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Research Article

Antimicrobial activity of *Micrococcus luteus* Cartenoid pigment

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Abstract

Cartenoids are group of pigments, with enormous types different structurally and functionally, have colors range from red to yellow found in a wide variety of plants, fungi, algae and bacteria. The animals took from food because they cannot make it, on contrary, the plants and microbes produce them due to subjection to environment.

The aim of the study is to isolate and characterize the cartenoid pigment from *Micrococcus luteus*. The pigment extraction was done by acetone, and then was characterized with UltraViolet-Visible spectroscopy (UV–Vis) and Fourier Transform Infrared (FTIR) spectroscopy. Then, it was tested for antibacterial activity against five different bacterial isolates and antifungal activity tests against six different fungal isolates by well diffusion method. The results found that, the extracted pigment having antibacterial activity and antifungal activity and having the ability to absorb UVA rays within the range of 300-500 nm. There was no significant difference in antimicrobial effect of pigment, even when the extraction and isolation were done by two culture mediums (Nutrient Broth and Luria Bertani Broth). There were considerable inhibition percentages of adhesion after subjection to Cartenoid pigment ranged between (5.71, 23.84) % for *Klebsiella spp.* and *Pseudomonas aeruginosa* respectively and all the 11 isolate changed from Biofilm producer to non-producer.

The isolated compound can be used against different bacterial and fungal infections. So they had a great future in medicine, cosmetics and as a sun protecting agent.

Keywords: *Micrococcus luteus*, Cartenoid piment and Biofilm.

الخلاصة

الكاروتينات هي مجموعه صبغات بعده انواع مختلفه تركيبيا ووظيفيا وبألوان مختلفه متضمنه الاحمر الى الاصفر موجوده في البكتريا، الطحالب ، الفطريات والنباتات. الكاروتينات منتشره بشكل كبير في الطبيعه وتنتج من النباتات والاحياء المجهريه كنتيجه لتأثير العوامل البيئيه، بينما الحيوانات لا يمكنها ان تصنعها لذا لا بد ان تحصل عليها من الغذاء.

هدف الدراسه كان عزل وتشخيص صبغه الكاروتينات الفعاله بايولوجيا من بكتريا*Micrococcus luteus*.استخلصت الصبغه بماده الاسيتون وشخصت باستخدام المطيافUltra Violet-visible spectroscopy وFourier Transform Infra-Red spectroscopy. تم التحري عن الفعاليه الضد بكتيريه والفعاليه الضد فطريه بعد تشخيص صبغه الكاروتينات بأستخدام طريقه الانتشار بالحفر.

اظهرت النتائج ان الصبغه لها فعاليه ضد بكتيريه وفعاليه ضد فطريه ولها القابليه على امتصاص الاشعه الفوق بنفسجيه ضمن المدى (300-500) نانوميتر. تم الاستخلاص والعزل للصبغه بأستخدام وسطين مختلفين (هما السائل المغذي Nutrient Broth ووسطLuria Bertani Broth)، الا انه لم يظهر اختلافا معنويا في فعاليه الصبغه الضد ميكروبيه. انخفضت بشكل ملحوظ نسب تثبيط التصاق العزلات البكتيريه بعد التعرض للصبغه اذ تراوحت بين (5.71, 23.84) لبكتريا Klebsiella spp. و*Pseudomonas aeruginosa*. استخدمت احد عشر عزله بكتيريه منتجه للغشاء الحيوي في الدراسه ، اذ تحولت الى غير منتجه بعد التعرض للصبغه. الصبغه الفعاله بايولوجيا المعزوله في هذه الدراسه يمكن ان تستخدم ضد اصابات بكتيريه وفطريه مختلفه ، وبذلك فأن لها مستقبل كبير في الطب وصناعات المكياج وكعامل واقي من التعرض لاشعه الشمس المضره.

Introduction

The genus *Micrococcus* is Gram-positive cocci, nonspore former, aerobic and rich in carotenoid pigments, which are known to have radio-protective and bioactive characters. The carotenoid pigments produced by *Micrococcus* are benefit for food industry, dye industries, pharmaceutical and cosmetic [1][2], the information about pigments and their different biological actions is lacking.

Pigments are bioactive secondary metabolite in microbes. Therefore, several bio prospecting

projects looking for bioactive molecules have been targeted to pigmented bacteria [3].

Demand for color from natural source has increased now-a-days, because people became very much aware about the toxicity of synthetic food color [4]. Microorganisms, seed roots, vegeTables and fruits were a source of "bio-colors" [5], which are safe and convent for human use because its biological origin. Seven hundred and fifteen structurally distinct carotenoids pigments are identified [6], and still new structures recorded now-a-days.

Because of carotenoids effect on UV radiation of sun and reactive oxygen species, they are a sun protector factor by light absorption at 350-500 nm [7].

The aim of this study was to isolate the carotenoid pigments from bacteria *Micrococcus luteus*, and investigate their antimicrobial activities against collection of bacterial and fungal isolates. The effect of pigment on adhesion and biofilm production of bacterial isolates was also studied.

Materials and Methods

Micrococcus luteus bacteria were taken from Mustansiriyah University – College of Science - laboratories of Biology Department. The bacteria were plated on nutrient agar medium and incubated at 37 ºC for one week. After incubation, the yellow colonies were picked out, purified by repeated streaking. The pure cultures of the bacterial colonies were inoculated into nutrient agar slants and stored at 4º C for further studies.

Extraction of Cartenoid Pigment from Micrococcus luteus

The extraction and isolation of pigments from *M. luteus* were carried out by two methods using two different culture mediums:

First, by follow the procedure of [8]. Briefly, 10ml of bacteria was transferred into flask containing 300ml of nutrient broth, and then incubated in rotary shaker up to 5d. After incubation, the cultures were centrifuged at 10, 000 rpm for 15 min at 4°C, and the cell pellets were collected.

The collected pellets were extracted with cold methanol, and then separated from the cells by centrifugation at 10, 000 rpm for 15 min at 4°C.

Second, the bacterial isolates were inoculated in Luria bertani broth and incubated in rotating incubator at 37 ºC at 120 rpm for 3d. The cultured media was centrifuged at 7500 rpm for 20 min. The supernatant was discarded and pellets were extracted using acetone in the ratio of (1:5) until the pellets become colorless. Extraction was done at dark, avoid the direct light exposure. Extracted pigment was covered with aluminum foil and stored in refrigerator for further studies [9].

Characterization of the Cartenoid Pigment

UV-Vis Spectrophotometry

Absorption spectra of pigment were taken using a UV-Vis biospectrophotometer [10].

FTIR spectroscopy

The purified pigment was characterized by FTIR spectroscopy, as described by [11]. The relative intensity of transmitted light was measured against the wavelength of absorption in the region 400-4000 cm-1.

Antimicrobial Activities of Carotenoid Pigment

The human pathogenic bacteria including (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) were obtained from College of Science, Mustansiriyah University laboratories. All the tested bacteria were maintained on Nutrient agar and were used for the antibacterial assay. Mueller Hinton Agar plates were prepared, wells were made in each plates using Pasteur pipette. Uniformly distribute the respective organisms on agar plates and add 50 µl of pigment extract to the wells. After 24 hr of incubation at 37ºC, zone of inhibition was measured in millimeter [12].

The fungal isolates include (*Aspergillus Niger, Aspergillus terreus, Alternaria sp., Cladosporium sp., Curvularia* and *Penicillium certum*) were obtained from College of Science, Mustansiriyah University laboratories. All tested fungi were maintained on Potato Dextrose Agar and were used for the antifungal assay by well diffusion method. Equally distribute the particular organism on each plate with a sterile cotton swab, add 50 µl pigment extract were added in to the well and incubate all plates for 7-14 days at 25 ºC [12].

Determination of Minimal Inhibitory Concentration (MIC)

The broth microdilution method was used to determine the MIC of carotenoid pigments of *M. luteus*, following the procedure of [13]. Briefly, 200µL of desired different concentrations of carotenoid pigments in Muller Hinton Broth (0.78-100 mg/ml) was added separately to the wells of a sterile 96-well microtiter plate (MTP) and inoculated with 15 µl of a microbial suspension containing 10^8 CFU/ml of test bacteria (*Staphylococcus aureus, Acinetobacter spp., Klebsiella spp., Enterobacter spp., Serratia spp.* and *Pseudomonas aeruginosa*), and incubated at 37°C for 24h. The MIC was defined as the lowest concentration of the pigment required for inhibiting the growth of bacteria.

Anti-Adhesion Assay

It was performed as described by [14]. Typically, 25µl of an overnight culture of tested bacteria (2 isolates of *Staphylococcus aureus*, 2 isolates of *Acintobacter spp*., 4 isolates of *Klebsiella spp*., 4 isolates of *Enterobacter spp.*, 4 isolates of *Serratia spp*. and 4 isolates of *Pseudomonas aeruginosa*) in Nutrient broth was used to inoculate at least 40 wells of MTP containing 175µl of sterile Nutrient Broth with and without 175µl of Cartenoid pigment (1mg/ml). The covered microtiter dish was sealed with parafilm during incubation at 37°C for 24h. Cultures were removed and the wells were rinsed with distilled water. After drying for 15 min, 200µl of crystal violet (1%) was added to the wells for 20 min. The stain were rinsed several times with distilled water, allowed to dry at room temperature for 15 min, and extracted with 200µl of 95% ethanol. The optical densities (OD) were estimated using ELISA reader spectrophotometer and inhibition percentages were calculated as mentioned below.

$$\text{Inhibition Percentages (\%)} = \frac{\text{ODc}-\text{ODt}}{\text{ODc}} \times 100 \quad (1)$$

ODc : Optical Density of control
ODt : Optical Density of test

Biofilm Formation and Quantification

Biofilm production of all the isolates was quantitatively investigated using the method of adherence to polystyrene MTP proposed by [15]. All the bacterial isolates were cultivated on Brain Heart Infusion Broth one at a time and incubated to initiate growth for 18 hours to reach an OD≤1 at 600 nm. The cultures were then diluted (1: 100) with fresh medium, then 200 µl were transferred to sterile 96-well polystyrene MTP with and without 200 µl of Cartenoid pigment (1mg/ml). After incubation for 24 hours at 37ºC, the cultures were discarded and MTP were washed with distilled water to eliminate the unattached cells. Attached cells were then fixed at 60ºC for 1 hour and stained with 1% crystal violet solution. Excess stain was removed by successive washings. The crystal violet in each well was solubilized with 200 µl of 96% ethanol, and microplate reader was used to measure the absorbance at 540 nm [16]. Negative control wells contained sterile broth. The interpretation of biofilm production was done according to the criteria of [17].

Results and Discussion

M. luteus isolates on nutrient agar plates showed yellowish colonies after incubation at 37ºC for 24 hr. It grew well in the nutrient medium, both on agar plates and in broth. By Gram-staining, it was cocci arranged in tetrads and in irregular tetrads clumps. So, it was identified as Gram-positive coccus non-sporeformer [18]. Characterization of the Cartenoid pigment was done by measuring absorption spectra by UV-Vis spectrophotometry as shown in Figure 1, and FTIR spectroscopy as shown in Figure 2. Pigment extract characterization with UV Vis spectroscopy showed the maximum absorbance at 350 nm, in UVA region.

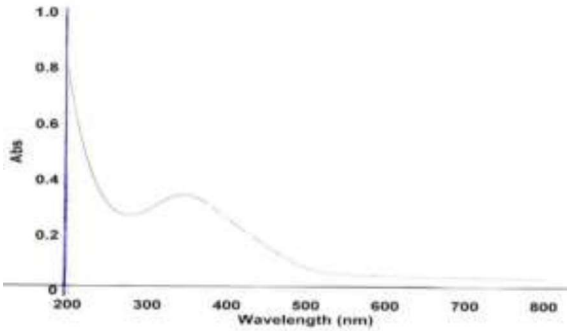
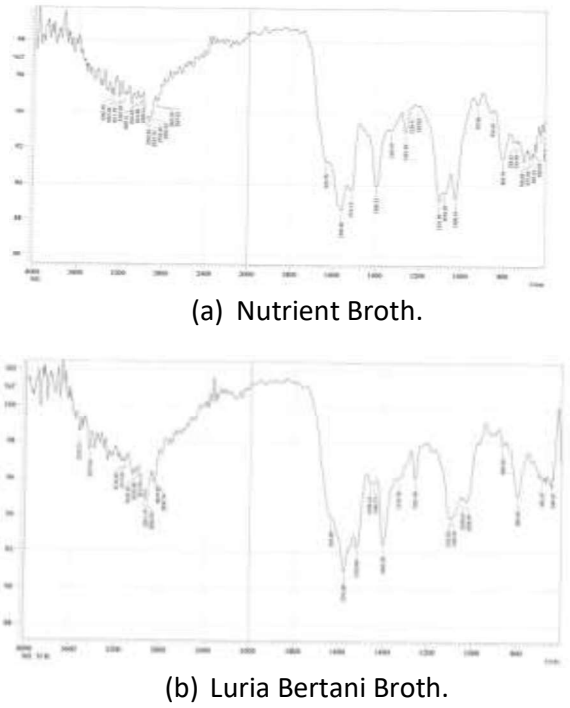


Figure 1: UV-Vis absorption spectra of carotenoid pigments.

Pigment extracted in different solvents, could be characterized by different techniques like gel permeation chromatography, thin layer chromatography, HPLC, UV-Vis and FTIR [19].



(a) Nutrient Broth.

(b) Luria Bertani Broth.

Figure 2: FTIR for Cartenoid pigment prepared in: (a) Nutrient Broth, and (b) Luria Bertani Broth.

UV-Vis absorption spectra of carotenoid pigments are of huge importance, since they aid a great deal in determining the structure of carotenoids [20].

FTIR absorption of the yellow pigment showed strong and broad peaks. The peaks correspond to different functional groups. FTIR helps to determine the functional groups in the sample, and different functional groups absorb characteristic frequencies of IR radiations differently [21].

From all these results, it may be concluded that the yellow color pigment isolated from *Micrococcus* sp, able to absorb UV rays and is a sun protecting pigment which can be used with sun protection cream after processing, instead of synthetic sun protection creams.

Although the extraction and isolation of pigments from *M. luteus* were carried out by two methods using two different culture medium (Nutrient Broth and Luria Bertani Broth), There were no significant difference of Cartenoid pigment antimicrobial effect as shown in Table 1. The Cartenoid pigment showed a promising effect on both the bacterial and fungal used isolates. For MIC results, it was 25 mg/ml for *Klebsiella spp.*, 50 mg/ml for *P. aeruginosa* and 6.25 mg/ml for *Enterobacter spp.*, 3.12 mg/ml for *Staphylococcus spp.*, 1.56 mg/ml for *Acintobacter spp.* and 0.78 mg/ml for *Serratia spp.* .

Table 1: Inhibtion zones in millimeter (by cm) against some bacterial and fungal isolates of Cartenoidpigment.

Microbial isolate	Cartenoid pigment Prepared in Nutrient Broth	Cartenoid pigmentPrepared in Luria Bertani Broth	Negative Control
Escherichia coli	-	1.1	-
Bacillus subtilis	-	-	-
Staph. aureus	1.1	-	-
Klebsiella spp.	1	0.4	-
Ps. aeruginosa	1.3	1	-
Alternaria spp.	1.5	-	-
Aspergillus niger	0.2	0.3	-
Cladosporium	0.2	0.3	-
Aspergillus terreus	-	-	-
Penicillium certum	0.2	2.5	-

The crude pigment from *M. luteus* was tested by [22], resulted high antibacterial effect with *Staphylococcus sp., Klebsiella sp., Pseudomonas sp*. isolates at 50, 100µl compared to *Escherichia sp*. isolates, That's was compatible with the study result.

They also found that Cartenoid pigment caused inhibition on Bacteria (*Ps. aeruginosa* by 12mm, *Klebsiella* by 9mm, *E.coli* by 14mm) and on fungi (*Aspergillus niger* by 17mm and *Penicillium* sp. by 19mm) [9]. This was compatible with the study result.

These results are of great importance, particularly for *S. aureus*, which is well known for being resistant to a number of antibiotics [23]. Therapy by usual antibiotics led to develop resistant strains and development of different sensitivity patterns, which in turn need to new effective therapy [24]. Cartenoids of *M. luteus*

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could be new agents for treating diseases must be investigated. There was significant lowering in OD measurements of adhesion of all bacterial isolates after subjection to Cartenoid pigment. Inhibition ranged between (5.71, 23.84) % for *Klebsiella* and *P. aeruginosa* respectively as shown in Figure 3.

There were 11 isolate out of 20 isolate biofilm producer. After subjection to cartenoid pigment all the 11 isolate changed from biofilm producer to non-producer.

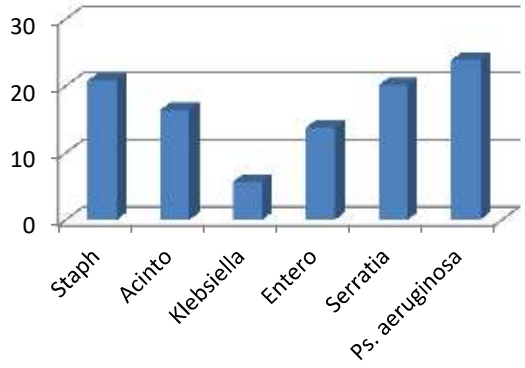

Figure 3: Inhibition percentages of cartenoid pigment on bacterial isolates.

Biofilm is a survival strategy for bacteria and fungi to adapt to their surroundings, especially in the host environment. Microbial cells in biofilm became tolerant to antibiotics and the immune responses, which increases the problems for the clinical treatment of biofilm infection (25). These natural alternatives such as cartenoids pigments are so useful for many different bacterial and fungal infections by their effects on the virulence factors such as biofilm, adhesion and etc., especially with the promising effect noticed in this study.

Conclusion

The pigment isolated from *Micrococcus luteus* can absorb UV radiation and may be used in sunscreen cosmetics. The pigment is a carotenoid, which can use as a vitamin source and also a natural dye.

The crude pigment produced from the strain *Micrococcus luteus* was found to contain antimicrobial activity. Further, purification may give better effect.

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