In vitro study of formulated 1% clotrimazole eye ointment

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الدراسة خارج الجسم لتركيبة مصيغة كمرهم للعين من ١% كلوترايمازول

بحث مقدم إلى
فرع الصيدلانيات في كلية الصيدلة /جامعة هولير الطبية
من قبل
هبة أنطوان فتوحي/ ماجستير صيدلانيات
و
أريان رزطار طنجو/ ماجستير في الأحياء المجهرية/ كلية الصيدلة /جامعة هولير الطبية

الخلاصة:
الاهداف: الالتهاب الفطري للعين يكاد يكون مزمن و يحتاج الى علاج طويل.
تم تحضيرمرهم كلوترايمازول ١% كتركيبة جديدة للعين (تركيبة أ) و دراستها خارج الجسم لاثبات مدى فاعليتها في علاج الالتهاب الفطري للعين عند الانسان، وكذلك لمعرفة مدى تاثير المواد المضافة الى التركيبة على فاعلية الكلوترايمازول نفسه.
الطرق: تم تحضير مرهم للعين من الكلوترايمازول بتركيز ١% (تركيبة أ) و دراسة منطقة منع النمو لهذه التركيبة ضد عائلة الكانديدا و دراسة درجة منع النمو ضد عائلة الاسثيرجيلاس و الفيوزاريوم ومقارنة كافة النتائج مع نتائج فاعلية منتج كلوترايمازول ١% للجلد المتوفر تجاريا في الاسواق (تركيبة ب).
النتائج: كلا" من تركيبة أ و ب لهما فاعلية جيدة من ناحية منطقة منع النمو ضد عائلة الكانديدا و كذلك فان تركيبة أ منعت نمو الفطريات من عائلة الاسثيرجيلاس كليا" في كل التراكيز المستخدمة، ولكنها منعت نمو الفيوزاريوم فقط في التراكيز العالية.
الخلاصة: تركيبة الكلوترايمازول ١% للعين المحضرة حديثا" كانت فاعلة خارج الجسم ضد الجراثيم التي تسبب التهاب العين الفطري و بالتالي الدراسة السريرية و مدى تأثير هذه التركيبة على العين البشرية هي الخطوة التالية للدراسة لاثبات فاعليتها في عين الانسان.
مفاتيح الكلمات: كلوترايمازول، مرهم، التهاب العين الفطري.

Abstract:
Background & objective: Fungal infections of the eye tend to be chronic and often require prolong therapy. A newly prepared formula of 1% clotrimazole eye ointment was formulated then in vitro study was done to assess its potential in treatment of human keratomycosis.
Methods: Clotrimazole eye ointment of 1% strength was formulated (formula A). The zone of inhibition of the prepared clotrimazole eye ointment for *Candida* species and the degree of growth inhibition for *Aspergillus* and *Fusarium* species were studied then compared with that of commercially available 1% topical clotrimazole (formula B).
Results: Both formula A & B has good inhibition zone for *Candida* species, formula A inhibits the growth of *Aspegillus* species in all concentrations used, while it inhibits the growth of *Fusarium* species in high concentrations only.
Conclusion: a newly prepared 1% clotrimazole eye ointment is effective in vitro against pathogens that cause keratomycosis, further in vivo study will be done to prove its effectiveness.
Key words: Clotrimazole, ointment, keratomycosis

Introduction:

Fungus considered as the first organism scientifically established to cause disease. Fungi were recognized as pathogens prior to bacteria; however the development of effective antifungal agents has been disappointingly slow[1]. Although invasive fungal diseases are now more frequent than during first half of the century, they are still difficult to diagnose clinically[2].With the increase in the number of patients compromised by human immunodeficiency virus, cancer chemotherapy, organ transplants and long-term antimicrobial therapy, the incidence of opportunistic fungal infections is increasing[1]. With the world wide decrease in trachoma & other traditional causes of blindness, the world health organization has recognized that corneal blindness resulting from fungal keratitis is emerging as an important cause of visual disability[3]. Filamentary fungal ulcers are thought to have a particularly poor prognosis[4], *Aspergillus* and *Fusarium* are the most common genera[5]. The use of antifungal ointment is effective in preventing the development of fungal ulcers after traumatic corneal abrasions[6], making it very important to search for antifungal compounds which would be more effective and cheaper, with wide antifungal spectrum, short time of use & minimum side effects[7].

The imidazoles represent one of the two major classes of antifungal azole derivatives[1]. Clotrimazole is a topical derivative of imidazole[8], which is (1-[(2-chlorophenyl)diphenyl methyl]-1-imidazole). The melting point of clotrimazole is 141-245 ^{o}C, practically insoluble in water[9], soluble in alcohols & well distributed in tissues[10]. It was the first imidazole derivative developed as an antimycotic agent[11], which is highly effective drug against a broad spectrum of fungi[12], several reports indicate that this agent has broad in vitro antifungal activity against both pathogenic yeast and filamentous fungi[13].

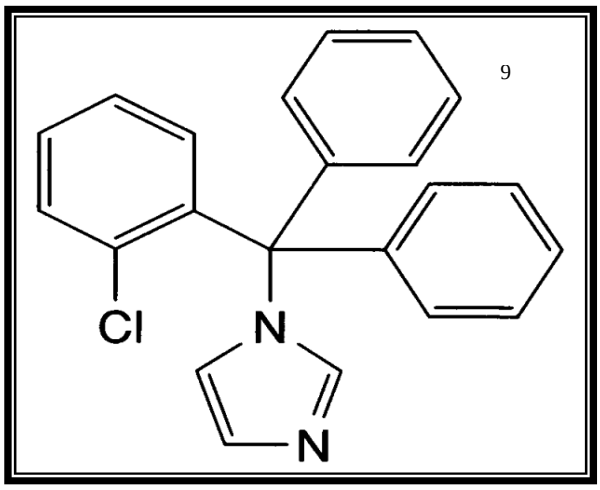

Figure (1): Structure of clotrimazole

Clotrimazole applied topically as a 1% cream, lotion or solution in the treatment of fungal skin infections, the 1% solutions is also used topically for fungal otitis externa. Clotrimazole is given as pessaries in treatment of vulvovaginal candidiasis also used as vaginal cream. Lozenges of clotrimazole used for the treatment of oral candidiasis[14].

Eye ointments are sterile semisolid preparations, contain medicaments dissolved or dispersed in a suitable non-irritant basis[15]. Ophthalmic ointments remain popular as a pediatric dosage form[16], it useful for treating children who may "cry out" topically

applied solutions[17]. Ophthalmic ointments can be used to obtain the effect of a variety of medicaments on the outside and edge of the eyelids, the conjunctiva and the cornea[18].

Materials and methods:

Materials: Clotrimazole powder (SDI), liquid paraffin (E, Merk, Darmstadt, Germany), wool fat & soft paraffin (Riedel-De-Haen AG, Seelze-Hannover, Germany), commercially available 1% topical clotrimazole (Epico). Culture media: Potato dextrose agar (PDA), this medium was used for growing of fungi and prepared by dissolving 39.0 g in one litter of (D.W.), then autoclaved at 15 pound / inch2and temperature of 121 ºC for 15 min.. Sabouraude dextrose agar (SDA), this medium was used for growing of fungi, yeasts and anti–Candidal test; also used in determining the fungal content of mycological evaluation, Prepared by dissolving 72 g in one litter of (D.W.), then autoclaved.

Methods:

Formulation of ointment base: Oleaginous and mainly anhydrous materials have commonly been used as bases for eye ointments, which should be non-irritant to the eye and should permit diffusion of the medicament when become in contact with the fluids at the eye surface[15].

Preparation of simple eye ointment base:

A Suitable base for eye ointments prepared using wool fat 10 gm, soft paraffin 80 gm and liquid paraffin sufficient quantity to produce 100 gm of the base. All these ingredients will melt together, filter, sterilized by heating for a sufficient time and allow to cool, taking precaution to avoid contamination with microorganisms[18].

Preparation of clotrimazole eye ointment:

Clotrimazole particles of pure powder was reduced to suitable particle size range, then triturate with small amount of simple eye ointment base, then add sufficient quantity of sterile simple eye ointment base required to prepare 1% clotrimazole eye ointment[18].

Adjusting the pH of the culture media:

pH for all culture media were adjusted to (7.2-7.4) by using pH meter[19].

Microorganisms:

The fungi which used for the study were *Candida albicans, Candid tropicalis, Aspergillus ochraceus* and *Fusarium oxysporum,* provided and identified in the Department of Biology/ College of Science/ University of Salahaddin/ Erbil-Iraq.

Preparation of paper discs:

Candida spp. sensitivity to prepared 1% clotrimazole eye ointment and commercially available formula was done by using filter paper disc diffusion method, the filter paper disc of 6 mm were prepared from whatman no. 3 filter paper by using ordinary office two-hole puncture, the discs placed in vials sterilized by oven and allowed to cool.

Anti yeast sensitivity test:

The stock solution (200 mg/ml) of commercial clotrimazole was prepared by dissolving 1gm in 5 ml of DMSO.

The concentrations (1,2,3,4 and 5 mg/ ml) were prepared from the stock solution then 1 ml added to 50 filter paper discs, then allowed to dry for 2 hrs. and another filter paper discs were soaked with DMSO only used as negative control.
Yeast suspension prepared from 24 h. colony by using phosphate buffer saline (PBS) in compare with standard control with concentration ($2.1x10^6$cell/ ml) of yeast suspension.
0.1 ml of yeast suspension were inoculated on SDA then spread using sterilized (L) shaped glass rod, then inoculated at 37 ^{o} C for 15 minutes, the prepared discs were placed on inoculated petri dish medium then incubated for 24-48 h. at 37 ^{o} C. Zones of inhibition were measured for each discs, that represent different concentrations of prepared clotrimazole 1 % eye ointment (formula A) and the commercially available topical clotrimazole 1 % (formula B) [20].

Preparation of spore suspension:
Pure culture of fungi (*Aspergillus ochraceus* and *Fusarium oxysporum*) were obtained by sub-culturing on to PDA agar and incubated for 3-5 days at 28 ºC to obtain freshly grown fungi. Then the spore suspension of selected fungi prepared (that used to test the effect of formula A and B on it), by adding 10 ml of Sterilized Distilled Water (SDW) on fungal plate, then scrape the spore by using sterilized glass rod, the spore mixture placed in a small sterilized vial put in stir bar for 10 minutes. The spores quantified using Hemocytometer and light microscope, then the spore suspension adjust to ideal concentration of $1x10^6$ spores/ ml[21].

Anti mycotic sensitivity test:
The stock solutions of (formula A and formula B) were prepared by adding (1 gm) of formula (A & B) in 5 ml of sterilized DMSO. The concentrations (1, 2, 3, 4 and 5 mg/ml) prepared from the stock solution then added to 500 ml of (CDA) zapeks dox agar and poured to sterilized petridishes then inoculated by (spore suspension) of each fungus, a sterilized plate with no addition of any formula was inoculated by spore suspension of each fungus, then the plates were incubated at 28 ± 1 ^{o}C for 5-7 days. The fungal growth measured by measuring colony diameter.

Results:
Both formula A and B have good inhibition zones against Candida albicans as shown in Fig.2.
Fig.3 (a) show that all concentrations of formula A have good inhibition zone for Candida tropicalis comparing with those of formula B which did not cause any inhibition zone at low concentrations as shown in Fig.3 (b).
Formula A with all concentrations used totally inhibited the growth of Aspergillus species compared with the commercially available formula B that have no effect at the lower two concentrations as shown in Fig.4(a) and (b) respectively.
While for Fusarium species, formula A inhibit the growth of this fungus only in high concentrations comparing with formula B which inhibit the growth of most plates except the lowest two concentrations, as shown in Fig.5 (a) and (b) respectively.

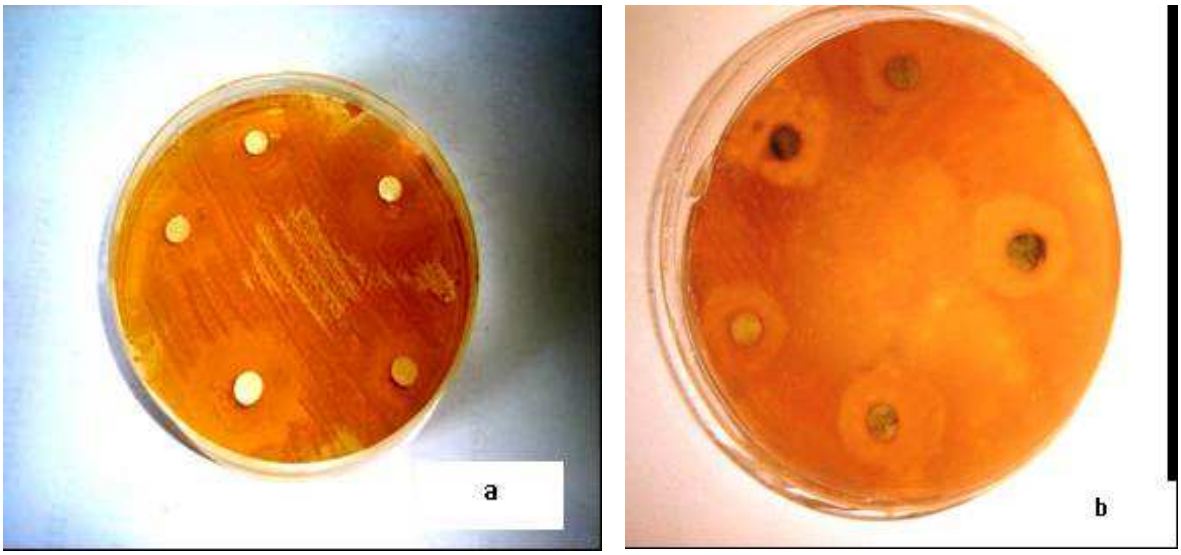

Figure (2): Inhibition zone of formula A (a) and formula B (b) on *Candida albicans*

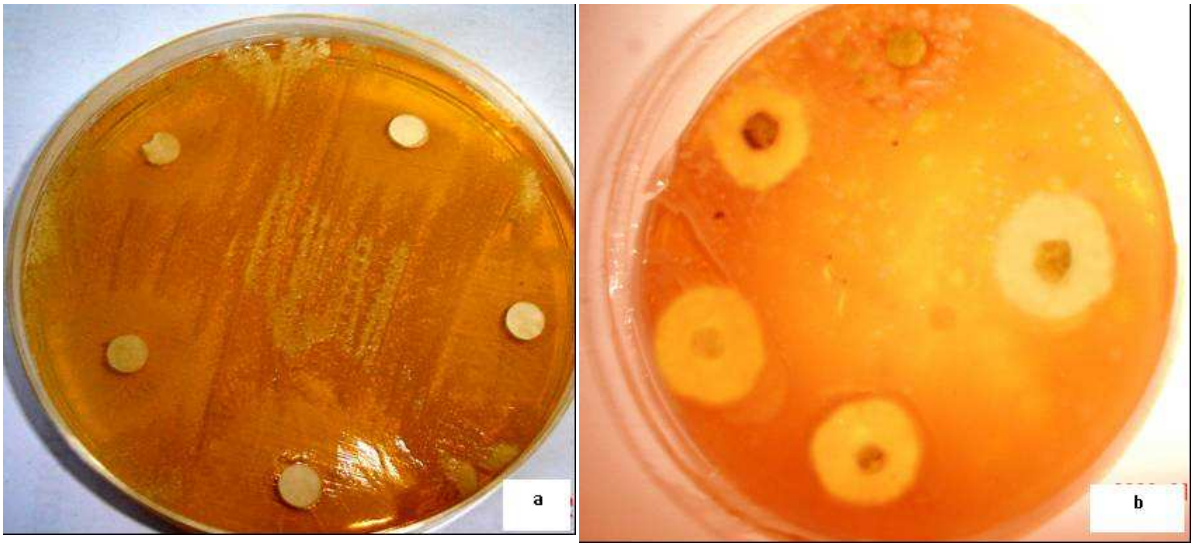

Figure (3): Inhibition zone of formula A (a) and formula B (b) on *Candida tropicalis*

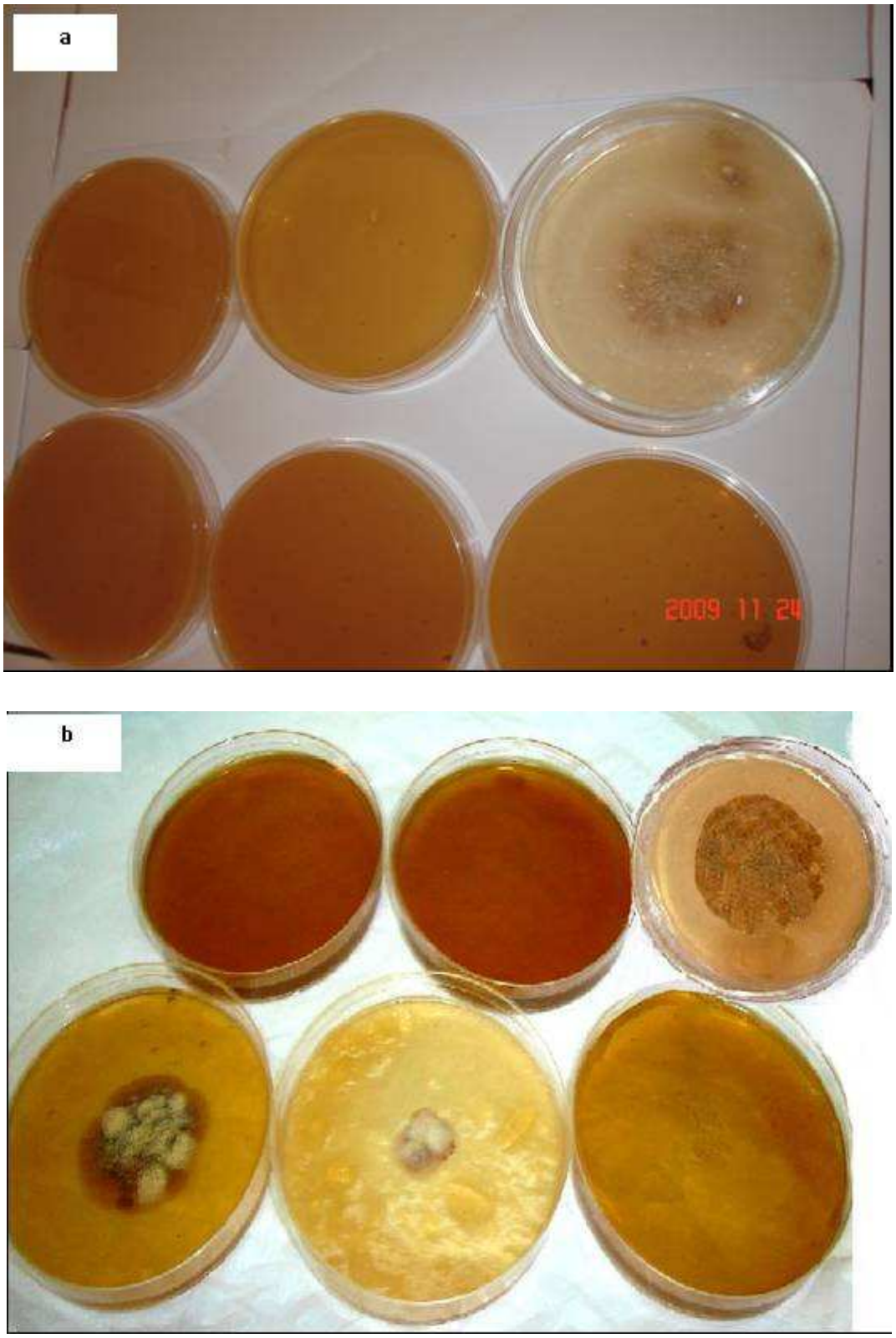

Figure (4): Growth inhibition for *Aspergillus* spp. of formula A (a) and formula B (b).

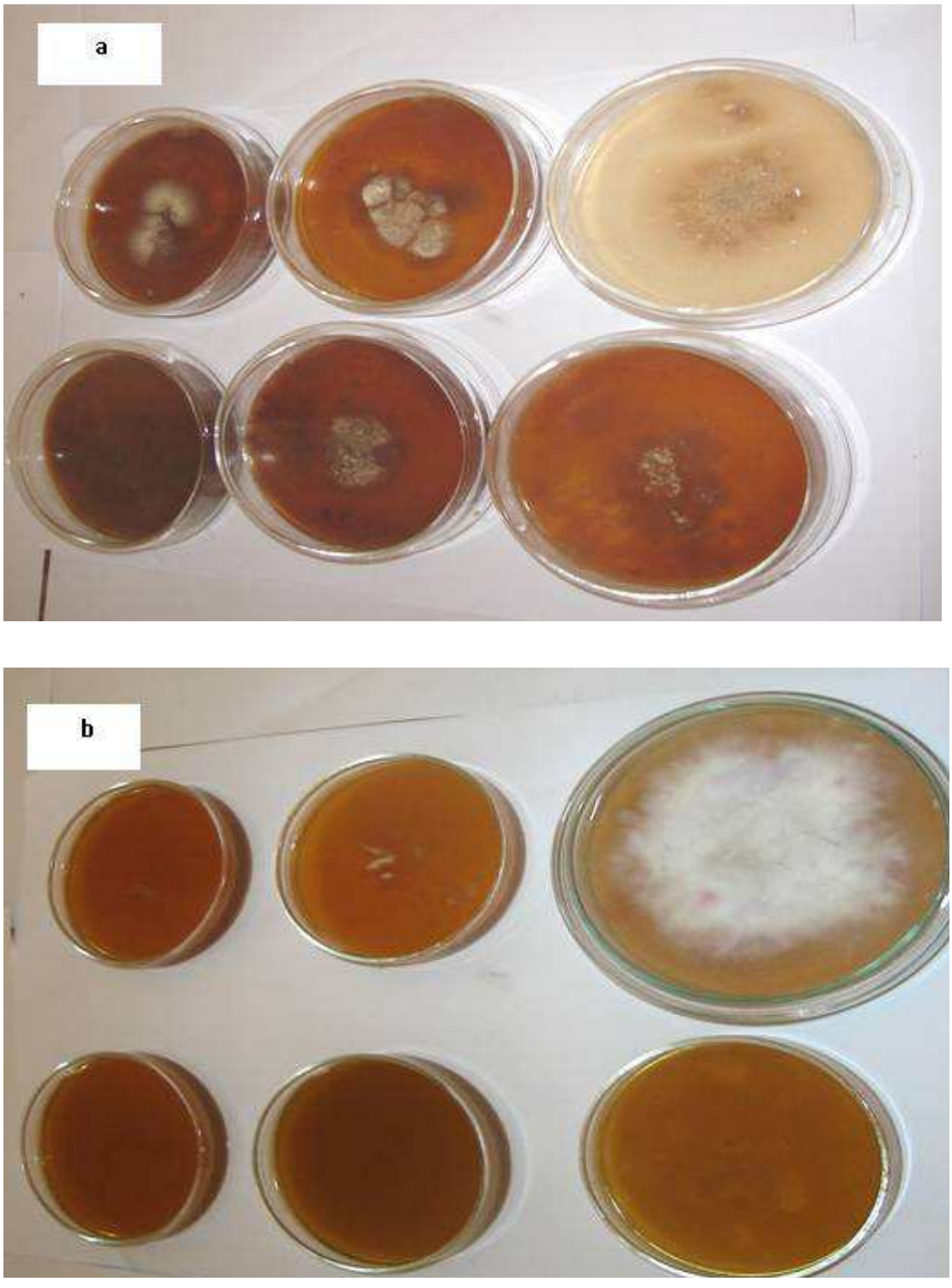

Figure (5): Growth inhibition for *Fusarium* spp of formula A (a) and formula B (b).

Discussion:

As seen from the results, formula A has good action on *Candida* species (albicans and tropicalis) which was consistent with the references that state, *Candida albicans* and *Candida tropicalis* are susceptible to clotrimazole[11], *Candida albicans* can be treated with imidazoles such as clotrimazole [22]. In vitro activity for both formula A and B is nearly the same; as a result we can use the newly prepared 1% clotrimazole eye ointment in the treatment of keratomycosis due to *Candida* species.

Regarding *Aspergillus* spp. formula A was very effective, it inhibits the growth of the fungus in all concentrations used as compared with formula B which was effective only in high concentrations, this result was consistent with the reference stated that clotrimazole would appear to be the drug of first choice for *Aspergillus* infections of the eye[23]. For *Fusarium* species formula A and B were effective in high concentrations this result consistent with reference stated that there is slow resolution of some cases infected with *Fusarium* species treated with diluted topical clotrimazole[8].

Depending on the results and from the pharmaceutical point of view, the antifungal activity of clotrimazole in formula A was not lost during formulation by any of the additives present in the formula.

Further clinical and in vivo study is needed to evaluate the use of newly prepared formula A for the treatment of human keratomycosis due to *Candida*, *Aspergillus* and *Fusarium* spp..

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