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Efficiency of Fungal β-carotene Against Some Causative Agents of Dermatomycoses

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ABSTRACT

Aims Considering the increase of dermatomycoses and the emergence of new strains resistant to antifungal treatment, it is important to find an alternative treatment of natural origin. This study aimed to extract and purify β-carotenoid from two types of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* and test its reactivity toward some yeasts isolated from dermatomycoses infection.
Materials & Methods Rhodotorula samples were isolated from extreme environmental soils, and after cultivation, they were identified genetically and apparently. Then their pigments were extracted and purified. Beta-carotene was detected by ultraviolet and infrared regions. The activity of β-carotene was tested using three different concentrations using the agar diffusion method against four isolates of Candida and Cutaneotrichosporon dermatis pathogenic species by comparing its reactivity with the antifungal Nystatin.
Findings All tested isolates, including Candida albicans HAM25, Candida dubliniensis, Candida tropicales HAM13, and Cutaneotrichosporon dermatis Judy 4, showed resistance against β-carotene purified from R. diobovata and Nystatin at the concentration 0.01mg/ml. However, β-carotene and Nystatin showed activity against all isolates with concentrations of 0.02 and 0.03mg/ml. The purified compound from R. mucilaginosa showed activity against all isolates in three concentrations, according to concentration and type. There was a significant difference between the antifungal activity of both purified β-carotene and Nystatin ($p<0.0001$).
Conclusion Both isolates of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* produce β-carotene, and the preference quantitatively is to isolate R. diobovata first. The activity of β-carotene against all tested yeast isolates are higher than the antifungal Nystatin.

Keywords Dermatomycoses; Beta-Carotene; Rhodotorula; Candida; Antifungal Agents

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Introduction

Dermatomycoses are infections of the skin, hair and nails, which are caused in most cases by dermatophytes, and in rarer cases by yeasts and molds. Fungal skin infections have been recognized as the fourth most common health disorder globally (after dental caries, tension-type headaches and migraine) [1], and dermatophyte infections of the skin, hair and nails are the most common fungal infections [2].

Dermatomycosis is characterized by both superficial and subcutaneous infections of keratinous tissues and mucous membranes caused by a variety of fungal agents, the two most common classes being dermatophytes and yeasts. Overall, the stepwise process of host infection is similar among the main dermatomycotic species; however, the species-specific ability to elicit a host reaction upon infection is distinct. Yeasts such as *Candida albicans* elicit a relatively low level of host tissue damage and inflammation during pathogenic infection, while dermatophytes may induce a higher level of tissue damage and inflammatory reaction. Both pathogens can, however, manipulate the host's immune response, ensuring survival and prolonging chronic infection [3].

The genus Candida includes about 200 different species, but only a few species are human opportunistic pathogens and cause infections when the host becomes debilitated or immunocompromised. Candida infections can be superficial or invasive. Superficial infections often affect the skin or mucous membranes and can be treated successfully with topical antifungal drugs. However, invasive fungal infections are often life-threatening, probably due to inefficient diagnostic methods and inappropriate initial antifungal therapies [4].

Although the antifungal drugs used in clinical treatments appear to be diverse and numerous, only a few classes of antifungal agents are currently available to treat infections with Candida spp. [5, 6].

The emergence of antifungal resistance, with an increasing number of reports of difficult-to-treat infections [7-9], has been highlighted as an issue of growing concern. Antifungal resistance is based on different mechanisms, namely, (i) reduced drug intracellular accumulation, (ii) decreased target affinity/processivity for the drug, and (iii) counteraction of the drug effect. Particularly, the mechanism of resistance will be different depending on the mode of action of antifungal compounds [10].

Fungal infections caused by Candida spp. are deemed increasingly challenging in human medicine, despite the availability of anti-fungal medications [11]. The antifungal medicine effect is not a new problem, even among persons who did not expose to antifungal medicine, it is reported the resistance of unsaturated hydrocarbon carotenoid dissolved in ether petroleum [12].

Pigments are colorants that are produced from plants, animals, and microorganisms and can also be synthesized by chemicals synthetically. The pigments have many applications in industries (food, textile, paper, cosmetics, plastic, paint) [13], agriculture, biology (antibiotics, antimicrobial agents, anticancer agents) [14, 15], etc.

Nowadays, pigments produced from living organisms gained more importance as synthetic pigments are toxic and show harmful effects [16]. The microbial productions of pigments are in many ways superior to the pigments produced from the plants and animal sources due to several reasons such as their rapid growth rate, easy downstream processing, cost-effectiveness, independent of season and geographical conditions, controllable, more stable and safe to use [17, 18]. Microbial pigments can be produced from bacteria, algae, fungi, and protozoa.

Carotenoids are a large group of organic, pigmented, isoprenoid-type compounds that play biological activities in plants and microorganisms (yeasts, bacteria, and microalgae).

Carotenoids also can act as antimicrobial agents, and few reports showed quantitative measurements of Minimal Inhibitory Concentrations against different pathogens. The demand for scale-up of different naturally obtained carotenoids has increased due to the concern about the detrimental health effects caused by synthetic molecules and antimicrobial resistance [19].

Carotenoid is produced in nature from different microbial and plant sources such as bacteria, molds, yeasts, and fungi [20, 21]. This pigment is applied in different fields so that it come very interested [22]. In the past years, the interest in this pigment has increased due to its many benefits and importance for human health because it is a source of vitamin A in the body and it is converted into a vitamin in the intestine, which is very necessary to maintain vision. It is also anti-tumor and anti-oxidation and protects the body against free radical initiators [23], and strengthens the immune system [24].

The production of carotenoids from plants has become limited due to the huge cost of production in comparison with revenues. Therefore, many researches and studies are conducted to obtain carotenoids from microorganisms, which have become an alternative source instead of plants due to their low production cost and limited impact on the environment [25]. In addition, carotenoids produced by these microorganisms are never affected by the seasonal and geographical changes that plants face [26].

Yeasts, due to their nature, are the most applied for carotenoid production compared to other microorganisms, as they are unicellular organisms in

addition to overgrowth. Yeasts that produce carotenoids are Rhodotorula, sporpbolomyces, Phaffia, Cryptococcus, and Rhodosporidium [27].

There are few studies on the antifungal activity of β-carotene. According to our knowledge, this is the first study in Iraq on extracting carotenoids from yeasts and testing their activity.

This study aimed to extract and purify β-carotenoid from two types of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* and test its reactivity toward some yeasts isolated from dermatomycoses infection.

Materials and Methods

Sampling

Six soil samples were collected from the extreme environments inside and outside Basra Governorate, including Jabal Sanam (Safwan Hill), Sawa Lake, Al-Faw Sediment, Al-Nasiriyah Desert, and Sulfur Lake in Heet. The samples were collected using a clean spatula after removing the surface layer of the soil to a depth of about 5-10cm and from several places so that the weight of each sample reached 500g. The samples were diluted in concentrations of 0.1, 0.01, and 0.001g/ml and cultured on Potato Dextrose Agar (PDA) medium at 25°C for seven days. After that, pigmented colonies of yeasts were isolated in pure cultures and apparently diagnosed [28].

Genetic diagnosis

The genetic diagnosis was performed in the genome extraction steps using the Presto Mini gDNA Yeast Kit (Geneaid; South Korea). ITS region was amplified using universal primers ITS1 and ITS4 using thermal Cycler (Bioneer Corporation; South Korea). The total volume of 25µl consisted of 3µl DNA Form, 1µl F. Primer, 1µl R. Primer, 12.5µl Mastermix, and 7.5µl Nuclease free water.

The PCR program was as follows: 94°C for 3min, followed by 35 cycles at 94°C for 45s, 52°C for 1min, 72°C for 1min, with a delay at 72°C for 7min.

PCR products were analyzed by 2% agarose gel electrophoresis alongside DNA Ladder (100 bp) after RedSafe™ staining. Sanger gene sequencing was performed at Macrogen (South Korea). A homology search was conducted using the Basic Local Alignment Search Tool (BLAST) program that is available at NCBI (online at https://www.ncbi.nlm.nih.gov), and the sequences saved at the Gene bank under Code No. LC472129.1 and LC463094.1.

Culturing the samples and extraction of pigments

Yeasts were cultured on Potato Dextrose Agar (PDA) and incubated at 25°C for five days. Then 6.5g of the grown yeast was collected using a sterilized blade and placed on a 500ml glass beaker containing 250ml of acetone and 2g of glass balls with a magnetic rod for 36 hours on a magnetic motor to destroy walls of cells and release carotenoids. After that, destroyed cells were rejected using a centrifugal device. The settlement was neglected, and the filtrate was placed on a separatory funnel, and a 50:50 mixture of ether and methanol with a small amount of NaCl was added. The separatory funnel was strongly stirred for two minutes to form two polar and non-polar layers. The upper layer contained ether and carotenoid, and the lower layer was ignored. The upper layer was evaporated using glass dishes, and the product was kept in opaque bottles [29].

Total purification

In this method, a glass column was used whose dimensions were 30x30mm. In the end, there was glass wool, the height of which was 2.5cm. After that, the column was filled with mesh emulsion, silica gel 100-200µ, and a mixture of ether and acetone (200:800, respectively). 0.25g of carotenoid was dissolved in 1mL of distilled water and then smoothly added to the surface of the mesh, silica gel, evenly. After that, the mixture was added to the column until the end of the separation process with some modifications [30]. The amount of runoff at the column was 1ml/20sec, and separated patterns were received and collected from the end of the column through 1ml test tubes. Then components of each one were selected using a thin-layer chromatography technique as they were tested and exposed to Iodine steam [31]. Similar products were collected together.

Beta-carotene diagnosis

Spectroscopy UV absorption

Spectroscopy UV absorption, the measurement of vision, and UT regions are important tools to specify pigments, including carotenoids. These spectra were registered in the vision and UT regions using Heliosαv4.60U.V-visible Spectrophotometer, England, in the regions ranging from 200 cm^{-1} to 600 cm^{-1} [32].

Infrared spectra (IR)

IR spectra were registered applying KBr discs technology in the regions ranged from 400 cm^{-1} to 4000 cm^{-1} using Fourier Transform Infrared Spectrophotometer (FTIR)-84005 (Shimadzu; Japan).

Antifungal activity of β-carotene

- Beta-carotene activity was investigated in four isolated yeasts: *Candida albicans* HAM25, *Candida dubliniensis*, *Candida tropicales* HAM13, and *Cutaneotrichosporon dermatis* Judy 4.

Yeasts were isolated from superficial fungal infections in the Fungal Research Laboratory, College of Science, University of Basra University, and kept in the gene bank with numbers LC722508.1, LC722507.1, LC7261991, and LC731319.1. Then they were activated in PDA medium at 35±2°C for 24 hours, and a suspension of grown fungi was prepared according to McFarland's second standard with a cell concentration of $6x10^6$ [33].

- The agar diffusion method was applied to evaluate the activity of β-carotene. 0.1ml of cell suspension (prepared in the first step) was diffused on a PDA medium. Three concentrations of β-carotene

including 0.01, 0.02, and 0.03mg/ml, and volume 300µl at the same concentrations and volumes of Nystatin as a standard compound and by three repetitions per each isolate with each concentration were used and then incubated at 37°C for 48h [34].

Toxicity

The toxicity of purified β-carotene was tested on two red blood cell isolates *in vitro* with a concentration of 0.03mg/ml, and a volume of 200µl per isolate was added to 800µl of blood separately. The tubes were then incubated at 37°C for 24h, with phosphate-buffered saline as the positive control and tap water as the negative control, according to the technique of Nair *et al*. [35], with some changes.

Findings

There was little difference between *R. diobovata* and *R. mucilaginosa* isolates, with filter weights of 0.27g for *R. diobovata* and 0.15g for *R. mucilaginosa*, while the total purification product for *R. diobovata* extraction was 0.2g. One spot of purified β-carotene collected from tube 58-121 with Relative Flow (RF) on thin-layer chromatography was about 0.93, while the product of total purification of the extract of *R. mucilaginosa* was 0.1g. Purified β-carotene from one spot of tube 58-112 of relative flow was equal to ca.0.92. Upon verifying the present of β-carotene, thin-layer chromatography techniques were applied, and measured RF was equal to ca. standard RF 0.95, using movable phase 800 petroleum ether: 200 acetone.

Analysis of produced β-carotene

The pigment demonstrated maximum absorbance at 438,386nm for *R. diobovata* and *R. mucilaginosa* (Figures 1 & 2).

In addition, FT-IR showed the types of pigments similar to the chromatogram that are well-known as standard carotenes (Figures 3 & 4).

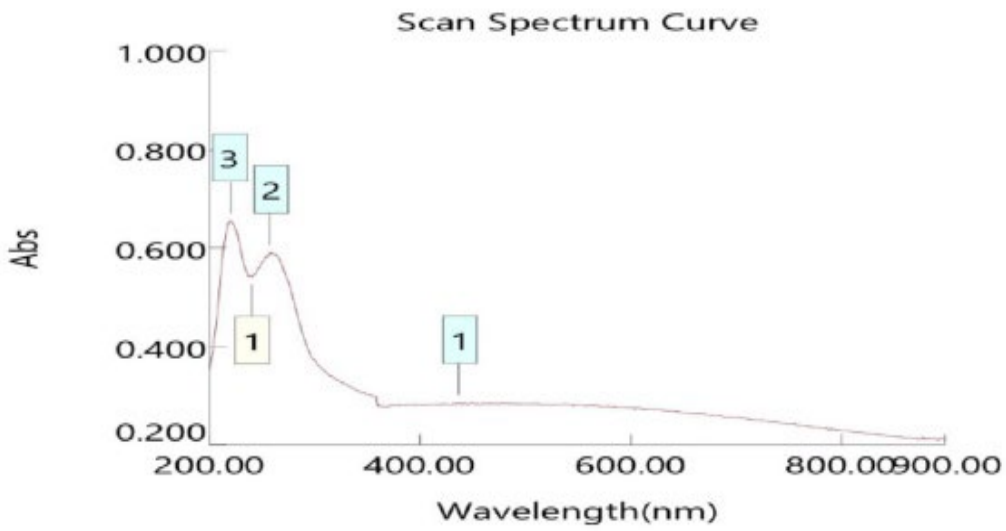


Figure 1. UV spectrum of β-carotene purified from *R. diobovata* isolate

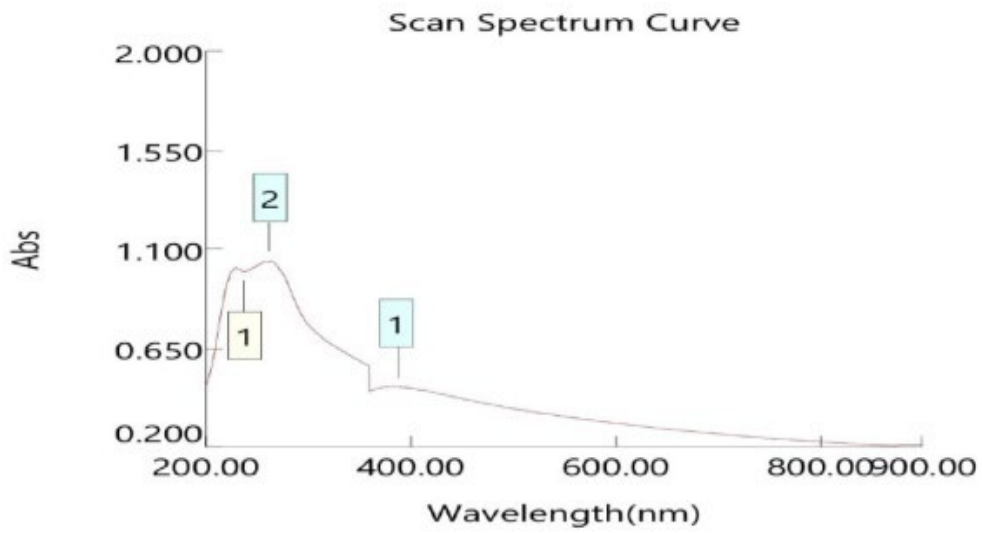


Figure 2. UV spectrum of β-carotene purified from *R. mucilaginosa* isolate

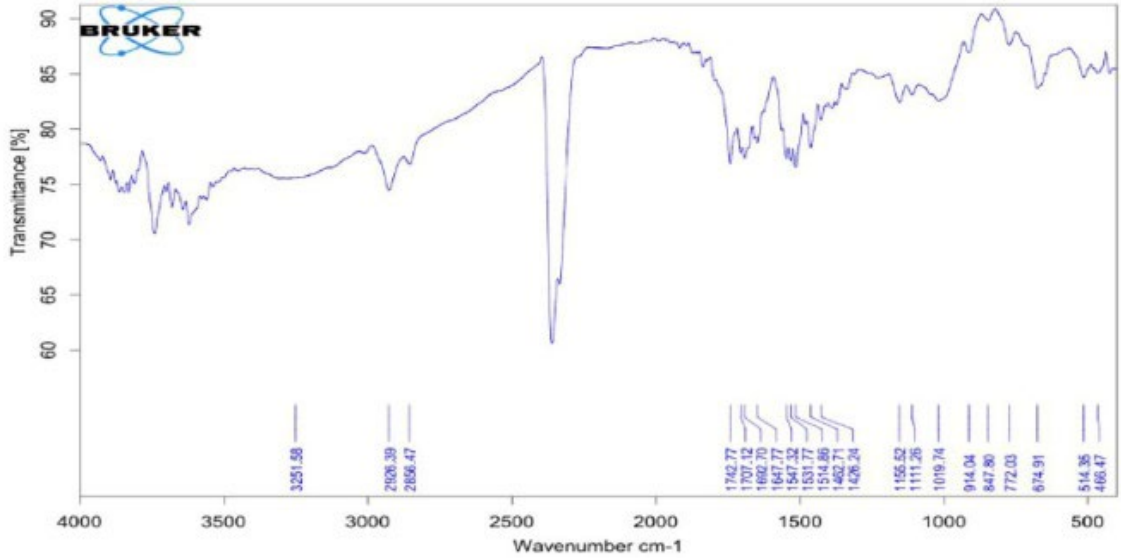


Figure 3. Infrared spectrum of β-carotene purified from *R. diobovata* isolate

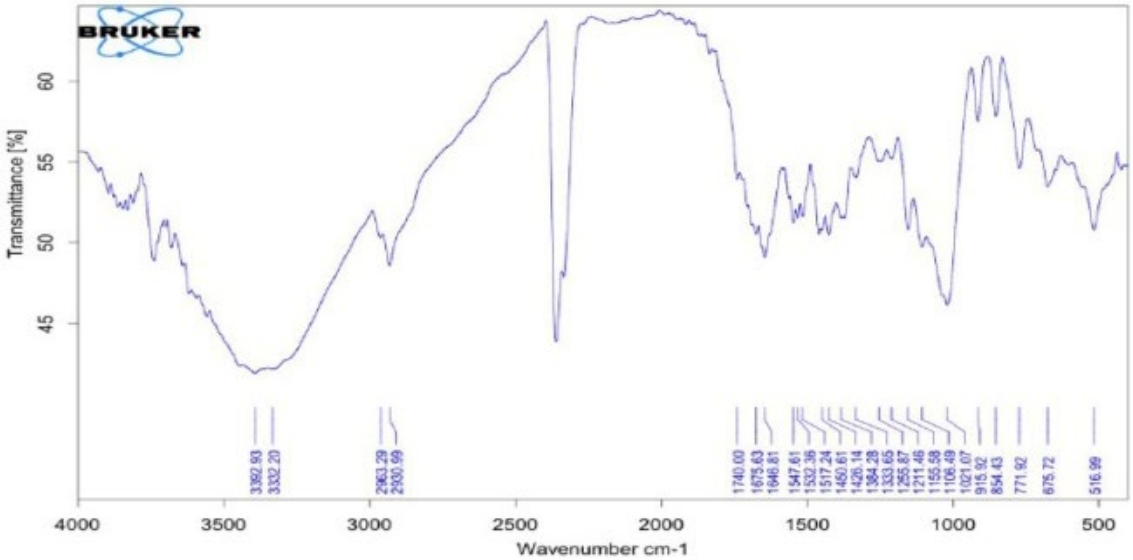


Figure 4. Infrared spectrum of β-carotene purified from *R. mucilaginosa* isolate

Table 1. Average inhibition diameters (mm) of β-carotene purified from *R. diobovata* (R1) against four yeast isolates at three different concentrations and compared with the standard compound Nystatin after 48h

Concentration (mg/ml)	***Candida albicans***	***Candida dubliniensis***	***Candida tropicalis***	***Cutaneotrichosporon dermatis***
β-Carotene (R1)				
0.01	0	0	0	0
0.02	27	25	28	22
0.03	28	29	27	23
Nystatin				
0.01	0	0	0	0
0.02	27	25	26	0
0.03	19	24	20	14

Table 2. Average inhibition diameters (mm) of β-carotene purified from *R. mucilaginosa* (R2) against four yeast isolates at three different concentrations, compared to the standard compound of Nystatin after 48h

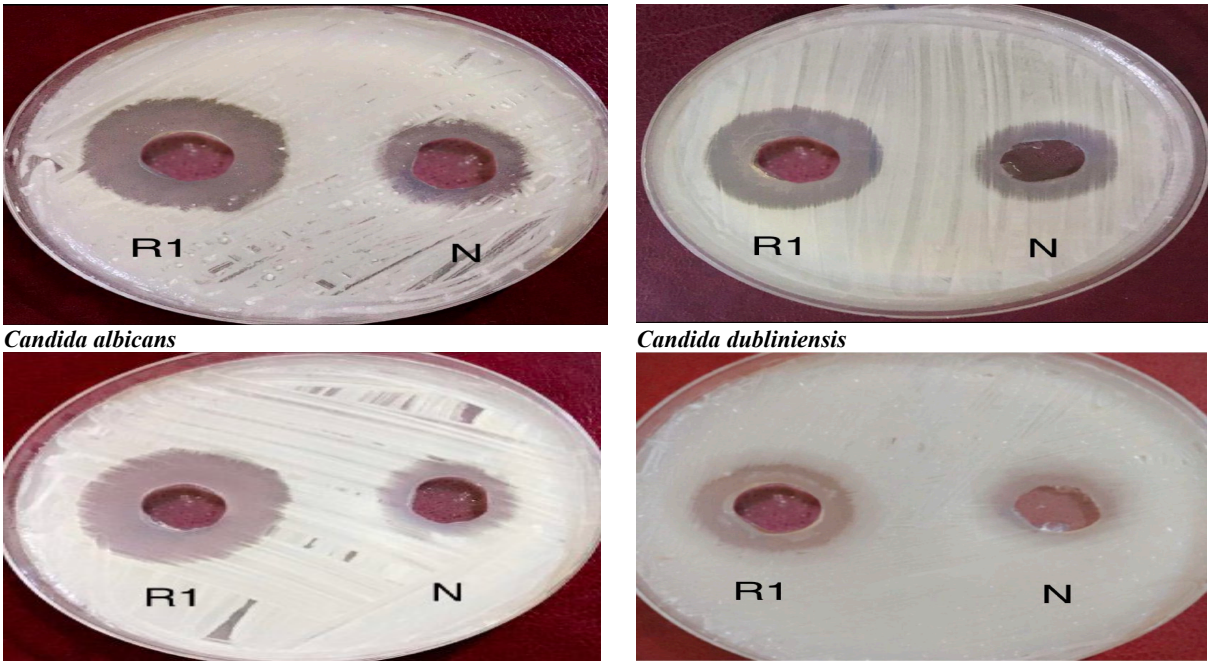
Concentration (mg/ml)	***Candida albicans***	***Candida dubliniensis***	***Candida tropicalis***	***Cutaneotrichosporon dermatis***
β-carotene (R2)				
0.01	29	27	24	15
0.02	30	26	29	24
0.03	30	27	29	24
Nystatin				
0.01	0	0	0	0
0.02	26	27	25	24
0.03	25	23	24	19

Beta-carotene activity

All tested isolates, including *Candida albicans* HAM25, *Candida dubliniensis*, *Candida tropicales* HAM13, and *Cutaneotrichosporon dermatis* Judy 4, showed resistance against β-carotene purified from *R. diobovata* (R1) and Nystatin at the concentration 0.01mg/ml. However, β-carotene and Nystatin showed activity against all isolates with concentrations of 0.02 and 0.03mg/ml. The purified compound from *R. mucilaginosa* (R2) showed activity against all isolates in three concentrations, according to concentration and type. The highest amount was related to *Candida albicans* HAM 25, and the lowest was related to *Cutaneotrichosporon dermatis* Judy 4. There was a significant difference between the antifungal activity of both purified β-carotene and Nystatin ($p<0.0001$; Tables 1 & 2; Figures 5 & 6).

Toxicity of β-carotene

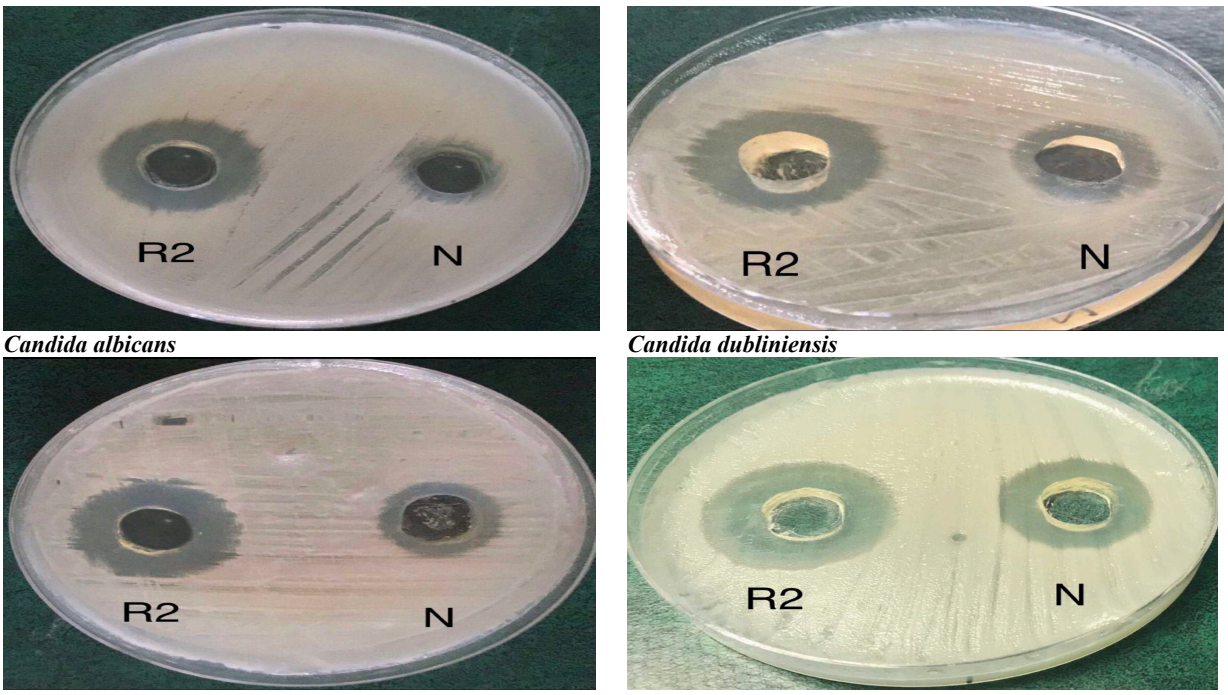
Purified β-carotene from two isolates showed no obvious changes or decay in blood cells *in vitro*.



Candida albicans ***Candida dubliniensis***

Candida tropicals ***Cutaneotrichosporon dermatis***

Figure 5. Antifungal activity of β-carotene purified from *R. diobovata* (R1) and nystatin (N) against *Candida albicans*, *C. dubliniensis*, *C. tropicalis*, and *Cutaneotrichosporon dermatis*



Candida albicans ***Candida dubliniensis***

Candida tropicals ***Cutaneotrichosporon dermatis***

Figure 6. Antifungal activity of β-carotene purified from *R. mucilaginosa* (R2) and nystatin (N) against *Candida albicans*, *C. dubliniensis*, *C. tropicalis*, and *Cutaneotrichosporon dermatis*

Discussion

Dermatomycoses are a common category of fungal infections affecting the skin and subcutaneous tissues. Recent advances in topical and systemic antifungal chemotherapy have provided us with satisfactory results in most superficial fungal infections, both clinically and mycologically. Despite the efficacy of antifungal agents, however, some dermatomycoses do not respond to our current standard regimens of treatment [36]. These infections have become recalcitrant to treatment which can be due to antifungal resistance [37].

Carotenoids are yellow, orange, and red pigments that are found in several animals, plants, and microorganisms [38]. Recent studies have shown that some of these pigments have important biological functions such as antibiotic, antifungal, and antitumor activities, and many of them have potential chemotherapeutic effects [39]. Carotenoid production from yeasts has been considered a safe alternative to chemically synthesized carotenoids since plant-based carotenoids are expensive and an irregular source for obtaining pigments [40].

This study aimed to extract and purify β-carotenoid from two types of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* and test its reactivity toward some yeasts isolated from dermatomycoses infection.

The results of the present study stated that there was little difference between *R. diobovata* and *R. mucilaginosa* isolates, with filter weights of 0.27g for *R. diobovata* and 0.15g for *R. mucilaginosa*, while the total purification product for *R. diobovata* extraction was 0.2g. One spot of purified β-carotene collected from tube 58-121 of RF on thin-layer chromatography was about 0.93, while the product of total purification of the extract of *R. mucilaginosa* was 0.1g. Purified β-carotene from one spot of tube 58-112 with relative flow was equal to ca.0.92. Upon verifying the present of β-carotene, thin-layer chromatography techniques were applied, and measured RF was equal to ca. standard RF 0.95, using movable phase 800 petroleum ether: 200 acetone.

This result complies with the findings of Latha & Jeebaratnam [31], who used a thin-layer chromatography technique and the same movable phase to obtain β-carotene from *R. glutinis* upon the same runoff rate of 0.92. Zeb & Murkovic [41] reported that petroleum ether, acetone, and Hexane are the main applied movable phases in the thin-layer chromatography technique, and it is proven that this mode is eligible to be the first choice to analysis carotenes in the biological samples.

The pigment demonstrated maximum absorbance at 438,386nm for *R. diobovata* and *R. mucilaginosa*. The maximum absorbance for the pigment is stated between 300-600nm, indicating the presence of carotenes [42]. Also, Madhukar stated that the maximum absorbance of β-carotene is 425-430nm and 470-489nm [43]. However, according to Finkel'shtein's study [44], most carotene pigments have maximum absorbance ranging between 375-505 nm.

According to the results of the present study, β-carotene of purified extracts had high activity against four yeast isolates, including *C. albicans* HAM25, C. *dubliniensis*, *C. tropicals* HAM13, and *Cutaneotrichosporon dermatis* Judy 4 on.

The activity of β-carotene has never been studied before except on *C. albicans* compared to echinocandins [45]. The results showed that with the increase in the concentration of β-carotene, its activity against all different isolates increases, so the combination of β-carotene has more antigenic activity than the standard antifungal nystatin, which may be the reason for its significant use.

Several studies reported that the carotenoids synthesized by yeasts of the Rhodotorula and Sporobolomyces genera show strong anti-microbial activity [46-49].

Manimala *et al*. [50] studied the activity of β-carotene produced from *R. mucilaginosa* YP 197 Yeast and stated that β-carotene shows anti-bacteria activity more than standard Chloramphenicol, and the most activity is appeared against *Bacillus subtills* and *Staphylococcus aureus*.

Manimala & Murugesan [46] reported similar action in carotene pigment extracted from Sporobolomyces sp. isolated from natural sources and stated that negative gram bacteria has higher level of resistance against β-carotene. Also, Madhukar [43] reported the antifungal activity of β-carotene pigment against some pathological fungi attack plants such as *Aspergillus niger* NCIM 1025, *Penicillium chrusogenum* NCIM 709, and *Fusarium oxysposrum* NCIM 1281, which its activity was at two concentrations of 50µl and 100µl.

The importance of β-carotene in inhibiting the growth of some pathological microorganisms, such as bacteria and fungi, is because it dissolves in the lipids of the protoplasm membrane and then increases its penetration through the membrane and stops the growth or kills the fungi.

Yoo *et al*. showed the antibacterial activity of glucosidal carotenoids from the yeast *R. mucilaginosa* AY-01 toward antibiotic-resistant bacteria isolated from the porcine semen [51]. In addition, the carotenoid contents of *Rhodotorula glutinis* M29 strain (1.07 mg L−1) showed antibacterial activity against a range of bacteria, e.g., *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Salmonella enteritidis*, and *Escherichia coli* at 10^3CFU/mL [47].

Keceli *et al*. concluded that carotenoids extracted from some strains of *Rhodotorula glutinis* (M21, 22, 24, 28, 29, 30, 31, 35, 37, 39, 40, 42, and 47) can effectively be used to obtain both antioxidant and antibacterial effect. These strains can be isolated best from tree leaves, parsley, and carrots, and these

strains produce high-quality carotenoids to be used as preservatives. The antioxidant or antibacterial effect of carotenoids significantly depends on source of isolation and carotenoid composition rather than carotenoid content [47].

Finally, the present study investigated the cellular toxicity of β-carotene. The cellular toxicity test is the most practical test used for bioassay and evaluation of blood cells, which observes the apparent effects of cells in the laboratory. In the present study, the purified β-carotene from two isolates showed no obvious changes or decay in blood cells *in vitro*, which is consistent with the findings of Burton *et al*. [52].

Conclusion

Both isolates of *Rhodotorula diobovata* and *Rhodotorula mucilaginosa* produce β-carotene, and the preference quantitatively is to isolate *R. diobovata* first. The activity of β-carotene against all tested yeast isolates are higher than the antifungal Nystatin. The non-toxicity of β-carotene was proven in the blood decay test *in vitro*.

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