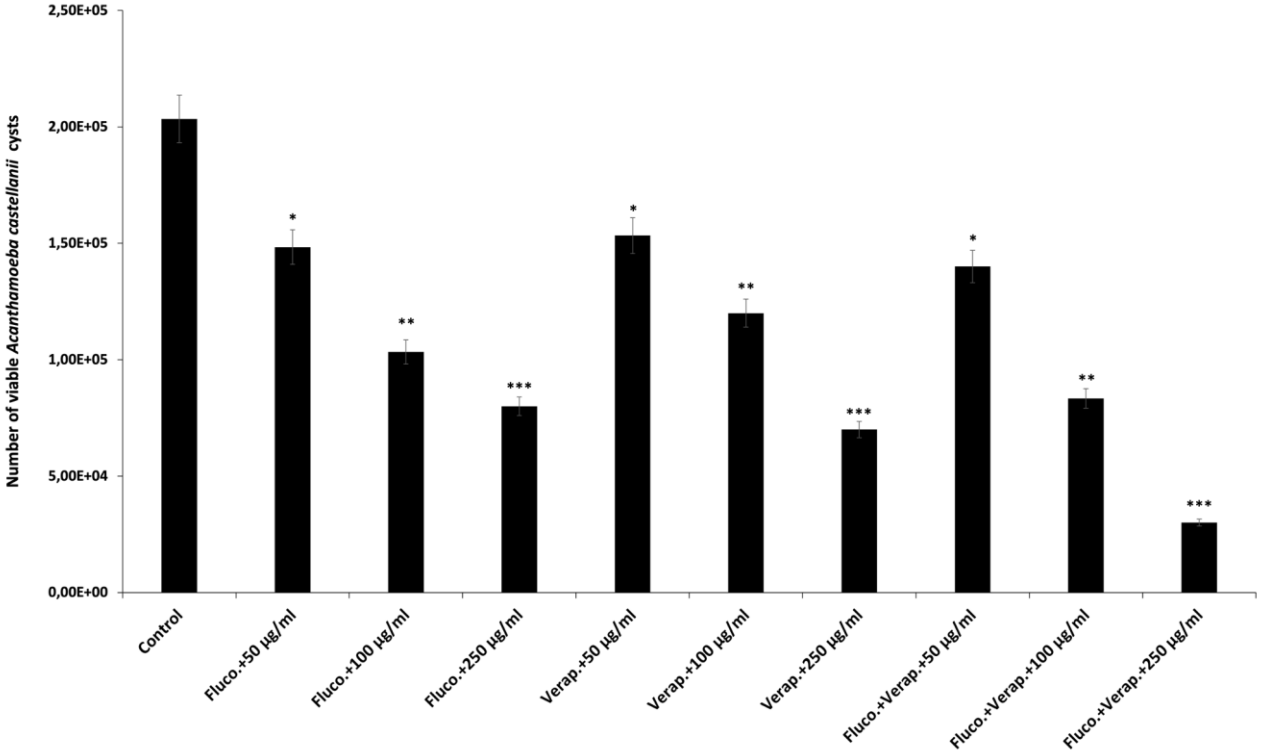

Figure 3. *Acanthamoeba castellanii* viability effects of various drugs were determined using Trypan blue staining. *Acanthamoeba castellanii* (2 x 105 cysts) was incubated with (50 µg/ml, 100 µg/ml and 250 µg/ml) fluconazole, verapamil and fluconazole +verapamil drugs at 37 °C for 24 h. The results show significant anti-*acanthamoebic* activity when compared to the control (***$p < 0.001$, **$p < 0.005$, *$p < 0.05$)

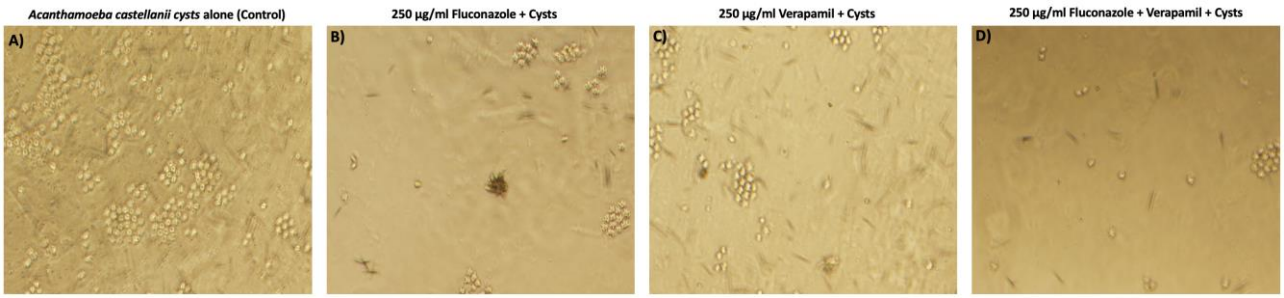

Figure 5. Representative effects of fluconazole, verapamil, and combination fluconazole + verapamil on inhibiting the viability of *Acanthamoeba castellanii* cysts. **A)** *Acanthamoeba castellanii* alone (control); **B)** Fluconazole +250 µg/ml; **C)** Verapamil +250 µg/ml; **D)** Fluconazole + Verapamil+250 µg/ml

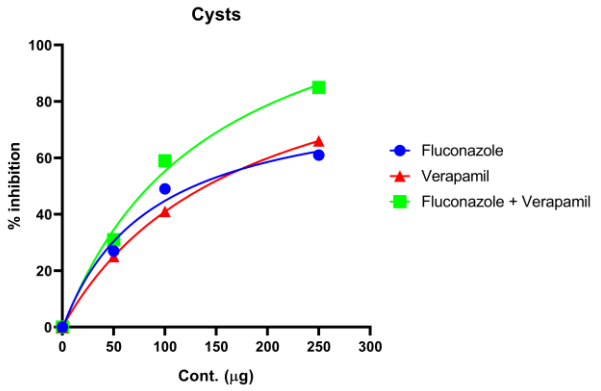

Figure 4. Growth inhibition curve of *Acanthamoeba castellanii* cysts at different concentrations. Growth inhibition curves of trophozoites treated with fluconazole, verapamil, and fluconazole + verapamil at 24 h.

The growth inhibition assay revealed that both fluconazole and verapamil alone and the combination of fluconazole + verapamil exhibited cysticidal effects on the growth of *Acanthamoeba castellanii*. The cysticidal activity analysis results are supported by representative images recorded at x100 magnification (Figure 5). Therefore, the combination effect of fluconazole and verapamil was observed to increase growth inhibition with increasing dose. In the previous studies, cysticidal activity of fluconazole on *Acanthamoeba* was reported as >500 µg/ml and >128 µg/ml [30, 33]. Nakaminami et al. reported that the effect of fluconazole on *Acanthamoeba* cysts showed the minimum cystic concentration range from 256 to 512 µg/ml [9]. Anti-infective effects of calcium channel blocking agents such as amlodipine have been reported against *Candida* spp. and *Acinetobacter baumannii* [34, 35]. The effects of amlodipine on various *Candida* spp. at doses of 256 µg/mL and on *Acinetobacter baumannii* at dose ranges of 40 to 320 µg/ml demonstrated the antibiotic potential of these calcium channel blockers [34, 35].

4. CONCLUSION

Recently, it was reported that the presence of calcium channels that are comparable to human calcium channels and the impact of calcium ions on *Acanthamoeba* viability and growth. Furthermore, calcium channel blockers (amlodipine, loperamide, nifedipine and verapamil) have been found to have amoebicidal effects on *Acanthamoeba*. In our study, although fluconazole and verapamil have influenced *Acanthamoeba* trophozoites and cysts, it was observed that the effect increased, especially with the increase in the dose of the drugs. Fluconazole alone had a minimal impact on the survivability of *Acanthamoeba* trophozoites, but when combined with verapamil, it completely inhibited proliferation. This study revealed that the combined effects of fluconazole and verapamil were very effective against *Acanthamoeba* trophozoites and cystic.

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