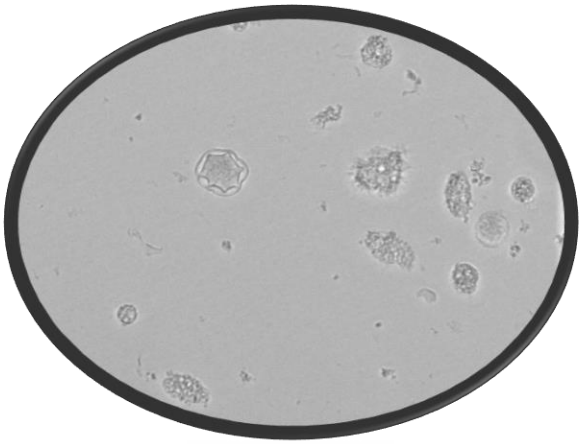
EVALUATION OF THE ACTIVITY OF DIFFERENT COMMERCIAL EYE DROPS AGAINST *Acanthamoeba*

Trabajo de fin de grado



18 DE ABRIL DE 2018

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1. Abstract

Free Living Amoebae belonging to *Acanthamoeba* genus has become increasingly important worldwide in the last decades as an emerging pathogen. This protozoan presents two stages: an active trophozoite phase and a dormant and highly resistant cyst one. These amoebae are the causative agents of a Granulomatous Amoebic Encephalitis (GAE) and *Acanthamoeba* keratitis (AK). Regarding therapy against AK, there are not current available treatments which are 100% effective and therefore, the need to find novel anti-amoebic agents. One of the main problems that oppose treatment is the parasite encystment process, which occurs in the event of harsh environmental conditions (pH, temperature, osmotic pressure) including treatment with currently used drugs. Lately, studies have been focused not only on the search of novel therapeutic options in order to treat AK but also to prevent infections. The evaluation of commercialised products seems to be an option for this case since no clinical assays would be required. Therefore, in this study the anti-*Acanthamoeba* activity of different commercialised eye drops in Spain was evaluated using the AlamarBlue™ method.

2. Introduction

2.1. Free-living amoeba

In the last two decades, Free-living Amoebae (FLA) have been associated with an increasing number of infections not only in developing countries but also in developed countries (Martinez and Visvesvara 1997). These ubiquitous and opportunistic protozoa are widely distributed in nature and can be found all over the world including soil, water and air samples. These amoebae have been also isolated from a wide variety of different sources including dust, seawater, drinking water, swimming pools, sewage, eyewash solutions, contact lens, dialysis units and dental treatment units (Schuster and Visvesvara 2004; Trabelsi et al. 2012). Moreover and due to their amphizoic ability, they are capable to live free in nature but also to live as parasites if they invade an animal host tissue (Visvesvara, Moura, and Schuster 2007). *Acanthamoeba spp., Naegleria fowleri, Balamuthia mandrillaris* and *Sappinia* spp. are the only four genera known to cause opportunistic and non-opportunistic infections (Trabelsi et al. 2012; Qvarnstrom et al. 2009; Visvesvara, Moura, and Schuster 2007). Whereas *N. fowleri* and *Sappinia* are only encephalitis causing agents, *Acanthamoeba* spp. and *B. mandrillaris* also cause epithelial disorders (Lorenzo-Morales et al. 2013).

2.2. *Acanthamoeba* spp.

Acanthamoeba spp. is the aetiological agent of Granulomatous Amoebic Encephalitis (GAE), a serious infection of the brain and spinal cord that typically occurs in persons with a compromised immune system, cutaneous acanthamoebiasis, *Acanthamoeba* pneumonitis and *Acanthamoeba* keratitis (AK), a painful eye infection that can lead to blindness (Marciano-Cabral and Cabral 2003; Juárez et al. 2017; Martinez and Visvesvara 1997; Chan et al. 2011; Nuprasert et al. 2010). The morphological identification of *Acanthamoeba* from other genera is relatively easy due to the presence of acanthopodia, spiny thorn-like surface projections, that allow parasite adhesion to surfaces, cellular

movement and also feeding (Marciano-Cabral and Cabral 2003; Trabelsi et al. 2012; Lorenzo-Morales et al. 2013). In 1977, Pussard and Pons tried to establish an *Acanthamoeba* classification based on their morphological features, dividing them into 3 different groups regarding only their cysts stage (Adamska 2016). However, as culture conditions can change the morphology of *Acanthamoeba*, identifications began to be based on the DF3 fragment from the 18s rRNA gene sequences. Then, 22 genotypes (T1-T22) have been discovered in both environmental and clinical samples (Corsaro et al. 2015; Nuprasert et al. 2010; Fuerst, Booton, and Crary 2015; Corsaro et al. 2017; Fuerst 2014; Tice et al. 2016; Di Cave et al. 2014). This identification has become highly relevant in the field not only for taxonomic and epidemiological studies but also related to virulence factors, pathogenic capacity, drug susceptibility and relationship between the genotype and disease phenotypes (Haniloo et al. 2017; Zhang et al. 2004; Ledee et al. 2009; Hajialilo et al. 2016).

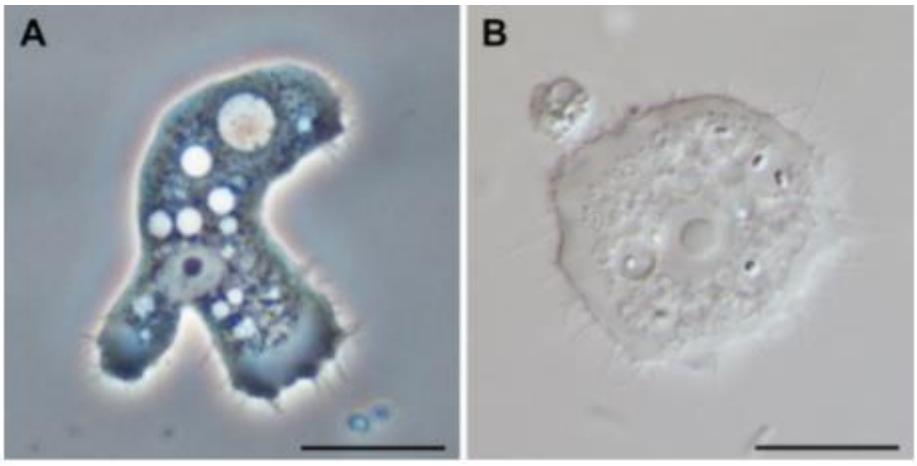


Figure 1 *Acanthamoeba* trophozoites with the characteristic acanthopodia. Scale bar: 10 µm ((Lorenzo-Morales, Khan, and Walochnik 2015)

2.3. Life cycle and morphology

Acanthamoeba can be found in two different stages: a motile and actively dividing trophozoite stage (20-40µm) responsible for feeding and a dormant resistant cyst stage (5-20µm) that appears upon severe environmental conditions for amoebic survival (Lorenzo-Morales et al. 2013). *Acanthamoeba* trophozoite feeds on bacteria, algae, yeasts or other small organic particles but can also grow axenically on nutrients present in liquid suspension taken up through pinocytosis (Marciano-Cabral and Cabral 2003; Lorenzo-Morales et al. 2013). On the other hand, when there is a lack of food or extreme conditions (pH, Temperature or hyper- or hypo-osmolarity) trophozoites differentiate into a highly resistant double-walled cyst with the ability of surviving for more than 20 years (Martín-Navarro et al. 2017; Lorenzo-Morales et al. 2013; Siddiqui, Dudley, and Khan 2012; Sriram et al. 2008). Concerning the composition of the cyst, the outer wall or exocyst is fibrous and mostly composed by proteins while the inner wall or endocyst is composed at least by 30% of cellulose (Martín-Navarro et al. 2017; Lorenzo-Morales et al. 2008; Dudley, Alsam, and Khan 2007).

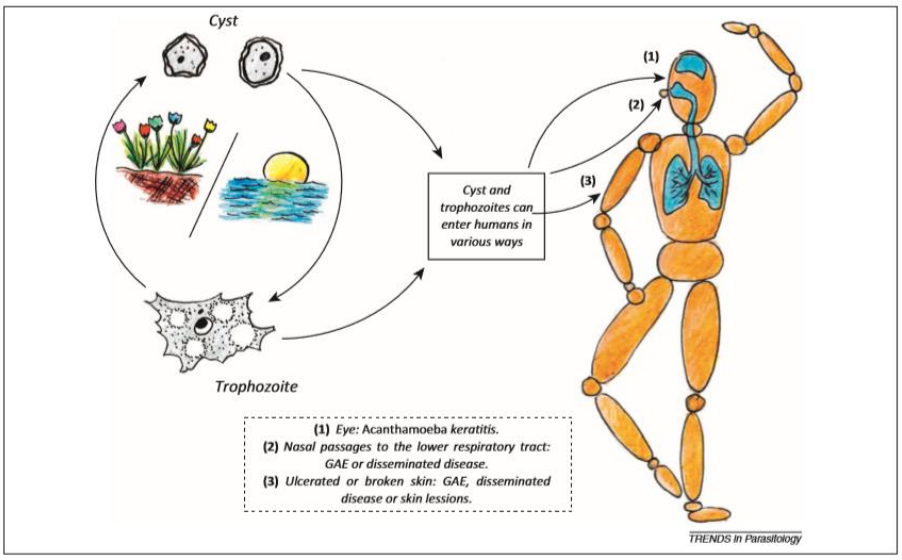


Figure 2 *Acanthamoeba* life cycle and infections. (Lorenzo-Morales *et al.*, 2013)

2.4.*Acanthamoeba* Keratitis (AK)

Acanthamoeba Keratitis is an infiltrative corneal infection caused by pathogenic *Acanthamoeba* strains which mostly affects immunocompetent individuals. It is known to be difficult to diagnose and treat, and, often, is misdiagnosed as herpetic and fungal infections. Risk factors include wearing contact lenses (CLs) for a long time, poor hygiene when handling and storage of CLs, corneal trauma and swimming while wearing CLs (Sifaoui et al. 2017; Juárez et al. 2017). CLs wearers usually ask for late medical help due to being used to small eye irritations (Lorenzo-Morales, Khan, and Walochnik 2015). Infection rate has been reported to be 1.2 per million adults and 0.2-1 per 10,000 CLs wearers per year. Recently however, rates increased to more than seven-fold in CLs wearers (Sifaoui et al. 2017; Pacella et al. 2013). Usually, only one of the eyes is involved and *Acanthamoeba* must be in the trophozoite stage to adhere to human corneal epithelium. The infection also depends on the parasite virulence and on the integrity of the cornea and host immune response (Trabelsi et al. 2012). Adhesins, particularly mannose-binding protein (MBP), expressed on the surface of *Acanthamoeba* play one of the most important roles in *Acanthamoeba* adherence to corneal epithelium cells (Garate et al. 2004). When adhered to corneal cells, *Acanthamoeba* parasites produce a diversity of proteases, facilitating corneal invasion and causing not only cytolysis of the cornea but also infiltration of inflammatory cells and, in final stages, can lead to formation of descemetocoele and perforation. Limbitis and Scleritis can also appear as a secondary immunological reaction (Illingworth et al. 1995; Khan and Tareen 2003). The first signs and symptoms of AK are redness, lacrimation, epiphora, conjunctival hyperhemia, pain, photophobia and foreign body sensation. However, the clinical signs, that mostly raise suspicion on this type of infection, are the presence of punctate epithelial erosions, pseudodendrites, epithelial opacities, and/or appearance of focal or diffuse subepithelial and perineural opacities especially when the infection becomes chronic. Sometimes, the epithelium can be entirely undamaged and corneal sensitivity may be reduced (Trabelsi et al. 2012; Lorenzo-Morales et al. 2013). In a more advanced stage of the disease, a ring-like abscess is formed due to the oedema caused by macrophages activation that reach the

corneal stroma being pathognomonic for AK. Loss of visual acuity and blindness can occur in the most serious cases (Patel and McGhee 2009; Trabelsi et al. 2012; Lorenzo-Morales et al. 2013).

2.4.1. AK Diagnosis

The most important step in the diagnosis of AK, since it is a less common disease, is to consider it, especially in CLs wearers and individuals presenting corneal and contact with potentially contaminated soil or water sources (Lorenzo-Morales, Khan, and Walochnik 2015). The most accepted method in the diagnosis of AK, excluding biopsies, is confocal microscopy because it is non-invasive and has a high sensitivity in cases of severe infection (Siddiqui, Dudley, and Khan 2012; da Rocha-Azevedo, Tanowitz, and Marciano-Cabral 2009; Vaddavalli et al. 2011). However, other laboratory tests should always be performed to increase the chances of making a correct diagnosis, since patients are often treated for viral, fungal or bacterial infections before the correct diagnosis of AK (Dart, Saw, and Kilvington 2009; Juárez et al. 2017). Among these tests, isolations from CLs, CLs cases and the cornea scraping should be performed, thus allowing the identification of *Acanthamoeba*. A definitive and more accurate diagnosis should be made by Polymerase Chain Reaction (PCR), allowing to know which genotype is causing the infection (Lorenzo-Morales, Khan, and Walochnik 2015; Lorenzo-Morales et al. 2013; Itahashi, Higaki, and Fukuda 2011).

2.4.2. AK Treatment

To date, there are no fully effective therapeutic agents against AK (Lorenzo-Morales, Khan, and Walochnik 2015; Lorenzo-Morales et al. 2013). Despite this, many drugs and active compounds have been and continue to be tested in both, *in vitro* and *in vivo* assays as an attempt to find a solution to this problem (Omaña-Molina et al. 2017). The current treatment of AK is based on the application of topical antimicrobials (eye drops) in order to obtain high concentrations at the site of infection. However, due to the existence of the cyst stage which gives more

resistance to chemotherapy, combinations of drugs are usually administered (Lorenzo-Morales, Khan, and Walochnik 2015). The most commonly used therapeutic regimens are biguanides, namely biguanide polyhexamethylene (PHMB) in concentrations of 0.02% and chlorhexidine, which has less adverse effects, also at 0.02%. In the case of chlorhexidine, it is common to combine its use with diamines or neomycin, having been demonstrated its efficacy when applied early in the progression of the disease (Lorenzo-Morales, Khan, and Walochnik 2015; Lorenzo-Morales et al. 2013; Roberts and Henriquez 2010).

3. Aims and Objectives

As it was discussed in the introduction section, there is a general lack of effective preventive and therapeutic options against *Acanthamoeba* keratitis. Moreover, an ideal option is to check if already commercialised products present this potential as antiamoebic agents.

Therefore, the objectives of this study were:

- To evaluate the activity against *Acanthamoeba* of seven commercialised eye drops.
- To calculate the Inhibitory Concentrations (IC_{50}) of the active eye drops.
- To establish whether the tested products could be further exploited against AK.

4. Materials and Methods

This study was performed at the laboratory of antiprotozoal chemotherapy of the University Institute of Tropical Diseases and Public Health of the Canary Islands (IUETSPC) in Universidad de La Laguna.

4.1. Materials

4.1.1. Parasites:

- *Acanthamoeba castellani* Neff strain ATCC (30010)

4.1.2. Culture medium:

For the maintenance and *in vitro* cultures of *Acanthamoeba* trophozoites:

- PYG medium (0.75% (w/v) proteose peptone, 0.75 (w/v) yeast extract and 1.5% (w/v) glucose) containing 20 µg gentamicin ml^{-1} (Biochrom AG, Cultek, Granollers, Barcelona, Spain).

4.1.3. Reagents:

4.1.3.1. Assay Reagent:

- AlamarBlue Assay Reagent® (Invitrogen, ThermoFisher Scientific)

4.1.3.2. Evaluated eye drops:

- Combigan® (Allergan S.A.): Brimonidine (2 mg/ml) + Timolol (5 mg/ml)
- Timolol Sandoz® (Sandoz, S.A.): Timolol (5 mg/ml)
- TobraDex® (Novartis, S.A.): Dexamethasone (1 mg/ml) + Tobramycin (3 mg/ml)
- Colircusi Antiedema® (Novartis, S.A.): Sodium Chloride (50 mg/ml)
- Voltaren® (Thea Laboratories, S.A.): Diclofenac Sodium (1 mg/ml)
- Duokopt® (Thea Laboratories, S.A.): Dorzolamide (20 mg/ml) + Timolol (5 mg/ml)
- Cusimolol® (Alcon Cusi, S.A.): Timolol (5 mg/ml)

4.1.4. Equipment:

- Multichannel automatic micropipette (Eppendorf)
- Incubator (Heraeus)
- EnSpire plate reader (PerkinElmer, Massachusetts, USA)
- Leica DMIL inverted microscope (Leica)
- Tali® image cytometer (Life Technologies)
- EVOS FL Cell Imaging System (Life Technologies)
- 96 well sterile plates
- Laminar flow chamber (TELSTAR AV)
- Plate Stirrer
- Culture flasks
- Parafilm
- Scrapes

4.1.5. Software:

- SigmaPlot 12.0 (Systat Software Inc.)
- Excel (Microsoft)

4.2. Methods

4.2.1. *In vitro* effect against the trophozoite stage of *Acanthamoeba castellani* Neff strain ATCC (30010).

The anti-*Acanthamoeba* activities of the eye drops were determined using the AlamarBlue™ assay previously described by McBride et al. (2005) based on the oxidoreduction of AlamarBlue™, measuring innate cellular metabolic activity. Metabolic products, such as NADPH, reduces the AlamarBlue™ dye and changes its colour as a measurable indicator of the amount of viable cells that are present in a test sample (Martín-Navarro et al. 2008; Mcbride et al. 2005). *Acanthamoeba castellani* Neff strain ATCC (30010) was grown axenically in culture flasks (25 cm^2) with PYG Medium. All assays were performed under sterile conditions in a laminar flow chamber. Briefly, *Acanthamoeba castellani* Neff strain was seeded in triplicate on a

96-well microtiter plate with 50 µl from a previously prepared solution of 10^4 cells ml^{-1}. Amoebae were allowed to adhere to the well bottom for 15 min, process which was checked using a Leica DMIL inverted microscope (Leica, Wetzlar, Germany). After that, serial dilutions of the tested eye drops were performed in a deep well plate and then, 50 µl of each dilution were collected and added to the correspondent well of the 96-well microtiter plate. Finally, the AlamarBlue™ was placed into each well at an amount equal to 10% of the total volume. Test plates containing AlamarBlue™ were then incubated for 96 h at 26 °C with a slight agitation. Later, after the 96h incubation, the plates were analysed with an EnSpire microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm.

4.2.1.1. Statistical analysis

The percentage of the growth inhibition, 50% inhibitory concentration (IC50 or CC50), was calculated by linear regression analysis with 95% confidence limits using Sigma Plot 12.0 statistical analysis software (Systat Software). All experiments were performed three times, and the mean values and the standard deviation were also calculated.

4.2.2. Effect of IC_{50} of each eye drops tested against *Acanthamoeba castellanii* Neff strain ATCC (30010)

After the statistical analysis, a new 96 wells plate with 10^4 cells ml^{-1} was incubated with the obtained IC50 values of each eye drop. To carry out this methodology we have used the EVOS FL Cell Imaging System (Life Technologies). The images were captured at 1h, 24 h and 96 h after the inoculation of the eye drops.

5. Results and Discussion

5.1. *In vitro* effect against the trophozoite stage of *Acanthamoeba castellanii* Neff strain ATCC (30010)

After carrying out the set of experiments, the IC_{50} values were calculated for each of the tested eye drops and are shown in table 1. All experiments were performed three times, obtaining the mean value along with its standard deviation.

Eye Drops	IC_{50}
Combigan®	6.37 ± 0.88
Timolol Sandoz®	3.53 ± 0.40
TobraDex®	1.56 ± 0.36
Colircusi antiedema®	9.14 ± 1.46
Voltaren®	15.86 ± 0.70
Duokopt®	27.20 ± 2.33
Cusimolol®	5.82 ± 0.35

Table 1 Anti-*Acanthamoeba* activity of the tested eye drops against *Acanthamoeba castellanii* Neff

The three eye drops that showed the lower IC_{50} values were TobraDex®, Timolol Sandoz® and Cusimolol®, respectively. After obtaining the fluorescence readings from the TobraDex® plate, it was detected that it emitted fluorescence, and because of that, a positive control was performed in order to subtract this value from the initially obtained value. Moreover, both Timolol Sandoz® and Cusimolol® contained timolol at a concentration of 0.5%. This compound is commonly used to lower eye pressure. As expected and according to previous studies, these two eye drops presented similar values in agreement with these studies (Sifaoui et al. 2017). On the other hand, TobraDex® does not have timolol in its composition. Furthermore, it is composed of an association of Tobramycin (3 mg/ml), an aminoglycoside used mainly to treat bacterial infections and Dexamethasone (1 mg/ml), a corticosteroid with anti-inflammatory effects. Although TobraDex® showed the lowest IC_{50}, many case reports where corticosteroids were used in an attempt to manage the AK, showed that its use is not associated with an improvement of the infection, probably due to its

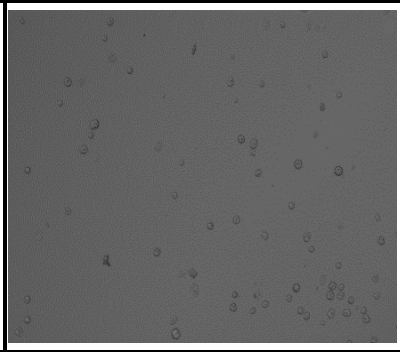
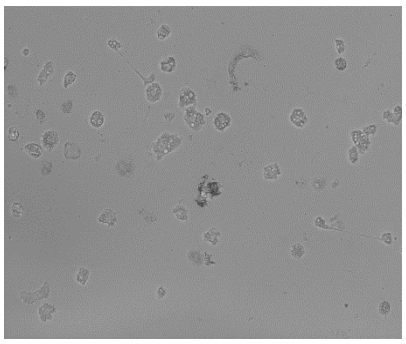
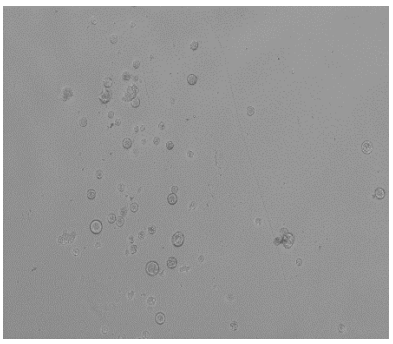
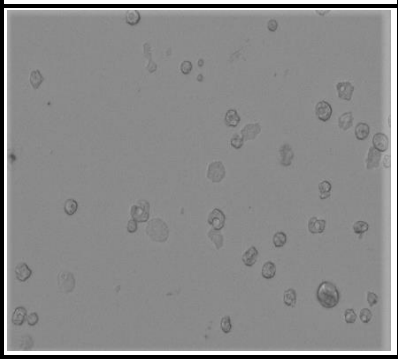
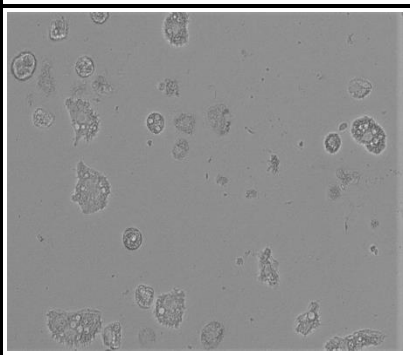
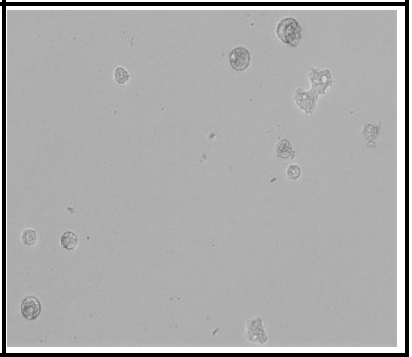
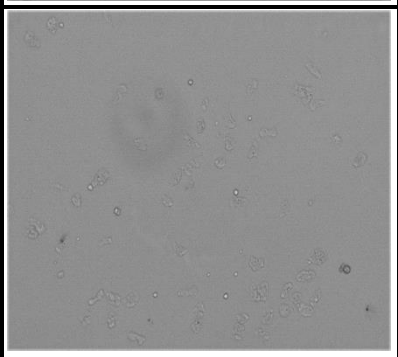
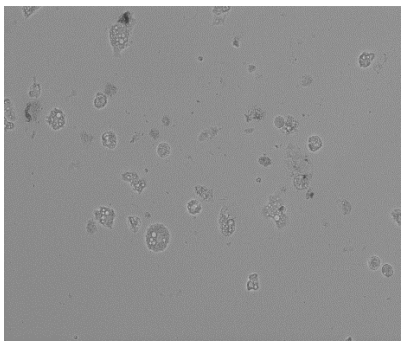
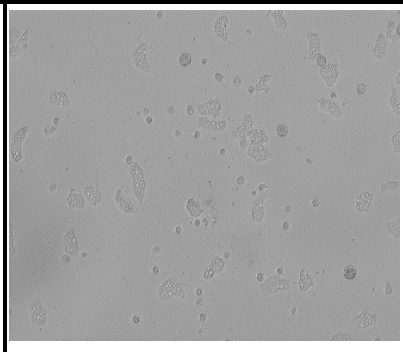
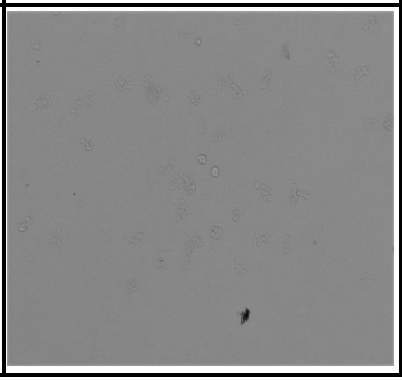
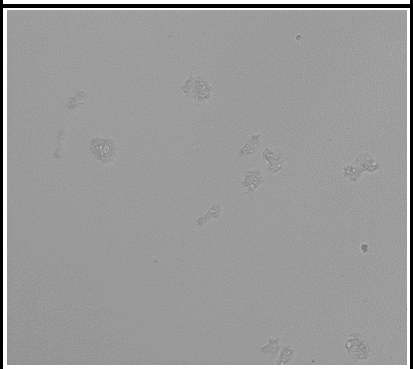
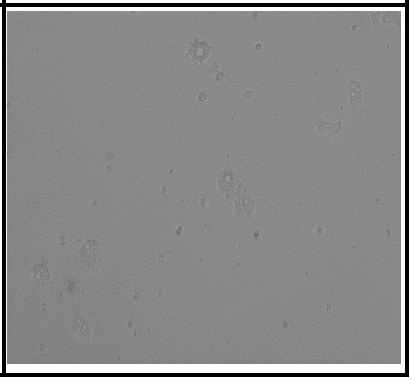
immunosuppressant effects and induction of encystation.(Lorenzo-Morales, Khan, and Walochnik 2015) Another reason that may explain such low IC_{50} values in this three eye drops, is the presence of benzalkonium chloride in the excipients used to formulate the three of them.

It has been previously described in prior studies that this specific excipient presents anti-*Acanthamoeba* activity (Tu et al. 2013). Concerning Voltaren® eye drops, it contains as the active substance, diclofenac sodium at 1 mg/ml, a NSAID used to reduce ocular inflammation and pain. Besides this, benzalkonium chloride and hydroxypropyl gamma-cyclodextrin are present as excipients, and here, lies a possible reason for the low activity against *Acanthamoeba*. When this two excipients are in the same formulation, the activity of the antimicrobial agent may be reduced or even neutralized by complexation with cyclodextrin (Loftsson et al. 1992). In contrast, Combigan® eye drops, composed by an association of two active substances, Brimonidine tartrate and Timolol, and also as excipients having the same concentration of benzalkonium chloride (0.05 mg/ml) as the one in Voltaren® but no cyclodextrins, presented an IC_{50} value of more than one-fold lower compared to Voltaren®. Regarding Duokopt®, although it has timolol at 5 mg/ml, the value of the IC_{50} obtained was the highest one. Once again, the most acceptable reason for this value appears to be, in this case, the absence of benzalkonium chloride in its composition. In a general analysis, TobraDex® and Timolol Sandoz® are the eye drops that appear to be more promising for the treatment of AK, and therefore to which further studies should be done to ascertain their mode of action against the parasite as well as cytotoxicity assays.

5.2. Effect of IC_{50} of each eye drops tested against *Acanthamoeba castellanii* Neff

In table 2, the phenotypical effects of the studied eye drops when applied at the IC_{50} concentration previously calculated are shown. The images obtained were taken 1h, 24h and 96h after adding the eye drops to the plate wells. It can be observed how the amoebae are rounded up 1h after adding the eye drops and, at this moment, it can also be observed how in the drops with the lowest IC_{50} there

are almost no parasites in trophozoite phase. Regarding Duokopt®, at 96 h a mature cyst was observed.

	1h	24h	96h
Combigan®			
Timolol Sandoz®			
TobraDex®			
Colircusi antiedema®			

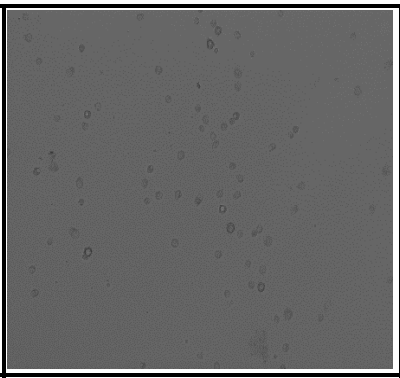
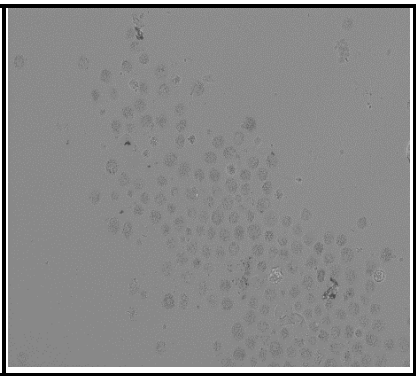
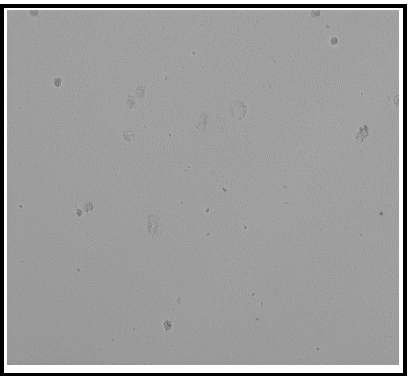
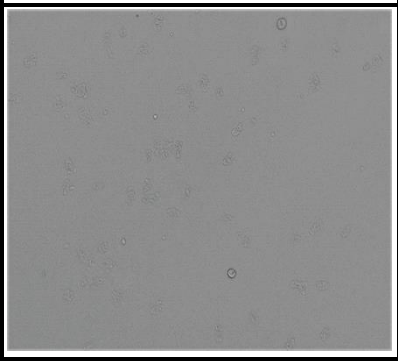
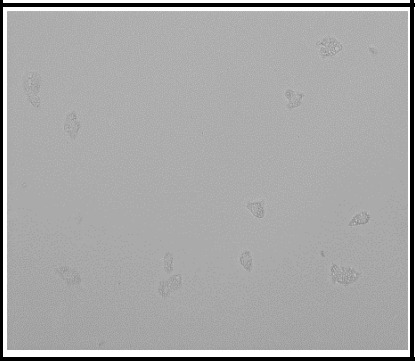
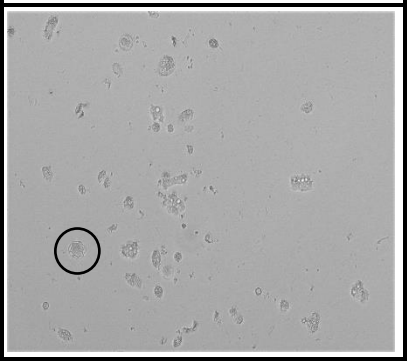
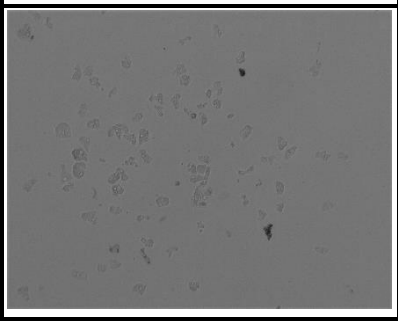
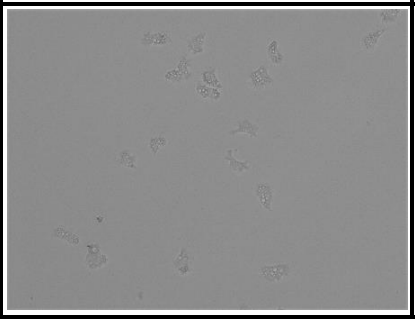
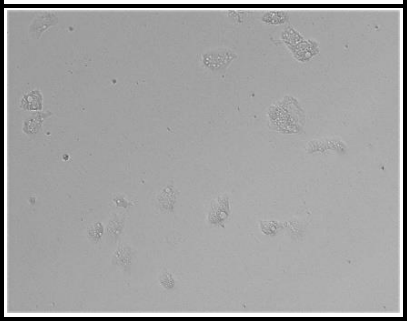
Voltaren®			
Duokopt®			
Control (-)			

Table 2 Effect of the tested eye drops on IC50 concentrations against *Acanthamoeba castellanii* Neff observed by inverted microscopy (x20).

6. Conclusions

- Commercialised eye drops are a good source of novel anti-*Acanthamoeba* agents.
- TobraDex® and Timolol Sandoz® eye drops could be a good option to treat and/or prevent AK in CLs wearers considering the obtained IC_{50} values.
- Additional *in vitro* and *in vivo* studies should be performed to elucidate the activity mechanism of these two eye drops against *Acanthamoeba* spp. as well as cytotoxicity assays.

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