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Utilizing Eco-Friendly Bioactive Fungal Pigment to Formulate an Anti-Cutaneous Candidiasis Ointment as an Alternative to Terbinafine Ointment

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Abstract

Microorganisms that produce pigment are natural resources that could aid in solving today's issues. Natural colours have numerous advantages, including being antibacterial and antifungal, and it's crucial to research the biological and therapeutic characteristics of these pigments. Thus, the pigment extracted from highly pigmented *Aspergillus niger* strains was tested as an antifungal agent against *Candida* sp. isolated from women patients with dermatomycosis. An experimental investigation using mice (in vivo) to treat *C. albicans* infection with a novel ointment made from the pigment verified the extracted melanin's antifungal activity. The outcomes show how well the demonstrated significant antifungal efficacy. Created an active formulation of the ointment produced specifically for the study. Sections of skin tissue from the affected group and the control group were compared, and the former displayed evidence of blood vessel flow, keratin layer reconstruction, and dermal and epidermal healing. The findings indicate that melanin plays a role in the healing of damaged skin.

Keywords: anti- candidiasis, fungal melanin, *A. niger*, dermatomycosis

استخدام صبغة فطرية حيوية صديقة للبيئة لتحضير مرهم مضاد لداء المبيضات الجلدي كبديل لمرهم تيربينافين

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الخلاصة

تُعدّ الكائنات الدقيقة المُنتجة للصبغة موارد طبيعية يُمكن أن تُساعد في حل مشاكلنا المعاصرة. للألوان الطبيعية مزايا عديدة، منها كونها مُضادة للبكتيريا والفطريات، ومن الضروري البحث في الخصائص البيولوجية والعلاجية لهذه الصبغات. لذلك، اختُبرت الصبغة المُستخرجة من سلالات فطر الرشاشيات السوداء عالية الصبغة كمضاد للفطريات ضد فطريات المبيضات، والتي جُمعت من مريضات يُعانين من داء الفطريات الجلدية. وقد أثبتت دراسة تجريبية أُجريت على الفئران (داخل الجسم الحي) لعلاج عدوى المبيضة البيضاء باستخدام مرهم جديد مُصنّع من الصبغة، فعالية الميلانين المُستخرج كمضاد للفطريات. وتُظهر النتائج مدى

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فعالية الصبغة المُستخرجة في التركيبة النشطة المُبتكرة حديثًا للمرهم المُنتج خصيصًا للدراسة. قُورنت مقاطع من أنسجة الجلد من المجموعة المُصابة والمجموعة الضابطة، وأظهرت الأولى أدلة على تدفق الأوعية الدموية، وإعادة بناء طبقة الكيراتين، وشفاء الجلد والبشرة. كما تُظهر النتائج أن الميلانين يلعب دورًا في شفاء الجلد التالف..

1. Introduction

Melanin is a pigment that contains phenolic or indole polymers and ranges in colour from brown to black. It is a unique class of multipurpose polymers that gives animals, plants, fungi, and bacteria their dark colouring. [1,2]. Almost every organism, especially microbes, has been shown to synthesize melanin. *Bacillus species, Pseudomonas species, Cryptococcus species, and Aspergillus* species are known to produce melanin. [3,4]. Melanin in fungi helps infect the host and keeps them from various stresses of the environment [5,6].

The infection known as cutaneous candidiasis is brought on by *Candida albicans* or other species of Candida. Intertrigo, diaper dermatitis, perianal dermatitis, erosio interdigitalis blastomycetica, and candidal balanitis are typical signs of a candidal skin infection [7]. Due to the emergence of antibiotic resistance, approximately 25% of healthcare infections are primarily resistant to common antibiotic treatment. [8,9]. The majority of synthetic antifungal medications are rather costly, and fungi are always becoming resistant to them. Others, however, have detrimental side effects like hepatotoxicity. Additionally, there is a high risk of certain antifungals interacting with other medications. Researchers are searching for novel antifungal agents and of identifying resistance due to of these factors. [10,11]. Consequently, the purpose of this study was to ascertain whether a fungal melanin had antifungal activity against Candida pathogens isolated from patients at the Children's Teaching Hospital in Diwaniyah, Iraq, as an alternative to the antifungal ointment (Terbinafine) that appears to be ineffective against *Candida* sp.

2-Material and Method

2-1. Candida strain

C.albicans strain was obtained from the Children's Teaching Hospital in Diwaniyah. Which is isolated from patients suffering from dermatophytosis

2-2. Melanin pigment extraction

A method for extracting melanin pigment from *A. niger*, as described by Rajagopal *et al.*, [12], was used for melanin pigment extraction**.**

2-3. Identification of the isolate

A chrome agar medium was used for the identification, as instructed by the company, 45.9 g of agar was placed in one litter of distilled water to produce a diagnostic medium for Candida. [13]

2-4. In vitro anti-Candida activity

The antimicrobial activities of the extracted dye were tested using the agar wells diffusion method described by Bonev *et al.,* [14]. The melanin concentrations used in this test were (50, 75, 100, and 200 µg/ml) which varied among *Candida albicans and Nakaseomyces glabrata*.

2-5. Anti-Candida activity in vivo

2-5.1. Experiments on infection

2-5.1. 1. Animal preparation

Laboratory mice (Mus musculus L., BALB/c) aged 6 to 8 weeks were obtained from the animal house of Al-Qadisiyah University, the College of Science. The ethics committee in the science college approved the study's animal recruitment under reference number 1 (on 10\7\2023) To begin the breeding program, all animals were housed in plastic cages measuring 30 × 12 × 11 cm and filled with sterile softwood shavings. The animals were housed in climate-controlled enclosures that featured light-dark cycles and a temperature of 22 °C, as shown in Figure 1.

Figure 1: Laboratory health animals used in the study before infection with yeast

2-5.1. 2.Induction of infection in laboratory animals

The back area is ideal location for the mouse to be injured and cause infection because it is difficult for the animal to reach, which prevents other infections from spreading to the wound area. Additionally, this location makes it easier to perform follow-up procedures, such as taking pictures, treating the infection, and tracking its progression. To guarantee uniform infection, the hair at the infection site was shaved with a sterile blade. Following hair removal (Figure 2). 70% ethanol disinfectant was used to cleanse the skin. The mouse was infected by swabbing from the yeast culture [15], as shown in Figure 3.

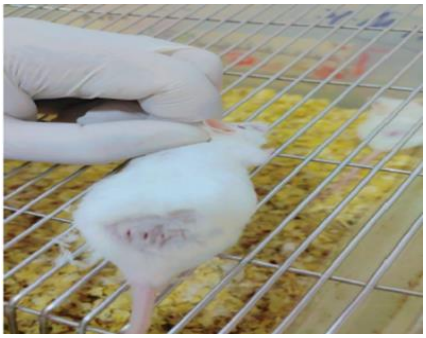


Figure 2: Animal wounding

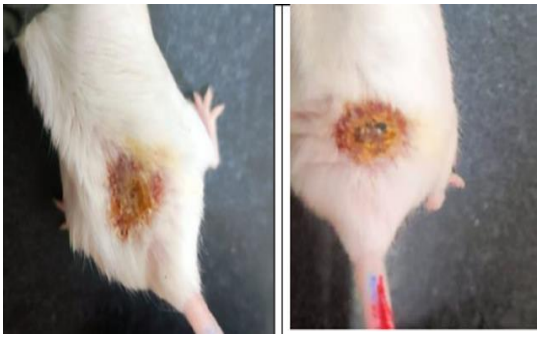


Figure 3: Induction of infection in laboratory animals

2-5.2. The ointment's preparation

The ointment (200%) was prepared pharmacologically by combining 160 g of white Vaseline with 40 g of extracted melanin. The drug's ingredients were combined while being stirred until the mixture was homogenous, and the preparation was created. The pigment was

absent from the control treatment, which consisted only of 200 g of white Vaseline, as shown in Table1.

Table 1: New ointment component

Treatment	Concentration	
	200% (treated ointment)	0% (control)
Extracted pigment	40g	0
White Vaseline	160g	200g

2-5.3. Animal groups

The animals were divided into four groups (4 each):

Group 1 - healthy animals.

Group 2 - untreated yeast-infected animals

Group 3 - yeast-infected animals treated with antifungal (Terbinafine).

Group 4 - yeast-infected animals treated with extracted tincture (new ointment) (twice daily).

Following two weeks of therapy, a biopsy had been taken from the site of infection and saved in formalin for analysis.

2-5.4. Histological analysis

The tissues were analysed using the method outlined by Jain, *et al*., [16]. After being fixed for 96 hours in buffered formalin, the pieces underwent standard laboratory processing. For histomorphological analysis, the specimens were embedded in paraffin, and sections of skin measuring 6 mm in thickness were cut apart and stained with hematoxylin and eosin

.2-5.5. Side effect of pigment

To conduct the test, 0.5 g (200% concentration) of the extracted pigment was applied to a 2 × 2 cm piece of sterile gauze on the healthy skin of six animals, and six more with skin erosion, (which was made by a sterile needle that was used to make three parallel incisions, each 0.5 cm apart, being cautious not to bleed). The gauze pieces were then positioned as in the healthy group. All animals' gauze pieces were tied with sterile gauze pieces in the shape of a X to prevent the pieces from moving. The animals were monitored every 24 hours and for 14 days during the treatment period, and the results were documented [17].

2-6. The haemolytic activity of the extracted pigment

Fifty microliters of the extracted pigment were applied as spots on the blood agar plates and incubated for 24 hours at 37°C to test the haemolytic activity in 5% sheep blood agar. The results were then documented after the decomposition that developed around the spots was examined [18].

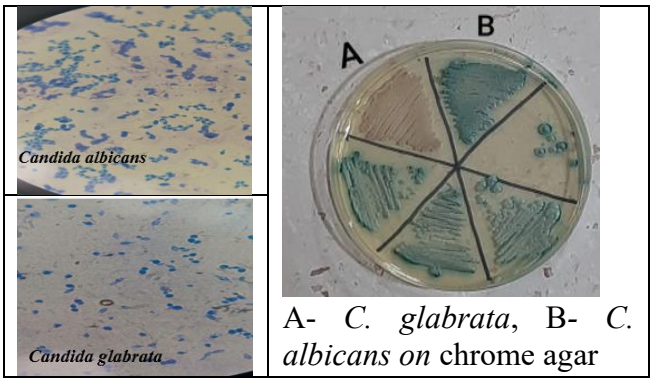
2-7. Analysis of statistics

The results are presented as mean ± standard deviation (SD). Three duplicates of each experiment were the conducted, and one-way ANOVA was used for statistical analysis using the SPSS statistical software, version 20.0.

3-Results and Discussion

Identification of Candida sp

The strains of *Candida albicans and Nakaseomyces glabrata* isolated from patients who had candidiasis infections are shown in Figure 4, and consistent with other studies [19,20].



A- *C. glabrata*, B- *C. albicans on* chrome agar

Figure 4: Species of *Candida* sp

In vitro anti-Candida activity

The activity of extracted melanin against the two isolated species of *Candida* is displayed in Table 2. There are notable variations in the effect between concentrations and species, and the effect grows as concentrations increase. Considering that the concentrations were 100 µg/ml and 200 µg/ml, respectively, this result is in agreement with the melanin concentration and the inhibition zone (mm). Inhibition zones for *C. albicans* are 23.22±0.11 and 15.32±0.18, while those for *C. glabrata* are 25.15±0.13 and 22.46±0.29. This was verified by Fuentes *et al.* [21], who found that melanin had an influence on four species of *Candida (C. albicans, C. parapsilosis, C. glabrata, and C. krusei)* and claimed that it was impossible to pinpoint the mechanism of action of melanin. However, due to their aromatic structure, reactive quinone intermediates formed during melanin synthesis through the production of hydrogen peroxide have been described as having antifungal and inhibitory properties. Numerous researchers have looked into the formation and use of pigments produced by microbes as natural sources [22-24].

Table 2: Antifungal activity of the extracted melanin pigment

strains	Concentration of Melanin and zone of Inhibition (mm)	
	100µg/ ML	200µg/ ML
Candida albicans	15.32±0.18*	23.22±0.11
Candida glabrata	22.46±0.29	25.15±0.13

In vivo anti-Candida activity

Clinical symptoms

The mice exhibited symptoms of the disease two weeks after the yeast infection (Figure 4). The symptoms of a Candida skin infection include skin thickening, hyperkeratosis (abnormal thickening of the skin's outer layer), redness, and inflammation. These symptoms are consistent with the remarks stated by Kühbacher *et al*., and Espinosa-Hernández *et al*., [25,26].

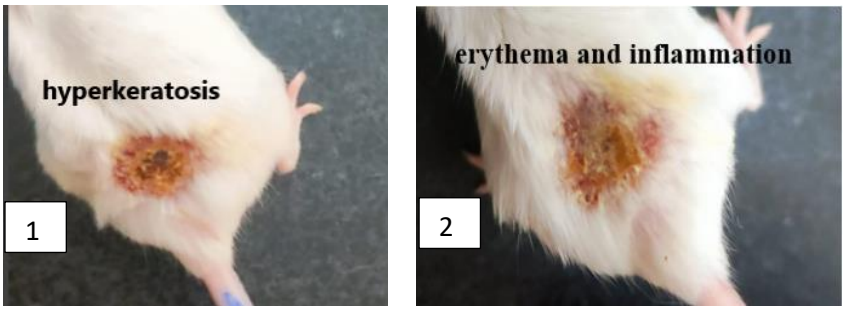


Figure 4: Clinical symptoms in mice infected with *C. albicans*
(1) Appear hyperkeratosis (the stratum corneum, the skin's outermost layer, becomes thicker.
(2) Erythema (any unusual skin or mucous membrane redness) and inflammation

Utilizing the new ointment for medication

Clinical symptoms disappeared, and a healing process was observed in the infected mice following four weeks of treatment with the novel ointment. This included hair growth, the disappear any lesion s or signs of inflammation, and the recovery of the injured area (Figure 5).

Figure 5: Treatment with a new ointment, A- Treated with a new ointment (hair growth), B- Treated with antifungal,(fewer inflammation signs), C- Control (without treatment), inflammation and ulcer

Histological study

In group 1, a healthy mouse's normal skin tissue section is depicted in Figure 6. It displays the fundamental histological structure of the skin, comprising the epidermis, dermis, hair follicles, normal dermal and epithelial layers, as well as the keratin layer. It further demonstrates the absence of notable infiltration by white blood cells, as well as the presence of a hair follicle close to the skin's surface. As mentioned by Abdullah *et al*., [27].

As illustrated in Figure 7, group 2 - skin tissue of untreated infected mice is characterized by parakeratosis (where epidermal keratinocytes do not fully mature, causing nuclei to be retained abnormally in the stratum corneum). The stratum corneum is infiltrated by neutrophils, the epidermis is thinned with loss of the follicular epidermis, and characteristic inflammation and necrosis are present. The histopathological alterations are concordant with Iwasawa, *et al.,* [28]. Figure 8 shows a section of group 3, a damaged mouse that received treatment with the new ointment developed in this study for four weeks, demonstrating epidermal thickening, dermal and epidermal healing, vascularization, and regeneration of the keratinized layer. When the skin is damaged, *Candida albicans* generates a significant amount of reactive oxygen species (ROS). Melanin effectively scavenges radical species in the skin. This fact illustrates melanin's role in skin repair. [29]. Figure 9 displays a cross-section of the skin from a mouse infected with group 4 and treated with the antifungal Terbinafine. The process of healing involves the thickening of the epidermis and the development of

granulation tissue. Although Terbinafine is the most effective antifungal for Candida infections, it can cause side effects such as burning and redness. As a result, researchers have evaluated options to discover alternative medications for this infection [30].

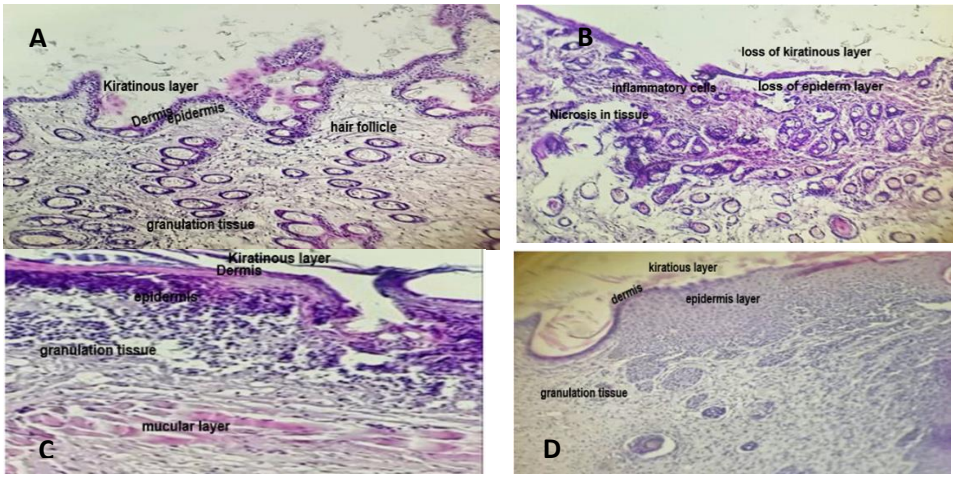


Figure 6: Section of skin tissue. A. Healthy skin tissue (the control group shows the skin's main histology, which includes hair follicles, the epidermis, and the keratinous layer). B. The infected mouse from group 2 (shows the skin tissue untreated). C. The group 3 mouse's infected skin section was treated with a new ointment. D Skin tissue section post-treatment with antifungal agent

Side effects of pigment

Accordant to Table 3, which shows the side effects of the extracted pigment on the skin of the mice used in the study, it appears that the pigment has no effect on the skin. There is no irritation, erythema, or edema. This result confirms that the pigment is safe for the skin and can be used in skin treatment.

Table 3: Effects of the extracted pigment on the skin of treated animals

Type of effect	acuteness level	No of animals not exposed to skin erosion	%	No of nimals exposed to skin erosion	%
irritation	No irritation	5	62.5	3	37.5
	slightly	1	12.5	2	25
	moderately	0	0	0	0
	severely	0	0	0	0
Erythema and Escher	No erythema	8	100	0	0
	slightly	0	0	0	0
	moderately	0	0	0	0
	severely	0	0	0	0
edema	No edema	8	100	0	0
	slightly	0	0	0	0
	moderately	0	0	0	0
	severely	0	0	0	0

The haemolyticus activity of the extracted pigment

The findings regarding the extracted pigment's haemolytic activity demonstrated that, following the incubation period, no regions of decomposition were observed surrounding the pigment spots. The most common technique for figuring out a biomaterial's hemocompatibility is haemolysis testing. This pigment is therefore regarded as a hemocompatible substance. [31].

4-Conclusion

The extracted melanin demonstrates antifungal activity *in vitro* (well diffusion) and *in vivo*. Laboratory mice with induced candidiasis responded well to the ointment made from the extracted pigment. In contrast to the antifungal effect, the mice treated with the prepared ointment experienced no adverse effects. Further research is needed on immunoassays, particularly on interferon levels, such as gamma interferon.

Conflicts of interest

There are no conflicts of interest

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