Determine the Antibacterial Activity of Staphyloxathin Produced by *Staphylococcus aureus* against some Bacteria

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Abstract

A total of 70 isolate of staphylococcus (22.8%) isolated from 306 sample of different clinical sources that is diagnosing as *S. aureus* depending on cultural morphological and biochemical test. 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 mannitol. Staphyloxanthin pigment was extracted by using different methods included procedure one using chloroform, procedure two using methanol and procedure three using ethyl acetate with ethanol was the best method. Staphyloxanthin purified by using coolum chromatography, purified extract of Staphyloxanthin that isolated from *S. aureus* as anti-bacterial activity ,which appeared inhibition zone against several gram negative bacteria at concentration (2,20)mg/ml , and the highly inhibition zone (15mm) of pigment was recorded against *Pseudomonas aeruginosa*.

***Keywords*:** Staphyloxanthin, antibacterial, purified extract, well diffusion.

تحديد الفعاليه المضادة للبكتريا لصبغة الستافيلوزانثين المنتجة من المكورات العنقودية الذهبية ضد بعض البكتريا

د. فاطمة عامر

الخلاصة

من مجموع 306 عينة تم عزل 70عزلة من بكتريا المكورات العنقودية الذهبية من مصادر سريرية مختلفة تبعا للفحوصات المظهرية والبايوكميائية .تشمل الفحوصات شكل المستعمرة وحجمها ولونها وتايرها .على الوسط الزرعي مثل تحلل وسط الدم وظهور الصبغة على وسط الحليب وتخمر سكر المانتول .صبغة الستافيلوزانثين تم استخلاصها بثلاث طرق تتضمن استخدام الكلوروفورم الميثانول والاثيل استيت مع الايثانول وتعتبر طريقة استخدام الاثيل استيت مع الايثانول افضل الطرق في استخلاص الصبغة. نقيت الصبغة باسخدام العمود الكرموتوغرافي ,المستخلص النقي للصبغة المعزولة من العنقوديات الذهبية له فعالية مثبطة للبكتريا وخاصة

ضد البكتريا السالبة لصبغة كرام عند التركيز (2و20 مع حدوث اعلى هاله تثبيط 15 ملم اذ سجلت ضد بكتريا *Pseudomonas aeruginosa*.

الكلمات المفتاحية : سافيلوزانين , الضد البكتيري , المستخلص النقي , الانتشار بالحفر.

Introduction

Staphylococcus aureus is a major global public health causing serious infections ranging from minor skin infections to life-threatening infections, such as bacteremia, endocarditis, necrotizing pneumonia and Toxic Shock Syndrome (TSS) in the community and hospital setting [1].

This bacterium express many potential virulence factors surface protein that promote colonization of host tissues , invasions Surface factors that inhibit phagocytic engulfment (capsule and protein A) and biochemical properties that enhance their survival in phagocytosis (carotenoids and catalase production) immunological disguises (protein A and coagulase) [2] Staphyloxanthine is produced in secondary phase; it scavenges free radicals with conjugated double bonds. Since Staphylolxanthin is located in the cell membrane, it protects lipids but might also be involved in protecting protein and DNA, and it plays an additional role in the defense against damage by ROS, thereby enhancing the virulence and fitness of the cells [3].

Staphyloxanthin was being a carotenoid pigment that was produced by some strains of *S. aureus* .Carotenoids are one among several classes of biologically active compounds that have been reported to possess greater antioxidant and anti-cancer activity [4] Staphyloxanthin is a membrane-bound carotenoid of *S. aureus*. And their chemical structures compounds which are all triterpenoid carotenoids, possessing a C30 chain instead of the C40 carotenoid structure found in most other organisms [5].

Extraction of metabolic compounds depending on nature of compounds that produced from microorganism or pigment characteristic and location in the cell, basically carotenoids are lip soluble, the use of organic solvents and alcohol lead to melt fat connected in the cell wall such as ethyl acetate**,** chloroform and methanol [6]

Column chromatography is a physical separation method based on the distribution of the bio components in a mixture, Wiktor *et al.*, (2016) [7] explained the

column chromatography step increased the purity of the isolated pigments, while the spectral quality of the main constituent remained.

Secondary metabolites like Statins, Naphthoquinones and Carotenoids produced from microorganism have pharmaceutical applications and possess antimicrobial, antioxidant and anticancer activities .Microbial carotenoids are of considerable interest in nutrition because of their role as antioxidants and potential for preventing or delaying degenerative diseases and for enhancing immune responses in animals and humans [8].

Collection of sample and diagnosing

A total of 70 isolate of Staphylococcus (22.8%) isolated from 306 sample of different clinical sources that is diagnosing as *S. aureus* depending on cultural morphological and biochemical test. 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 mannitol. Then bacterial isolates were examined and identified by microscopic, cultural, biochemical test and Vitek 2 system characteristics (9).as well as the 16 isolate of different genes of pathogenic bacteria *(Pseudomonas aeruginosa , Serratia marcescens . Klebsiella pneumonia, Enterobacter species*) isolated from different clinical source depending on cultural morphological and Vitek2 system characteristics.

Staphyloxanthin pigment assay

According to method described by Tao *et al.,* (2010) [10] the Staphyloxanthin pigment was measured as follows:

$$\text{Total carotenoid unite / cell} = \frac{V\,(A-0.0051)}{0.175W}$$

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 carotenoid.

Purification of Staphyloxanthin by column chromatography

After extraction of Staphyloxanthin by ethanol and ethyl acetate from *S.aureus* (S25) isolate.,the pigment purified by using column chromatography.The crude extract was dissolved in ethyl acetate and subjected to silica gel (20gm , Merck) column chromatography (1.5× 60) cm. The colored fractions were eluted with ethyl acetate, and the individual fractions were evaporated to dryness. Because of the light sensitivity of the pigments, all further purification steps were carried out in the dark. The fraction 5ml were collected and assayed for absorbance at 450nm .The purified pigment was stored at -20°C. The Staphyloxanthin peak fraction was pooled to assay pigment concentration [11].

Staphyloxanthin as anti-bacterial agent

Antibacterial activity of purified staphyioxanthin was determined according to [12].Qualitative and quantitative screening method as following: Four bacterial isolates *(Pseudomonas aeruginosa, Serratia marcescens, Klebsiella pneumonia, and Enterobacter species*) were selected and the inoculum was prepared compared with McFarland turbidity standard. The 100 µL of the concentrations (2, 20) mg/ml from purified stapyloxanthin were extracted from isolate *S. Aureus* (S.25) using via D.W.

Well-diffusion methods

Qualitative screening of antibacterial activity was carried out by inoculating of indicator bacteria on MHA plate, the wells were prepared in the plate with 4mm diameter sterile corn borer and the wells for each culture of indicator bacteria were filled with 100 µl of purified extracts with concentration (2, 20) mg/ml and then the plates were incubated at 37°C for 24hrs and inhibition rate was detected by zone of clearing around the purified Staphyloxanthin extract [12].

Turbidity method

The turbidity method was based on the inhibition of growth of a microbial culture in a fluid medium containing a uniform distribution of an antimicrobial compounds. The turbidity assay was employed to evaluate the sensitivity of the test pathogen in liquid culture according to [13.] In this assay 100 µl of inoculum of indicator bacteria (1.5×108 CFU/ml was added to the test tubes containing 5ml of nutrient broth, then added 100 µl of purified extract of Staphyloxanthin at concentration

(20)mg/ml ,and incubated at 37°C for 24hrs . After incubation the growth in terms of turbidity of the bacterial cultures was measured spectrophotometrically at 600 nm. The readings were measured by using spectrophotometer and compare between the control (tube containing nutrient broth and indicator bacteria) and inhibition of bacteria growth in tubes (containing the indicator bacteria and purified extract).

Results and Discussion

Cultural characteristics

Cultural characteristics for *S. aureus* isolates appeared when isolated bacteria grown on its selective media, the colony morphology of isolates on blood agar and mannitol salt agar, and these isolates were characterized by raised, smooth, glistering, and translucent with varied pigmentation production [14]

Detection the ability of isolates to produce Staphyloxanthin

The ability of *S. aureus us* isolates for Staphyloxanthin production was identified by culturing of *S. aureus* isolates (70) on skim milk agar ,In the assay figure (1) only ten isolates from 70 isolate of *S. aureus* that producing Staphyloxanthin ,and this isolate (S.25) was isolated from burn infection was recorded high productivity (1.56) and this isolate (S.25) with excellent identification(96%) by Vitek2 System.

Medium component supported the production of Staphyloxanthin and might have been critical for production of pigment, that the fatty acid , glucose, sucrose and xylose supported the carbon sources as well as the variation in the productivity of pigment may be the complexity of medium component, some being complicated are hard to profit from it [11].

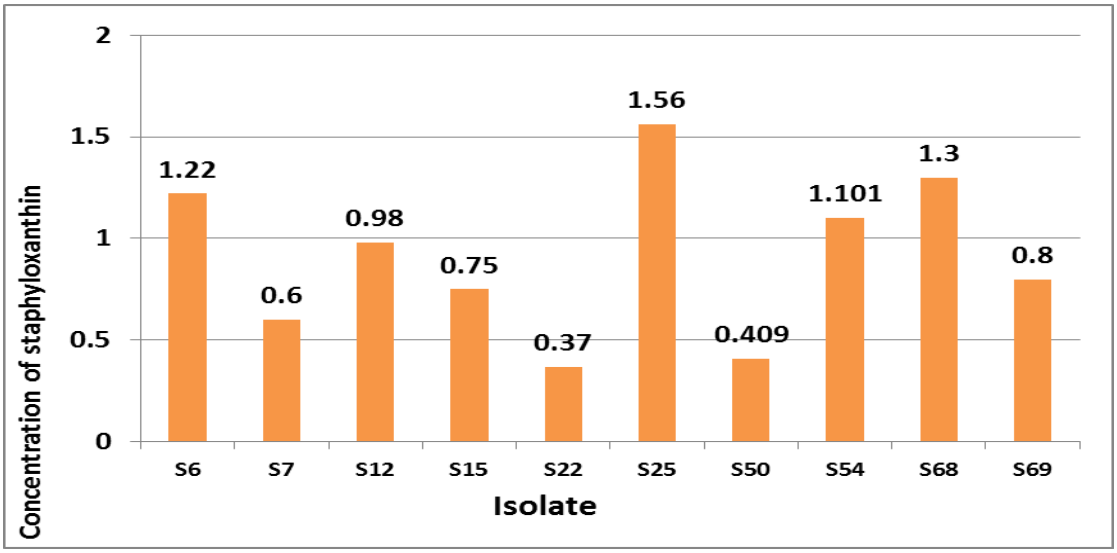


Figure (1): Productively of Staphyloxanthin from Different Isolate of *S .Aureus*

Extraction of Staphyloxanthin from *S. aureus* by using different methods.

Staphyloxanthin was extracted by using different solvent included chloroform according to [15]ethyl acetate and ethanol [11] , and methanol as according by [16] Table (1) showed the significant differences in methods of extraction ,as well as the highly concentration of Staphyloxanthin was obtained from extraction by ethyl acetate and ethanol ,with absorbance of Staphyloxanthin that was recorded (0.683) and the amount of pigment produced by *S. aureus* (S.25) isolate (154.948)unit /cell (4 cuvate) , and the results showed the significant difference at (P<0.05) in extraction of pigment, also the concentration of pigment was determined at 450nm, the high peak of pure pigment absorbance. And extraction of pigment by ethanol and ethyl acetate was the best method.

Table (1): Extraction of Staphyloxanthin from *S.aureus* (S.25) using different methods.

Extraction methods	**Means (O.D.)±S.E.**
Chloroform	0.226 ± 0.002 b
Methanol	0.345 ± 0.009 b
Ethyl acetate and ethanol	0.683 ± 0.006 a
LSD value	0.208 *
*** (P<0.05).** **Data with the different letters in same column differed significantly,**	

Column Chromatography was one of the most important and widely used techniques available for the separation and analyses of complex organic mixtures. By loading the mixture into a column packed with finely powdered adsorbent and then developing the column with a solvent by using Silica Gel may be regarded as a typical polar sorbent was used in separation and purification of steroids, lipids, amino acids and dyes [17].

Purification carried out according to [11] by a column chromatography using open glass Column (1.5 x 60) cm filled with silica. gel (20g) special for column chromatography. The residue was dissolved, mixed and saturated with the Ethanol (5 ml) and pooled in column then developing the column with a solvent, the pigment fractions were pooled and passed through silica gel column. Presence of one peak, represented in fraction (5-11) and other fraction (1-13) containing the purified extract of Staphyloxanthin (3ml) were read at 450 nm and the curve was plotted between the absorbance and fraction number.

Column chromatography step increases the purity of the extracted pigments, while the spectral quality of the main constituent remains comparable [18] .The results were showed in figure (2) and table (2) gave significant difference in concentration of Staphyloxanthin in tubes containing the purified extract and the extracts (in tube 7) recorded (0.998) highly concentration of Staphyloxanthin (highly purified).

Table (2): Purification of Staphyloxanthin crude extracts using column chromatograph

Number of Extract Tube	Means (O.D) of Purified Extract at 450nm ±S.E.
1	**0.0 ± 0.00 c**
2	**0.0 ± 0.00 c**
3	**0.0 ± 0.00 c**
4	**0.0 ± 0.00 c**
5	**0.208 ± 0.005 bc**
6	**0.581 ± 0.018 b**
7	**0.998 ± 0.041 a**
8	**0.970 ± 0.033 a**
9	**0.668 ± 0.026 ab**
10	**0.439 ± 0.012 b**
11	**0.344 ± 0.007 bc**
12	**0.028 ± 0.001 c**
13	**0.00 ± 0.00 c**
LSD value	**0.361 ***
* ($P<0.05$). Means having with the different letters in same column differed significantly.	

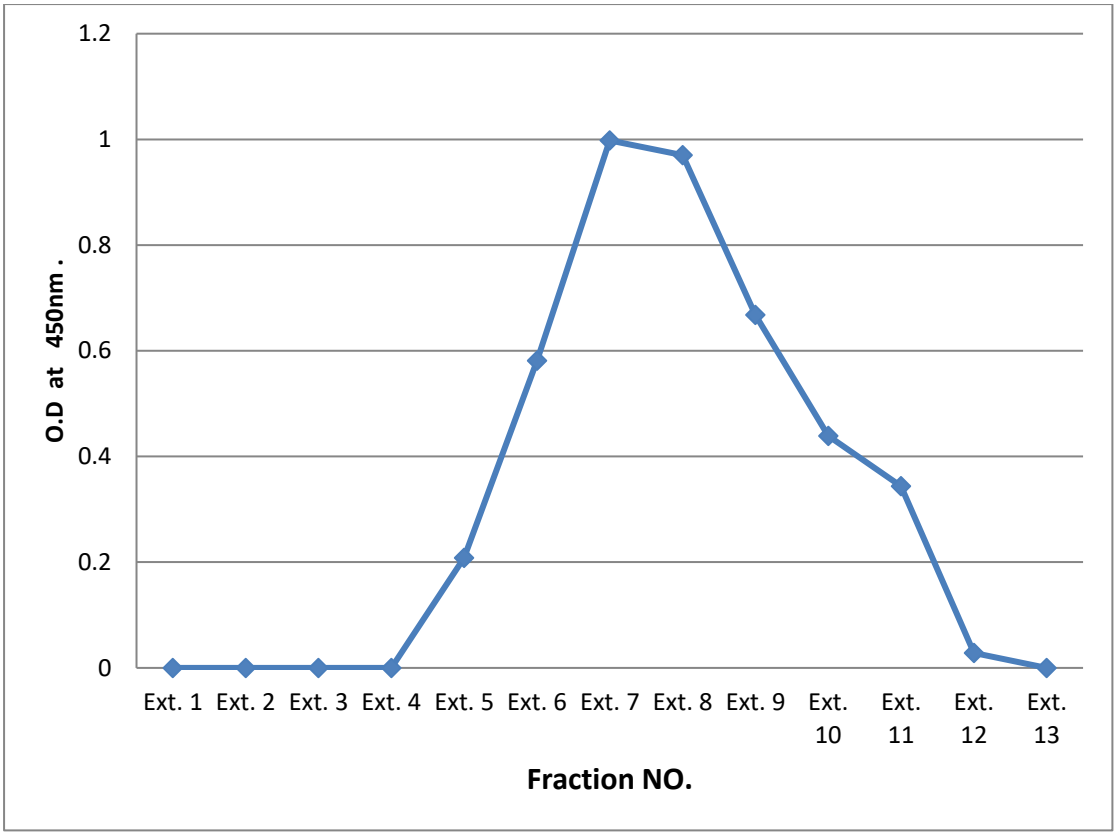


Figure (2) Purification of Staphyloxanthin by column chromatography silica gel .
Antibacterial activity of Staphyloxanthin

Agar well diffusion method

The anti-bacterial activity of Staphyloxanthin was examined against several bacteria *(Pseudomonas aeruginosa, Klebsiella pneumonia Serratia marcescens and Enterobacter species)*

Results shows in table (3) and figure (3) the significant activity of purified extract of Staphyloxanthin that isolated from *S. aureus* (S.25) as anti-bacterial activity ,which appeared inhibition zone against several Gram negative bacteria at concentration (2,20)mg/ml , and the highly inhibition zone (15mm) of pigment was recorded against *Pseudomonas aeruginosa*, therefore this result was agreement with (19) was demonstrated the anti-bacterial activity of carotenoid isolated from *Rhodotorula glutinis* strains against different types of pathogenic bacteria.

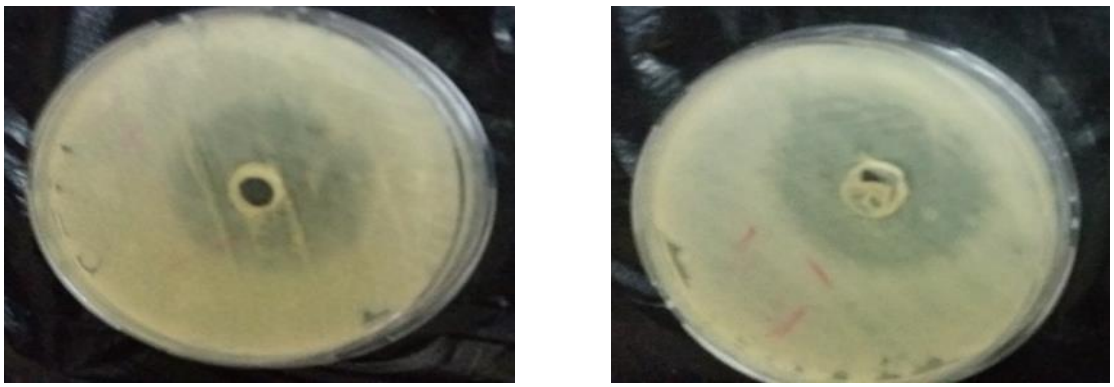
As well as Carotenoid pigment extracted from Micrococcus yunnanensis showed high antibacterial activity against *Pseudomonas aeruginosa* , Devihalli *et al.*,[20] and

Boontosaeng *et al*.,[21] showed the capability of carotenoid pigments to inhibit pathogenic bacteria by agar well diffusion method.

as well as the results showed in quantitative screening method of Staphyloxanthin, that (purified) extract was gave strong bactericidal activity at concentration (20 mg/ml) toward Pseudomonas isolates ,this result was similar to that report by [22] he showed that *Streptomyces* produced a yellow color sugar containing pigment with antimicrobial activity against drug resistant pathogens such as β-lactamase producing culture of *E. coli, Pseudomonas aeruginosa*, and *Klebsiella* . Also, Manimala and Murugesan [23] found that carotenoid pigment more effective against *E. coli* , *S. aureus* and *P. aeruginosa.*

Table (3) Anti-bacterial activity of purified extract of Staphyloxanthin toward different isolates of Gram negative bacteria

Type of bacteria	**Concentration of purified extract** / **Inhibition zone (mm)**	
	2mg/ ml	20mg/ml
Pseudomonas aeruginosa	*8*	*15*
Klebsiella pneumonia	*7*	*13*
Serratia marcescens.	*7*	*12*
Enterobacter species	*8*	*10*



Klebsiella pneumonia ***Pseudomonas aeruginosa***

Figure (3) Antibacterial activity of Staphyloxanthin against different Gram negative isolates

The Turbidity Method

Turbidity method is based on the inhibition microbial growth cultured in a fluid medium (B.H.I.B.) containing a uniform distribution of an antimicrobial compounds. The results in table(4) and figure (4) showed the antibacterial activities of purified extract of staphyloxanhin with a significant differences in imbibition rate of pathogenic bacteria specially Pseudomonas aeruginosa that was recorded (0.451) , also the results indicated the inhibition rate of Staphyloxanthin extract against *Klebsiella pneumonia* (0.096) and against *Serratia marcescens* (0.115) as well as against Enterobacter species (0.077). As well as *Pseudomonas aeruginosa* very sensitive to purified extract and used for infection of mice in immunity procedure.

Sanjay [24] indicated that the antimicrobial activity of xanthine pigment at higher concentration (400 µg/ ml) leading to Results in lysis of pathogenic bacterial cells, As well as the Astaxanthinas pigment has also health benefits in cardiovascular disease prevention, immune system boosting, bioactivity against Helicobacter pylori, and cataract prevention due to its high antioxidant activity [25].

Table (4) Inhibition rate of Staphyloxanthin extract (purified) by using turbidity method against Different Gram negative isolates

Type of bacteria	**Mean ± SE**			**LSD value (inhibition)**
	O.D. of purified extract and bacteria	**O.D. of bacteria (control)**	**Control negative**	
Klebsiella pneumonia	1.028 ± 0.06	1.131 ± 0.05	0.0	0.096 *
Pseudomonas aeruginosa	1.083 ± 0.08	1.264 ± 0.05	0.0	0.451 *
Enterobacter sp.	1.005 ± 0.05	1.109 ± 0.07	0.0	0.077 *
Serratia marscence	0.911 ± 0.05	1.093 ± 0.04	0.0	0.115 *
	* (P<0.05).			

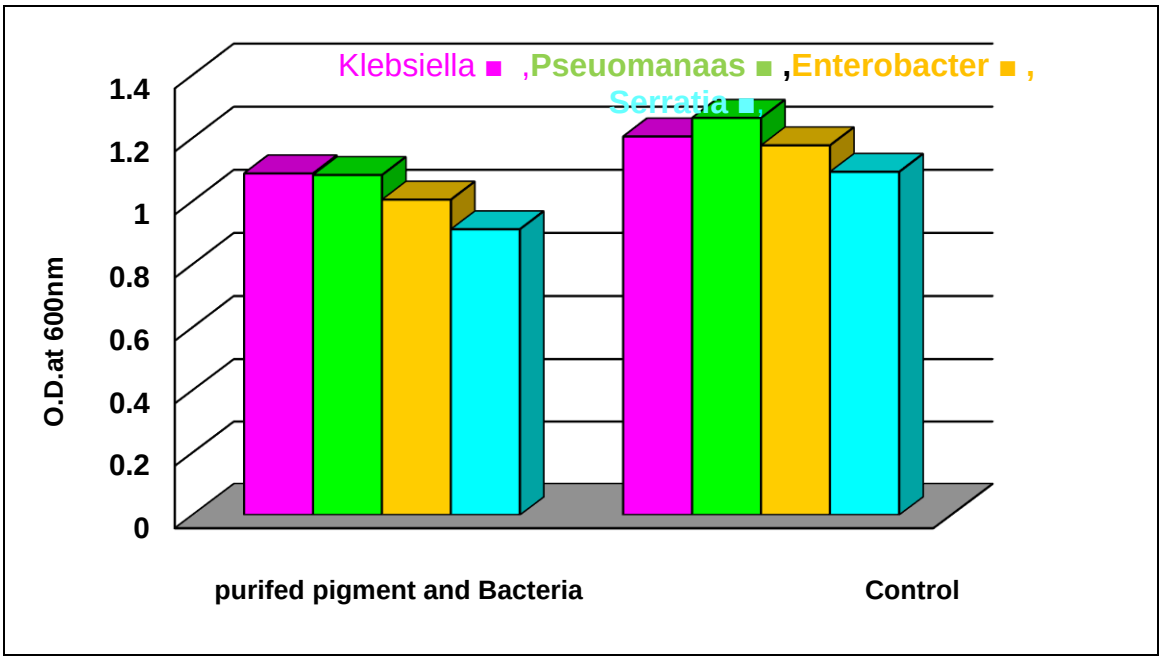


Figure (4) Inhibition rate of Staphyloxanthin extract (purified) by using turbidity method against Different Gram negative isolates.

Despite the availability of a variety of natural and synthetic carotenoid pigments, there is interest in microbial pigments due to their natural character, medicinal properties and nutritive value. Microbial production is being independent of season, geographical conditions, controllable and predictable yield and safety to use [26].

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