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Original paper

Determination of the anti-protozoal activity of medicinal agents using the phenomenon of plaque formation by *Acanthamoeba* spp.

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ABSTRACT. Representatives of the genus *Acanthamoeba* are among the most widespread protists in the environment. They have a ubiquitous distribution and can sometimes cause quite serious pathologies in humans. The treatment of protozoal infections caused by free-living amoebae is currently limited and often unsuccessful. In the presented investigation, amebicidal activity was determined against both the trophozoites and cysts of *Acanthamoeba* spp., which were isolated during the microbiological examination of environmental objects. The inhibitory activity of drugs *in vitro* was determined using the authors' proposed method, which is based on the plaque formation phenomenon: this is initiated by free-living amoebae when cultured in agar containing the bacteria *Cellulosimicrobium* sp. strain bent-1. Based on a series of experimental studies, the paper proposes a reliable and inexpensive method for determining the anti-protozoal activity of medicinal agents, which will significantly complement the current screening method system when studying existing drugs, or new drugs during their development stage.

Keywords: *Acanthamoeba* spp., anti-protozoal activity, plaque formation, *Cellulosimicrobium* sp., screening method

1. Introduction

Human parasitic diseases caused by protozoa are of significant social and economic importance. In recent years, there has been an increasing trend in the virulence of certain protozoa, leading many parasitic infections to have severe and chronic courses. For example, free-living amoebae such as *Acanthamoeba* and *Naegleria* can cause pneumonia, acute respiratory infections, keratitis, and rare but fatal conditions like granulomatous amoebic encephalitis and disseminated granulomatous amoebic disease (skin infections, lung infections, etc.) [1, 2]. The ability of the *Acanthamoeba* species to cause serious, opportunistic infections is associated with a growing number of immunocompromised patients [3]. Following the worldwide growth in popularity of contact lenses in the 1980s, there was a sharp increase in cases of amoebic keratitis [4]. *Acanthamoeba* keratitis is a local infection that can lead to progressive vision loss, ulcer formation, secondary anterior uveitis, abscess formation, scleritis, glaucoma, cataracts, and, possibly, corneal perforation. The prognosis worsens if therapy is delayed by more than three weeks [5], which may necessitate surgical intervention and/or a corneal transplant.

Furthermore, free-living amoebae can also serve as a source/reservoir for other microorganisms that are pathogenic to humans: microscopic fungi, viruses, and bacteria, which can not only survive but also proliferate within amoeba cells [6].

Acanthamoeba spp. can inhabit almost any environmental object, including human-made water distribution systems such as pools, hospital water supply networks, drinking water pipes, etc. Free-living amoebae of the genus *Acanthamoeba* have a two-phase life cycle, consisting of large (up to

30 μm) active trophozoites and smaller (up to 20 μm) inactive cysts with protective walls. The transition from the trophozoite stage to the cyst stage (a form with minimal metabolism) is mainly associated with adverse environmental factors [3, 7].

Acanthamoeba are among the most common protozoa in the environment and have been classified into at least 20 genotypes based on 18S rRNA sequencing, denoted as T1–T20. Additionally, based on morphological and/or molecular criteria, up to 30 species of *Acanthamoeba* have been described. The most common pathogens that are capable of causing human diseases belong to genotype T4 and correlate with the *A. castellanii* complex. The groups T1, T2a, T3–T6, T10–T12, and T15 are also associated with human illnesses [3, 8].

Drug therapy that targets *Acanthamoeba* is an important component in the treatment of infections caused by representatives of *Acanthamoeba*. So far, no chemotherapeutic agent has been described that is a singular effective treatment for amoebic invasions, regardless of the isolate or genotype causing it. Often, a specific therapy for amoebic invasions involves the use of combination strategies to provide a synergistic effect and to improve treatment outcomes. However, given the limited available set of chemotherapeutic agents, treating patients who have amoebic keratitis and, especially, granulomatous amoebic encephalitis, remains a healthcare challenge [9].

Determining the sensitivity of clinical amoebic isolates to antiparasitic drugs in laboratory conditions, although rarely used, can be beneficial for clinicians. Sensitivity to anti-amoebic drugs may not only differ across different amoebic strains but also the same strain may become resistant to previously effective medications. Moreover, the results of sensitivity testing may vary between laboratories, as there are currently no reference methods for investigation and, in some cases, the results may not correlate with the clinical course. Despite these challenges, testing sensitivity to anti-amoebic agents can be crucial for treatment and should be used when possible [10].

To effectively implement a program for identifying new, promising drugs that have anti-protozoal activity, there is a need to develop fast, inexpensive and efficient screening methods. Currently, there is no standardized methodology for determining anti-amoebic activity. Several methods have been proposed for the primary screening of drugs, using representatives of *Acanthamoeba* spp. or *Naegleria* spp. as test cultures. Most of these methods are based on defining the viability of amoebic trophozoites and/or cysts *in vitro* by examining their morphology and visually counting amoebae, and then assessing their viability after exposure to potential anti-amoebic agents [11–14]. This method of investigation (microscopic analysis and visual counting every 24 hours) was proposed as early as 1969 by R.F. Carter when studying the amebicidal properties of the antifungal drug amphotericin B against *Naegleria* sp. [15]. The drawbacks of these methods include significant labor intensity, prolonged duration of study (which can take several weeks), and inaccuracies in the results. Antonio Ortega-Rivas *et al*. [16] proposed a colorimetric analysis based on staining amoebae with sulforhodamine B and adapted the method for use with 96-hole plates. They also proposed methods for determining amoebic viability after exposure to experimental drugs using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which resulted in a reduction in the study time and increased efficiency [17].

Taking the above into consideration, the aim of this research was to develop a fast and cost-effective method for assessing the anti-protozoal effect of potential drugs based on inhibiting the formation of amoebic plaque in an agar medium.

2. Materials and Methods

2.1 Test microorganisms (amoebae and bacteria)

In this study, test cultures of various amoeba species belonging to the genus *Acanthamoeba* were utilized (*Acanthamoeba* sp. strain krym, GenBank: MH620776.1; *Acanthamoeba* sp. strain karpaty, GenBank: MH620775.1; *Acanthamoeba* sp. strain cherkasy, GenBank: MH620777.1), as well as bacteria of the genus *Cellulosimicrobium* sp. strain bent-1 (GenBank: MH517543.1). The microorganism *Cellulosimicrobium* sp. (utilized by the amoebae to fulfill their trophic needs) and all the amoeba strains were isolated from environmental samples [18, 19].

2.2 Bacterial cultivation

The *Cellulosimicrobium* sp. strain bent-1 microorganisms were maintained under laboratory conditions using tryptone soy agar (Merck; pancreatic digest of casein, 15.0 g/l; peptic digest of soybean, 5.0 g/l; sodium chloride, 5.0 g/l; agar,

15.0 g/l) and nutrient agar for microbial cultivation (HiMedia, peptic digest of animal tissue, 5 g/l; meat extract, 1.5 g/l; yeast extract, 1.5 g/l; sodium chloride, 5.0 g/l; agar, 15 g/l).

2.3 Amoebic cultivation

A monoxenic cultivation method with prior inoculation of the medium with *Cellulosimicrobium* sp. strain bent-1 bacteria was employed to support amoebic growth. Co-cultivation was carried out on the following medium: peptic digest of animal tissue, 5 g/l; meat extract, 1.5 g/l; yeast extract, 1.5 g/l; sodium chloride, 5.0 g/l; agar, 10 g/l.

2.4 Obtaining trophozoites of the Acanthamoeba *sp. strains*

Trophozoites were obtained by culturing amoebae on a dense nutrient medium previously inoculated with *Cellulosimicrobium* sp. strain bent-1. Cultivation lasted 1–2 days at a temperature of 36 ± 1°C. After microscopic confirmation that trophozoites were present, they were washed off the nutrient medium using a phosphate buffer (pH = 7.0) and centrifuged at 500 ×g for 10 minutes. The sediment was re-suspended in a phosphate buffer and microscopy was performed again to confirm the presence of trophozoites.

2.5 Obtaining cysts of the Acanthamoeba *sp. strains*

The same methodology described above was used for cyst production, with some modifications. Cultivation lasted 5–7 days until the trophozoites fully transformed into cysts. The transition was also confirmed by phase-contrast microscopy.

2.6 The concentration and viability of cysts and trophozoites

Two methods were used to determine the concentration and viability of cysts and trophozoites. A count of cysts and trophozoites was performed using a Goryaev chamber, and cell viability was assessed using trypan blue (0.2%) and the "plaque formation" phenomenon.

2.7 Microscopy

Phase-contrast microscopy (Carl Zeiss, Axioplan, magnification ×1800) was used to determine the stages of the amoebic cycle.

2.8 Chemotherapeutic agents

Synthetic chemotherapeutic agents from the group of substances containing adamantyl, and

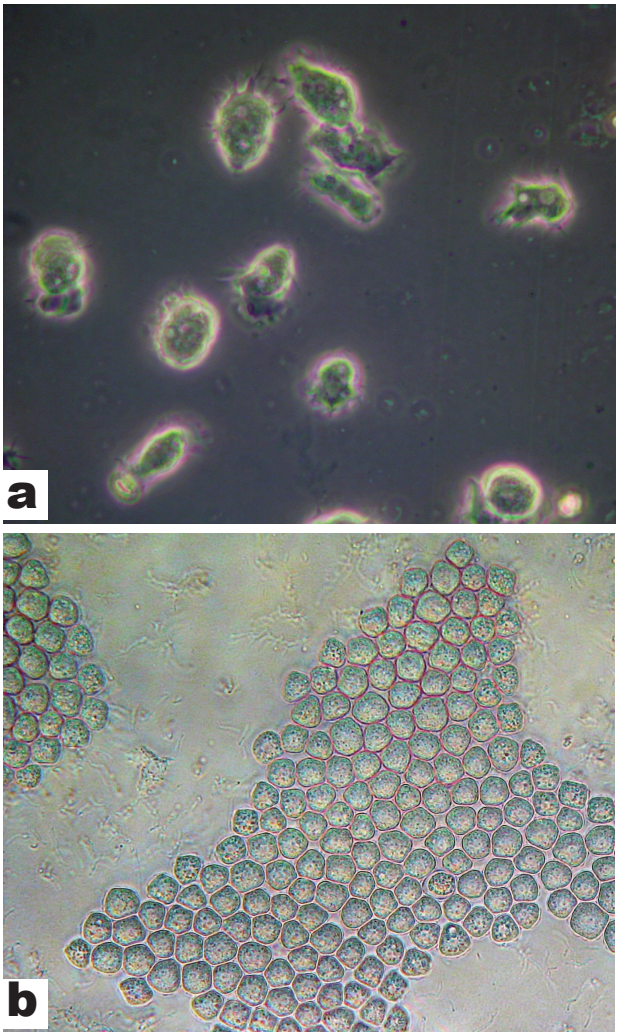


Figure 1. Motile trophozoites (a) and cysts (b) of amoebae. Phase contrast (original magnification ×1800)

compounds containing 7-bornyl (norbornyl) were used in the study, which, in previous research, had demonstrated broad spectrum antimicrobial properties [20]. Commercial antimicrobial agents were also used, including clotrimazole [21] and clarithromycin [22], which have been described as having amebicidal effects.

3. Results and Discussion

To assess the amebicidal activity we used amoebae of the genus *Acanthamoeba* as our model. It is known that free-living amoebae carry out their life cycle in various environments, feeding on microorganisms and small organic particles. The principle concerning the rapid and efficient cultivation of amoebae *in vitro* using *Cellulosimicrobium* sp. bacteria, formed the basis of the model developed to determine the amebicidal activity of the test substances.

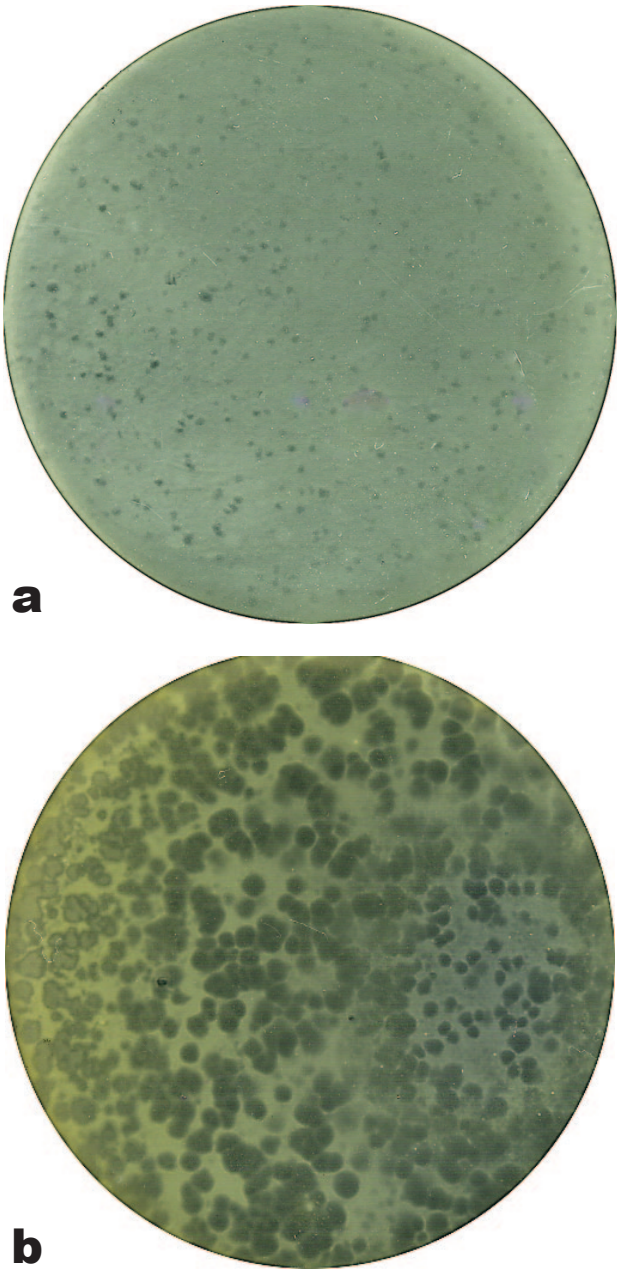


Figure 2. Growth of amoebic plaques: a, 3 days of cultivation; b, 5 days of cultivation

3.1 Obtaining Acanthamoeba *sp. cells at the different stages of development.*

To investigate the cytotoxic effect of the test compounds *in vitro*, amoebae were obtained at two different stages of their development.

It was observed that the optimal growth of amoebae on a lawn of *Cellulosimicrobium* sp. strain bent-1 microorganisms occurred on a dense nutrient agar for microbial cultivation containing 1% glucose (GNA).

Experimental studies have shown that the trophozoites of *Acanthamoeba* sp. appear in the first few hours after cyst germination when on a previously cultivated lawn of bacteria (1–3 days from the start of cultivation). They often have an elongated form, although other variants are also encountered. They are characterized by intense mobility, which is associated with the formation of pseudopods (Fig. 1a). With prolonged cultivation, amoebae trophozoites spread over the entire surface of the agar medium and eventually transform into cysts (5–7 days from the start of cultivation) (Fig. 1b).

3.2 Validation of methods for determining the amebicidal activity of promising or existing chemotherapeutic agents

As demonstrated in our previous studies [18, 19], amoebae isolated from environmental samples are capable of forming specific plaques on a dense nutrient medium.

By employing the agar layers technique proposed for bacteriophage detection [23, 24], we were also able to obtain plaques of free-living amoebae. These plaques exhibited clear boundaries, appearing 2–3 days after the introduction of cysts into the nutrient medium, and 1–2 days after the introduction of *Acanthamoeba* sp. trophozoites into a 0.7–0.8% GNA layer along with *Cellulosimicrobium* sp. strain bent-1 bacteria. A distinguishing feature of amoebic plaques is their ability to grow (increase in diameter) during cultivation (Fig. 2). Theoretically, a plaque results from the proliferation of a single amoebic cell, and their quantity indicates the concentration of amoebae in the investigated material.

The plaques form crater-like depressions in the agar's surface, apparently due to the compaction of the agar layer under the influence of the amoebas (Fig. 3).

The appearance of plaques is directly linked to the presence of carbohydrates in the nutrient medium—monosaccharides from the hexose group (aldohexoses), monosaccharides from the pentose group, and disaccharides. Glucose has been identified as the optimal carbohydrate for use in the nutrient medium for the manifestation of plaque formation. The density of the nutrient medium and the origin of agar also significantly affect the rate of plaque formation. Increasing the concentration of agar in the nutrient medium slows down the rate of plaque appearance. The best results are observed with an agar content of 0.7–1.0%; plaque formation is not observed at agar concentrations of 3%. There is a correlation between plaque formation and the pH of the medium. When the pH shifts towards

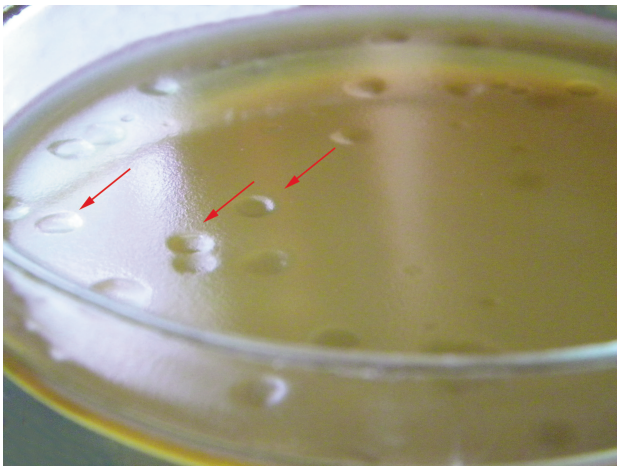


Figure 3. Crater-like depressions in agar at the site of amoebic plaques (0.8 % (by agar content) nutrient agar for microbial cultivation with 1% glucose added)

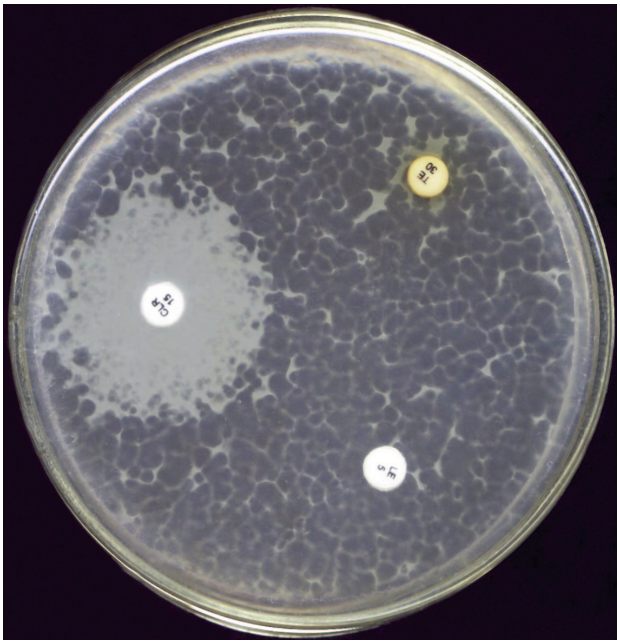


Figure 4. An example of the inhibition of amoeba plaque formation under the action of clarithromycin (standard commercial antibiotic disks from HiMedia were used in the study, with a load of 15 μg per disk)

acidity, the number of plaques decrease. This property should be taken into account when testing potential drugs with acidic properties that may affect the pH of the medium.

In addition, the phenomenon of amoebic plaque formation was utilized to determine the antiprotozoal activity of potential antimicrobial agents. The obtained results could serve as a basis for the further study and utilization of the phenomenon.

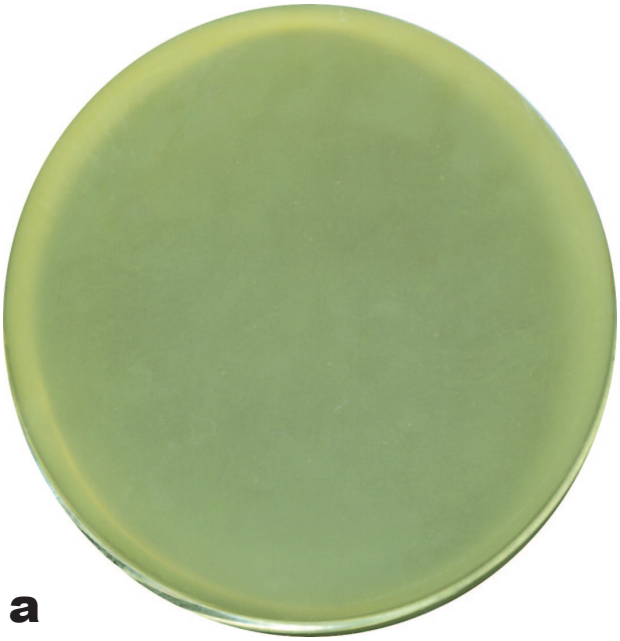
To detect the anti-protozoal effect, several different approaches can be employed.

(1) ***Agar diffusion method.*** This method is a modification of the Kirby-Bauer method, which is one of the standard techniques used in clinical microbiology to determine the susceptibility of microorganisms to antimicrobial agents [25].

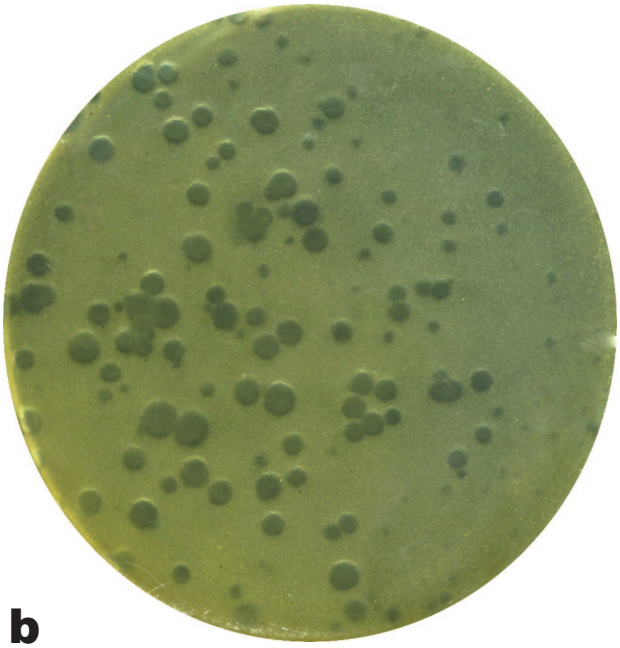
A nutrient medium of 1% agar with glucose, previously poured into test tubes, is melted and then cooled to 45°C. A suspension of amoeba (>10^3 cells/ml) and *Cellulosimicrobium* sp. strain bent-1 bacteria (10^8 CFU/ml) is added to this and is intensively mixed, after which it is poured evenly into sterile Petri dishes. After the medium has solidified, it is placed in a thermostat and incubated at 36 ± 1°C for 24 hours. After 18–24 hours of cultivation, paper disks soaked with various concentrations of the chemotherapeutic agent are placed on the surface of the medium. The delay in applying the disks to the medium's surface is due to the experimental substances potentially inhibiting the bacteria, which would prevent the evaluation of the amebicidal activity. Preliminary assessment is carried out on the third day of cultivation, and the final assessment on the fifth day.

If the experimental substance possesses an anti-protozoal effect, an active growth zone of *Cellulosimicrobium* sp. bacteria forms around the disk without lysed spots (lysed spots appear under the action of amoebae). This represents the inhibition of plaque formation by the drug (Fig. 4).

(2) ***Contact method.*** A suspension of amoebae (trophozoites or cysts) is mixed with the experimental preparation, incubated for several hours at room temperature. This is then added to a previously melted 1% nutrient medium with glucose containing a bacteria feeder and is then cooled to 45°C (1 ml of bacteria at a concentration of 10^8 CFU/ml). The mixture is vigorously stirred and then poured into a sterile Petri dish, which is then placed in a thermostat at 36 ± 1ºC. A primary assessment is carried out on the third day from the start of cultivation, and the final assessment on the fifth day. This is done by counting the number of plaques formed by living amoebae (CFU per dish). The number of plaques formed in the presence of different concentrations of experimental substances relative to a control is presented in percentages (Fig. 5). Dose-dependent curves can then be graphically constructed based on the obtained data.



a



b

Figure 5. An example of the of plaques under the action of antiprotozoal drugs (using the example of adamantane-containing substances: 1-adamantylmethyl-2,6-dimethylpiperidine): a, action of the drug at a concentration of 300 μg/ml; b, control

The percentage reduction in plaques under the action of experimental substances can be determined using the following formula [26]:

$$R = \frac{C - E}{C} \times 100$$

R, percentage reduction in plaques;
E, number of plaques in the experiment;
C, number of plaques in the control.

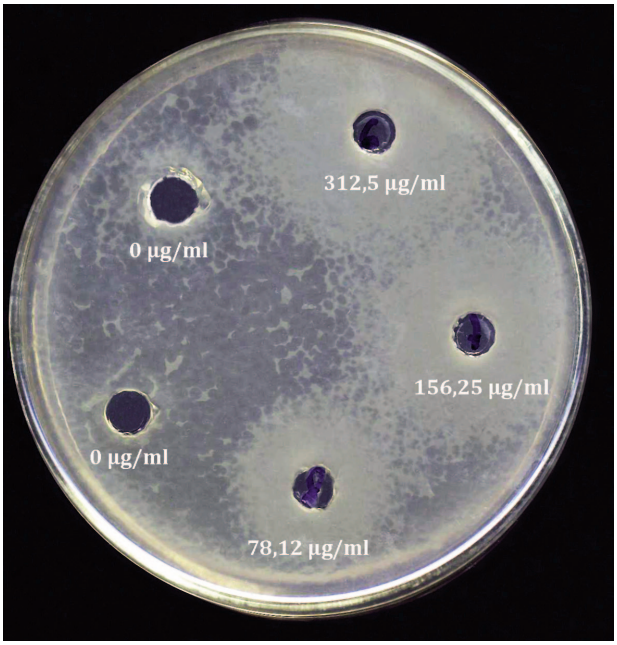


Figure 6. Example of the influence of the commercial antifungal drug clotrimazole (alcohol solution for external use)

(3) ***Modification of the agar well diffusion assay*** [27]. The essence of the method and the technique are as follows: at the beginning of the experiment, 10 ml of 1.5% agarose gel is poured into sterile Petri dishes and placed in a thermostat at 36 ± 1°C to allow the surface of the medium to dry. Subsequently, bases are set in the agarose gel for the formation of wells. Sterile dispenser tips are convenient for this purpose. This construction is then filled with 10 ml of 0.8% glucose nutrient agar for the cultivation of microorganisms that has been melted and then cooled to 45–50°C, which contains (<10^3 CFU/ml) and *Cellulosimicrobium* sp. strain bent-1 bacteria (10^8 CFU/ml). After the second layer of the medium solidifies, the dispenser tips are removed, leaving well-defined holes. The dishes are then placed in a thermostat for a day at 36 ± 1°C to enable the growth of bacteria, which are used by the amoebae as a trophic element. After 18–24 hours of incubation, various concentrations of the same drug or different antimicrobial agents/potential substances are introduced into the wells. The dishes are then incubated for 3–4 days at a temperature of 36 ± 1°C until plaques form. If the experimental drug exhibits antiprotozoal effects, intense growth of *Cellulosimicrobium* sp. bacteria occurs near the wells containing the drug, which are without plaque formation. This method can be used both qualitatively and quantitatively (Fig. 6).

In conclusion, we arrive at three points: (1) based on a series of conducted studies, effective and inexpensive models for the preclinical research of potential medicinal products that have amebicidal effects, have been developed; (2) the proposed methods allow for the determination of anti-protozoal activity within a short period of time at minimal cost; and (3) the developed methods can also be applied in the future to determine the sensitivity of clinical isolates of *Acanthamoeba* spp. to medicinal products.

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