Extraction of Staphyloxanthin from *Staphylococcus aureus* Isolated from Clinical Sources to Determine its Antibacterial Activity Against other Bacteria

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Abstract

Fourty three isolates (20.7%) characterized as *Staphylococcus aureus* , were isolated from 207 different clinical sources (blood , nose, , wound , urine , vaginal, ear and eye) in different percentages (30.23, 18.60, 16.28, 13.95, 15.15, 6.96 and 2.33 %), respectively. The staphyloxanthin (STX) production of *S. aureus* isolate was estimated 72.1% .The optimal conditions for pigment production by *S. aureus* AE_{36} , were detected and was noticed that the milk agar medium revealed the highest production of pigment which was estimated to be 165.21unit/cell, at pH 8 for 72 hr at 37^0C. The Staphyloxanthin pigment was extracted using methanol and was purified partially by organic solvents and Thin Layer Chromatography (TLC). The results revealed three peaks with a highest peak at 450 nm .No antibacterial activity of STX was detected against the bacteria used in this study (*Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Klebsiella* spp , *Proteus* spp. *Pseudomonas fluorescens* , *Pseudomonas putida* , *Staphylococcus aureus*).

Keywords: *Staphylococcus aureus* , Staphyloxanthin ,

استخلاص صبغة Staphyloxanthin من بكتريا Staphylococcus aureus المعزوله من مصادر سريريه و بيان تأثيرها على ممرضات بكتيرية اخرى

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الخلاصة

تم الحصول على 43 عزله بكتيرية (20,7%) تعود الى بكتريا *Staphylococcus aureus* من مجموع 207 عينة سريريه مختلفة شملت (الدم ، الأنف ، الجروح، الإدرار، المهبل، الاذن و العين) و بنسب تواجد مختلفة بلغت (30.23 ، 18.60 ، 16.28 ، 13.95 ، 15.15 ، 6.96 و 2.33) % على التوالي. اختبرت قابلية العزلات على انتاج صبغة الستافيلوزانثين في اوساط زرعية مختلفة وأظهرت النتائج ان نسبه العنقوديات الذهبية المنتجه لصبغة الستافيلوزانثين كانت 72,1% على الوسط الزرعي الامثل اكار الحليب. . استخلصت الصبغة باستخدام عدة مذيبات كان الامثل بالاستخلاص الميثانول ثم نقيت جزئيا باستخدام تقنية كروماتوغرافيا الطبقة الرقيقة وبينت النتائج وجود ثلاثة قمم للصبغة بينما كانت اعلى قمة لها عند 450 نانوميتر .حددت الظروف المثلى لانتاج الصبغة من اللعزلة المحلية المنتخبة AE_{36} كانت اعلى انتاجيه لها على اكار الحليب 165.21 وحده / خليه عند الاس الهيدروجيني 8 ولمده حضانة 72 ساعة في

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درجة حرارة 37^{0}م . اختبرت فعالية صبغه الستافيلوزانثين ضد انواع مختلفة من البكتيريا (*Staphylococcus epidermidis*, *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., *Escherichia coli, Klebsiella* spp , *Proteus* spp. *Pseudomonas fluorescens* , *Pseudomonas putidae* , *Staphylococcus aureus*). و تبين ان ليس لها اي تاثير ضد اي نوع من الانواع البكترية المذكورة اعلاه .

Introduction

The pathogen *Staphylococcus aureus* is a gram-positive, gold-colored colony and all available β-lactam antibiotics [1] . The yellow-to-orange colony color of *S. aureus* is one of the classical criteria for identification of this species.[2] Staphyloxanthin is membrane-bound carotenoid which plays a role in the environmental fitness of *S. aureus* [3 ,4]. Membrane pigments have also been hypothesized to be virulence factors in *S. aureus*, potentially by detoxifying reactive oxygen species produced by phagocytes [5]. Carotenoids may also stabilize the *S. aureus* membrane during infection and pathogenesis [6].

Staphyloxanthin is a typical secondary metabolite [7] It is not necessary for the growth and reproduction of *S. aureus* but might serve a role in survival in infected hosts and in combating the immune system, staphyloxanthin is mainly producted in stationary phase, it's chemical formula($C_{51}H_{78}O_8$). Staphyloxanthin is a neutral molecule [1,8,9] and light had no effect on it's synthesis [10]. Consequently, the aim of the present study was to detect the role of staphyloxanthin pigment production from *S. aureus* isolates from different clinical sources as anti bacterial agent against some pathogenic bacteria used in this study.

Materials and methods

Bacterial characterization

The colonies appeared on nutrient agar , blood agar and mannitol salt agar were selected for further diagnostic tests. Diagnosis of *S. aureus*. was determined according to Bergey's manual of systematic bacteriology [11].

These characteristics include; colonial morphology ,size of colony, color and the effect on the media such as blood hemolysis, pigments appear on Milk agar and ability to ferment mannito [11] and examination of shape using gram-stain reaction and arrangement of cells . Biochemical tests and api staph system were used to identify the bacteria.

Detecting the ability of *S.aureus* isolates to produce staphyloxanthin

In order to identify the ability of *S.aureus* isolates for the staphyloxanthin production , 10 ml of Brain-Heart Infusion Broth (BHIB)was inoculated with 100µl of *S.aureus* isolate (43 isolates) and incubated at 37°C for 18 hour in order to get 10^8 cell\ml (Bacterial growth was determined by measuring the absorbency at 620 nm). A volume of 100 µl of the inoculum from each isolate was streaked on different culture media including: Milk agar medium ,Peanut seeds medium , Sunflower seeds medium, Sesame seed medium , Trypticase yeast medium, Carotinoid expression medium and Trypticase soya medium incubated at 37°C for two days and then incubated at 20°C for two days . Appearance of growth with pigment (orange ,yellow) indicates a positive result [12] .

Determination of optimal conditions for staphyloxanthin production

The effect of some factors on pigment production were studied to determine the optimum conditions for the production :

- Effect of medium composition

A volume of 100µl of bacterial inoculum (10^5 cell/ml) was cultured on different prepared media and in ready made media .

- Determination of optimum temperature for staphyloxanthin production

A volume of 100µl of bacterial inoculum (10^5 cell/ml) was cultured at different temperatures (20, 25, 28, 37, 40^{0}C)for 24 hr .

- Determination of optimum pH for pigment production

A volume of 100µl of bacterial inoculum (10^5 cell/ml) was cultured at different pH values (5, 6, 7, 8, 9) using NaOH and HCL .

- Determination of optimum incubation time for pigment production

A volume of 100µl of bacterial inoculum (10^5 cell/ml) was cultured and incubated at 37^0C for different times (18, 24, 36, 48, 72 hr).

- Effect of aeration in staphyloxanthin production

A volume of 100µl of bacterial inoculum (10^5cell/ml)was cultured in different volumes of milk broth (50, 100 150 ml).

Extraction and purification of staphyloxanthin pigment

The pigment of *Staphylococcus aureus* (STX) was extracted by using methanol [13],as follows:

Bacterial cells were recovered from the growth on milk agar plate for 72hr at 37°C .Agar surfaces were rinsed with sterile double distilled water (each rinse with 3 ml). Then the bacterial cells were centrifuged at 6000rpm for 15 min .The supernatant was discarded and the pellet was resuspended with double distilled water and then centrifuged again at 6000 rpm for 15 min. Staphyloxanthin extraction from the pellet containing the bacterial cells was collected.

The pellet was mixed with 8 ml of 99.9% methanol wrapped with aluminum foil to prevent exposure to light.The packed cells were resuspended in 3 ml of methanol, held at 55°C in water bath for 5 min and cooled for 10 min , and then, the extract was collected by centrifugation (6000 rpm for 15 min). The extraction was repeated twice, until no further pigment could be extracted. Carotenoids were estimated quantitatively by measuring the absorbance of the solution at 450 nm.

Then extracted pigment was partial purified by Thin-layer chromatography (TLC). Solvent systems was used . The TLC plates were spotted with staphyloxanthin extracts and developed with the following:

benzene-methanol-acetic acid 87:11:2 (vol/vol/vol) [14].

The R_f value was calculated according to the following equation

$$R_f = \frac{\text{Distance of spot sample movement}}{\text{Distance of spot solvent movement}} \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

After developing, the spots from thin-layer plate were recovered by scraping the appropriate portion of silica gel into a tube and eluting with methanol [14]. The quantity of the pigment was determined using the equation [15].

$$\text{Total carotenoids unit/cell} = \frac{V\,(A - 0.0051)}{0.175W} \quad \ldots\ldots\ldots\ldots.. (2)$$

Where as:-

- A : Is the absorbance value of the diluted staphyloxanthin extraction at 450nm.
- V: Is the final volume of the extract staphyloxanthin .
- W(g): Is the weight of the dried powder of staphyloxanthin.
- 0.175: Is the extraction coefficient of carotenoids

But the concentration of the pigment was determined using the equation:-

$$\text{Concentration (g/ ml)} = \frac{W}{V} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots.(3)$$

W: Weight of the staphyloxant ng the appropriate portion of silica gel.

V: Volume of methanol will added

Statiscal analysis

The optimization of staphyloxanthin production were statistically analyzed by one way analysis of variance (ANOVA) [16].

Staphyloxanthin as an antibacterial agent

The activity of staphyloxanthin as antibacterial agent was tested by the well-diffusion [17] two replicates of each plate as follows:

The Mueller-Hinton Agar (MHA) plates were inoculated with 10^8cell/ml of indicator isolates (*Pseudomonas aeruginosa*, *Pseudomonas putidae*, *Pseudomonas fluorescence*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella* spp, *Salmonella* spp, *Shigella* spp., *Proteus* spp.)(obtained from department of biotechnology / university of Baghdad). Wells were prepared in the plates with 6 mm sterile cork borer. The wells for each culture of indicator bacteria were filled with 100 µl of staphyloxanthin solutions extracted from isolate AE_{36} (final concentration 0.2 g\ml by diluted 0.4 gm staphyloxanthin partial purified powder with 2 ml methanol)

The plates were incubated at 37^0C for 24 hr. Inhibition was detected by a zone of clearing around the partial purified staphyloxanthin extract.

Results and Discussion

The results of the biochemical test table-1 were compared with the characteristics of *S. aureus* documented by Bergey's Manual of Systematic Bacteriology [11].

Table 1- The biochemical tests of *Staphylococcus aureus*

Test	Result
Oxidase	-
Catalase	+
Hemolysis	β and α
Coagulase	+
Growth on NaCl agar:10%	+
Deoxyribonuclease (DNase test)	+
Protease	+
Urease	+ W
Gelatin liquefaction	+
Mannitol fermentation	+
Mannose fermentation	+
Melebioze fermentation	-
Raffinose fermentation	-
Sucrose fermentation	+
Trehalose fermentation	+
Xylose fermentation	-
Arabinose	-

(+) positive test , (-) negative test , + w (positive to weak reaction)

Table-2 shows the presence of *S. aureus* according to the site of infection . The results indicated that the percentage of *S.aureus* was 20.7% from the total samples (207)) in this study .Nasal infection (40%) was the most accessible site for *S. aureus* and this agrees with the results of [18] who found that the percentage of *S. aureus* in nasal infection was (39.1%). This may be due to the high distribution of this organism around the hospital environment [19]. In addition, our results are in agreement with the results obtained by other studies [20-22]

Furthermore, the present study revealed that the presence of *S. aureus* in eye samples (3.7%) was less than in blood(37.14%), nose(40%), wounds (21.87%), urine(19.35%), vagina(15.15%) and ear infections(10.3%) .These results are compatible with the results reported by [23] who mentioned that the presences of S. aureus in eye samples were less than in other samples.

The differences in the percentage of infection according to the site obtained in the present study may be due to the fact that these samples were not taken from the same site. However, these results are still agreed with those that says *S. aureus* is an opportunistic pathogen that causes human infections and can be isolated from different sites of infections [24]. Whereas the percentage of infection with *S.*

aureus obtained in other studies were 21.87% [25], 30.1% [26] , 18% [27] and 3.01% [28] results. There were significant differences ($p< 0.05$).

Table 2 - Frequency of *S. aureus* according to the site of infection

Type of samples	Total	No. of isolates	Percentage % *	Percentage %**	Percentage %***
Blood	35	13	37.14%	30.23	6.28
Nose swab	20	8	40%	18.60	3.86
Wounds swab	32	7	21.87%	16.28	3.38
Urine	31	6	19.35%	13.95	2.89
Vaginal swab	33	5	15.15%	15.15	2.42
Ear swab	29	3	10.3%	6.98	1.45
Eyes swab	27	1	3.70%	2.33	0.48
Total	207	43		100%	

*Percentage of *S. aureus* according to source ** Percentage of *S. aureus* according to total no. of *S. aureus* isolate *** Percentage of *S. aureus* according to total no. of total sample.

Detecting the ability of *S.aureus* isolates to produce Staphyloxanthin

The results of identifying the ability of *S.aureus* isolates (43 isolate) for staphyloxanthin production on different culture media found that milk agar medium was the best medium for the staphyloxanthin production which gave the highest percentage of orange pigment production 44.1 %. While, trypticase yeast medium and trypticase soya medium revealed percentage 37.2%.Peanut seeds medium and sesame seed medium appeared percentage 30.2 % for orange staphyloxanthin production. No production of orange pigment were observed in several media such as sunflower seeds medium, brain heart infusion agar, carotinoid expression medium , muller hinton agar and nutrient agar .

These results suggested that specific nutrients are required for staphyloxanthin production. According to the results pigment producer isolate $AE_{36.}$ Medium components may be critical for production of staphyloxanthin .The fatty acid as a carbon source is a better substrate for the growth of bacteria than sugars .Based on the comparison between the composition of different fatty acid containing seeds and oils . The saturated form of fatty acid could be a better choice of carbon source for the maximum production of pigment [29] as shown in figure-1.

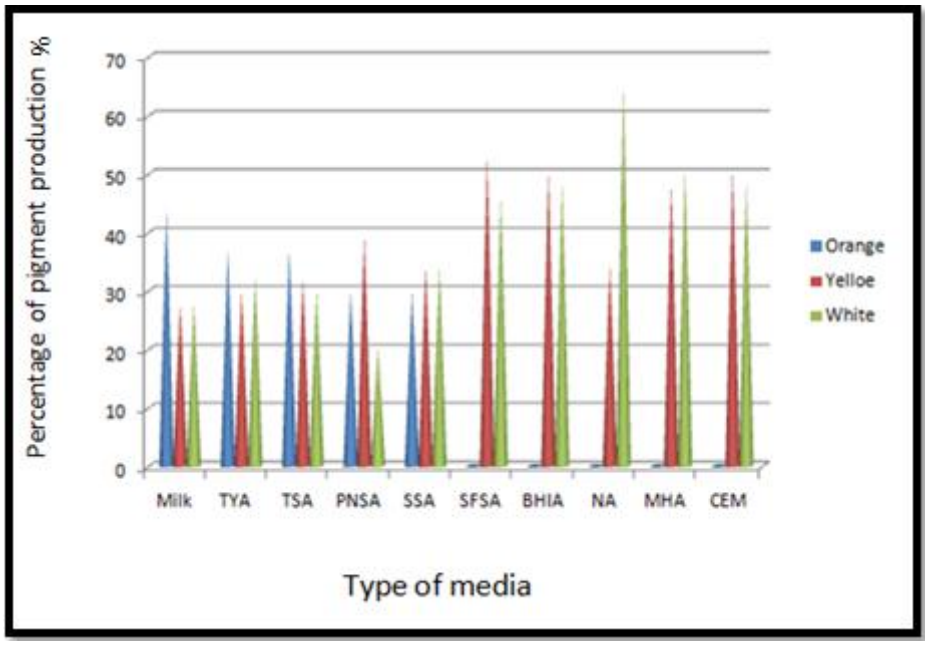

Figure 1- Influence of different culture media on the production of staphyloxanthin

*Milk: Milk agar medium , TYA Trypticase yeast medium ,TSA :Trypticase soya medium ,PNSA: Peanut seeds medium , SSA :Sesame seed medium , SFSA: Sunflower seeds medium , BHIA: Brain-Heart Infusion Agar, N.A: Nutrient Agar MHA: Mueller-Hinton Agar CEM: Carotinoid expression medium,

Determination of optimal conditions for staphyloxanthin production

The results found that the best medium for pigment production was milk agar with an amount of pigment produced for AE_{36} isolate (165.21) unit/cell compared with the pigment produced in TSA and TYA, which was (89.22 , 77.22) unit / cell respectively .In the CEM the production was less than 37.9 unit/cell These results showed similarity with the results mentioned by Grinsted and Laccy, (1972). the pigment production was increased at 37^{o}C, whereas decreased at the low temperatures (less than 20^{o}C) and in high temperature (more than 37^{o}C) . Optimum temperature for pigment production was 37^{0}C, and this result was similar to the results of [30,31]. The result revealed that the optimum pH was 8. Generally the pigment production was higher in alkaline media, this agrees with [14] finding. This is because at pH 8 the activity of proline oxidase enzyme is inhibited which cause anabolism of proline the basic amino acid for pigment production , whereas lower or higher pH than 8 lead to imbalanced or break in the biological pathway that lead to pigment production which affected by the activity of the enzyme responsible for pigment production [14,32]. The optimum incubation time for pigment production was found that 72 hr was the optimum incubation time for pigment production, because the pigment was secondary metabolite [3,33] which required time to produce. The results demonstrated that the higher production of pigment was obtained when 50 ml medium was used (165.21) unit/cell, while the production decreased when the volume of the media was more or less than 50 ml/flask . When the volume of culture media was 25 ml the ratio of surface area to the volume of media was high which provided high ratio of oxygen that made bacterial growth higher than the pigment production because the pigment is a secondary metabolite and is not necessary for bacterial growth [34]. In addition the small volumes of media do not provide the suitable amount of substance which when consumed cause decreased in pigment production [35]. The results showed significant differences ($p< 0.05$). The best results of extraction the pigment was with methanol, with good amounts to 0.2 g/ml. The RF value was 0.38, which was similar to the results of [13] .Figure- 2 shows the optimal conditions of staphyloxanthin production and figure-3 shows the result of TLC partial purification .

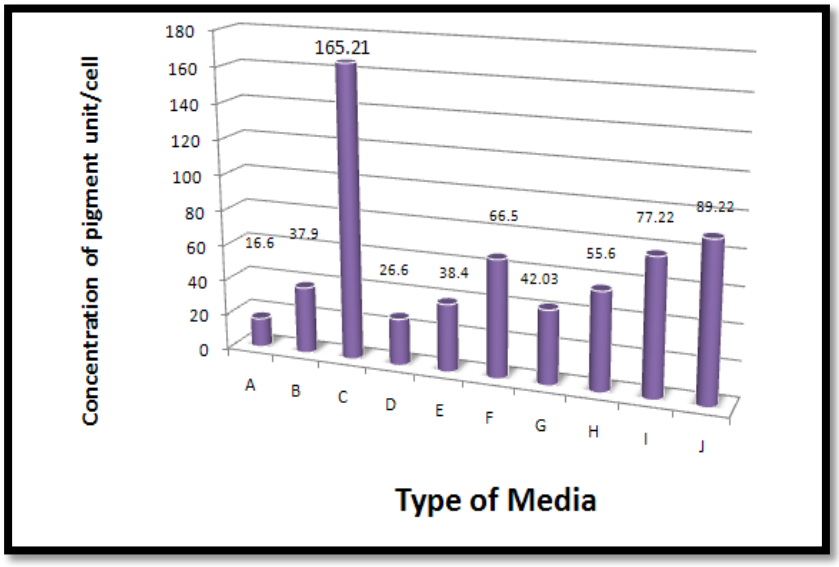

Figure 2a- Staphyloxanthin production from *S. aureus* AE_{36} isolate grown in different media

A:Brain-Heart Infusion Agar, B:Carotinoid Expression Medium , C : Milk agar , D: Mueller-Hinton Agar , E: Nutrient Agar , F : Peanut seeds mediumG: Sunflower seeds medium , H : Sesame seed medium , I : Tripticase soya medium, J: Tripticase yeast agar

* Each value represents the mean ± SD , where (n=20) .The SD value = 0.06

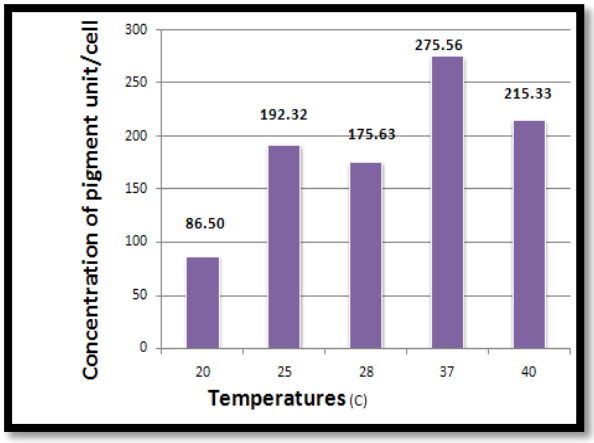

Figure 2b- Staphyloxanthin production from *S. aureus* AE_{36} isolate grown in different temperatures

* Each value represents the mean ± SD , where (n=20) .The SD value = 5.90

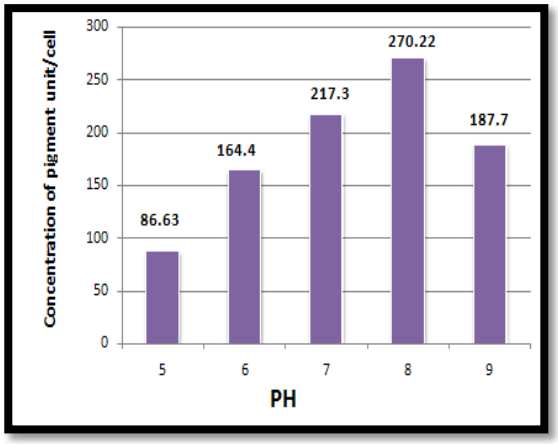

Figure 2c- Staphyloxanthin production from *S. aureus* AE_{36} isolate grown in different pH values

* Each value represents the mean ± SD , where (n=20) .The SD value = 9.77

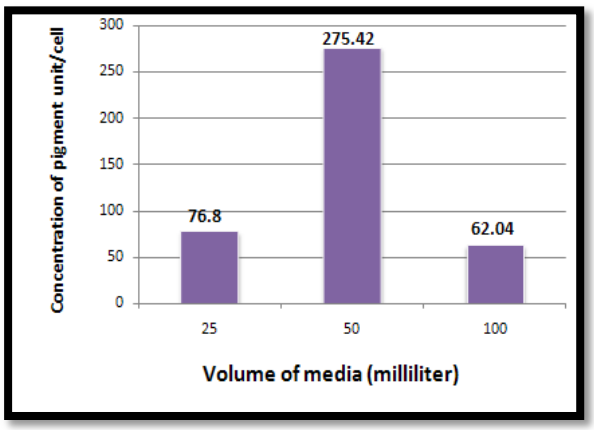

Figure 2d- Staphyloxanthin production from *S. aureus* AE_{36} isolate grown in milk agar at different incubation periods

* Each value represents the mean ± SD , where (n=20) .The SD value = 8.575

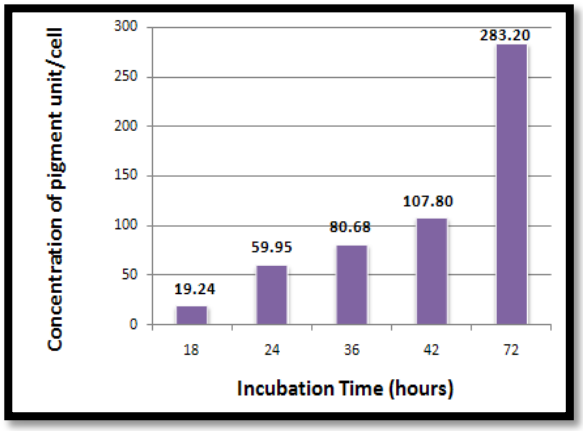

Figure 2e-Staphyloxanthin production from *S. aureus* AE_{36} isolate grown in different volumes of milk medium

* Each value represents the mean ± SD , where (n=20) .The SD value = 17.53

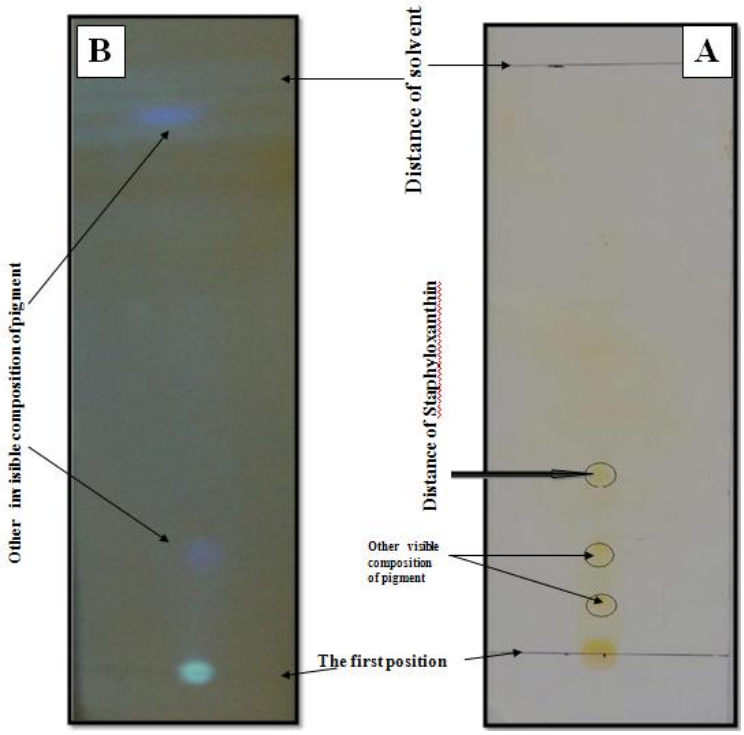

Figure 3- Partial purification of Staphyloxanthin from *S.aureus* AE_{36} by Thin Layer Chromatography
A: Visible Spots. B: Invisible Spots.

Anti bacterial activity of staphyloxanthin against bacteria

The antibacterial activity of the pigment was examined against several bacterial genera (*Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Klebsiella* spp , *Proteus* spp. *Pseudomonas fluorescens* , *Pseudomonas putida* , *Staphylococcus aureus* isolate AE_{36}, *Staphylococcus aureus* isolate AE_{32} and *Staphylococcus aureus* isolate AE_{38} .
The results revealed that staphyloxanthin has no activity against tested bacteria that used in this study.

Other study by [30] reported the staphyloxanthin pigment extraction did not show antibacterial activities against several gram positive and gram negative bacteria at concentration 0.25 mg/ml, the results in this study confirmed by results of [17] which revealed that the staphyloxanthin pigment extract has no activity against *Klebsiella* spp ., due to that STX pigment is a virulence factor help bacteria to cause a disease after inter human body.

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