

The Role of Extracted Carotenoid from Staphylococci as Antioxidant and Antibacterial

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ABSTRACT

The ability of some Staphylococci isolates producing and non-producing carotenoid pigments to resist the different concentrations of H_2O_2 and *Staphylococcus aureus* ranged between 0.6-1.5% studied in dark and light had been studied. The results showed that staphyloxanthin producing strains were more resistant than carotenoid producing pigmented and non-pigmented strains and this resistance was better in dark than light due to the pigment oxidation. Extraction and partial purification of the pigment was done and some biochemical properties such as conductivity and UV-spectra were performed. High conductivity had been registered at 469.25, 391.25 ms besides UV-spectra determination revealed peaks at 450-468 nm for staphyloxanthin and orange-carotenoid pigments respectively. Antibacterial ability of partially purified pigments had been tested against different bacterial isolates, results indicated that partially purified staphyloxanthin had highest antibacterial activity against *Staphylococcus epidermidis*, *Acinetobacter baumannii* and *Proteus mirabilis*, the lowest activity appeared against both *Pseudomonas aeruginosa* and *Shigella dysenteriae*, the other carotenoid pigment on the other hand, had no antibacterial effects against these isolates.

Keywords: Staphyloxanthin, carotenoid pigment, *Staphylococcus*, antioxidant

%1.6-%0.5

Staphyloxanthin

uv-spectrophotometry

electrical conductivity

478 466

390.25 469.25

Staphyloxanthin

Staphyloxanthin

Proteus mirabilis *Acinetobacter baumannii* *Staphylococcus epidermidis*

Shigella dysenteriae *Pseudomonas aeruginosa*

INTRODUCTION

Staphyloxanthin (STX) is an orange-red Triterpenoid, membrane-bound carotenoid which plays a role in the environmental fitness of *Staphylococcus aureus* (Clauditz *et al.*, 2006) besides it provides integrity to its cell membrane (CM) and limits oxidative host defense mechanism (Mishra *et al.*, 2011). Staphyloxanthin consists of a c30 polyene carbon backbone with alternating single and double bonds typical of carotenoid pigments, these alternating bonds are able to absorb excess energy from reactive oxygen species ROS (El-Agamey *et al.*, 2004).

Most *Staphylococcus aureus* produce the orange carotenoid staphyloxanthin in which biosynthetic genes are organized in an operon, *crtOPQMN*, with a σ^B -dependent promoter upstream of *crtO* and a termination region downstream of *crtN*. The first step in staphyloxanthin biosynthesis is the head-to-head. Condensation of two molecules of farnesyl diphosphate to form dehydrosqualene (4,4'-diapophytoene). Catalyzed by the dehydrosqualenesynthase CrtM (Pelz *et al.*, 2005).

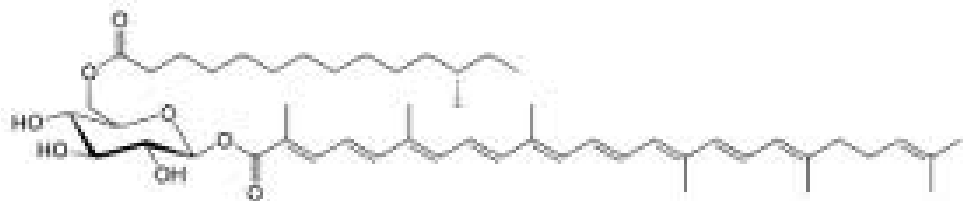


Fig. 1: Chemical structure of staphyloxanthin (Liu *et al.*, 2008)

One important mechanism by which phagocytic cells eliminate pathogens is through the release of reactive oxygen species generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Fang, 2004). It has been suggested that bacterial carotenoids such as those expressed by *Staphylococcus aureus* and *Staphylococcus capitis* could serve as a protective function against these defense molecules (Liu *et al.*, 2005). The research aimed to extract and partially purify carotenoid pigments and reveal its role as an antioxidant factor, study its chemical and physical properties, the antibacterial effect of staphyloxanthin with imipenem a broad spectrum antibiotic against the selected bacterial isolates.

MATERIAL AND METHODS

Bacterial isolates: Golden and non-golden *Staphylococcus aureus* isolates were obtained from AL-Zahrawi teaching hospital, laboratory definitive identification was done by using biochemical tests after Gram staining had been performed first. Bacterial isolates were cultured on a selective media (mannitol salt agar with 7-9% NaCl) which allow *Staphylococcus aureus* to produce yellow-colored colonies, as a result of mannitol fermentation. Biochemical tests such as catalase, coagulase, DNase for final differentiation of *Staphylococcus aureus* (Ryan, Ray, 2004). *Staphylococcus capitis* strain and other selected bacterial strains (mentioned later) were obtained from Bacterial Strains Bank, Department of Biology/ College of Science/Mosul university.

Growth condition and pigment extraction: Staphyloxanthin was isolated from golden *Staphylococcus aureus*, while other carotenoid pigments were isolated from orange *Staphylococcus capitis*. Bacterial strains were grown on tryptic soy agar (TSA) plates pH 7.2±0.2 adjusted by pH meter (OAKTON/SINGAPORE), these plates were inoculated by streaking method three independent parallel plating were prepared. After incubation at 37°C for 24h. Cells were scraped from the (TSA) plate placed in test tube containing 3ml of methanol and incubated at 50°C for 5min in water bath then the extracted pigment was centrifuged (REMI/INDIA) at 3000 rpm for 10 min. This process was repeated twice until no further pigment was extracted (Schmidt *et al.*, 1994). All manipulations during preparations of the pigments were carried out rapidly and the effects of light were avoided by carrying out the extractions in glass covered, with aluminum foil. After centrifugation the supernatant containing the pigment was concentrated to small volumes in vacuo (BINDER/USA) and then extracted with ethyl acetate (Pelz *et al.*, 2005).

Partial purification of staphyloxanthin and other pigments: The extracted pigments were subjected to silica gel 60(1.5,4.0 μ m) Colum chromatography. The colored fraction was eluted with ethyl acetate, and the individual fractions were evaporated to dryness (Plez *et al.*, 2005).

Oxidant suscepility assay: Three sets of duplicate cultured plates with 10^8 cells of pigmented and non-pigmented *Staphylococcus aureus* strains as well as *Staphylococcus capitis* were incubated for 1h in dark wrapped in foil in the presence of various concentration of discs H_2O_2 ranging from 0.6-1.5%, after incubation at $37^\circ C$ for 24h, the effect was assessed by growth inhibition zones. Control plates situated exactly 10cm from 100w light source to determine the evidence of bacterial killing (Liu *et al.*, 2005).

Spectral measurements: Spectra of extracted pigments were recorded from 190.0 to 1100.0 nm with a 2 nm sampling interval using 1601uv-visible spectrophotometer (UV-1650PC/SHIMADZU/JAPAN) Chemistry Department/ College of Science /Mosul University).

Conductivity measurements: The Conductivity of the extracted pigments were measured by using conductivity instrument (HANA/EUROPE).

Antibacterial activity of staphyloxanthin: Antibacterial susceptibility testing of staphyloxanthin pigment and antibiotic imipemen (10 mcg) were carried out by Kirby-Bauer's disc diffusion techniques, against the selected bacterial isolates: *Acinetobacter baumannii*, *Escherichia coli*, *Proteus mirabilis*, *klebsiella penumoniae*, *Shigella dysenteri*, *Salmonella enteritis*, *Psedomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus epidermidis*. The extracted carotenoid pigments were dissolved in DMSO and used for bioassay against these bacteria. Sterile discs 6 mm diameter were impregnated with 400 mg/ml extracts and introduced into the nutrient agar medium inoculated with the tested selected bacterial strains. The plates were incubated at $37^\circ C$ for 24 hours. Antimicrobial activity was evaluated by measuring the zone of inhibition (Chung *et al.*, 2011).

RESULTS AND DISCUSSION

Pigmented and non –pigmented *Staphylococcus aureus* as well as the isolate of *Staphylococcus capitis* that produce orange carotenoid pigment other than staphyloxanthin had been identified and utilized.

Fig. (2) revealed the morphological characteristic of these isolates. Yellowish golden staphyloxanthin producing strain, *Staphylococcus aureus* and orange carotenoid producing strain *Staphylococcus capitis* as well as non-pigmented strain *Staphylococcus aureus* incubation of these strains with different concentration of H_2O_2 saturated discs had been accomplished as shown in Fig (3-5) that indicate the decreased viability of *Staphylococcus aureus* and *Staphylococcus capitis* with increasing H_2O_2 concentration to different extent. The viability of *Staphylococcus aureus* producing staphyloxanthin more than *Staphylococcus capitis* and non pigmented strain at different concentrations of H_2O_2 . The viability of these strains had also been investigated in these figures after incubation with different concentrations of H_2O_2 in both dark and light conditions. This indicates the survival ability of *Staphylococcus aureus* producing staphyloxanthin in dark was better than light culturing condition due to the oxidation of staphyloxanthin, and thus this strain shows better survival ability than the other strains *Staphylococcus capitis* and non-pigmented strain of *Staphylococcus aureus* which is more susceptible to killing by hydrogen peroxide. These results had been accepted with other researches such as (Liu *et al.*, 2008) that indicates the role of golden carotenoid pigment of *Staphylococcus aureus*, staphyloxanthin to promotes resistance to reactive oxygen species and host neutrophil-based killing. The early enzymatic steps in staphyloxanthin production resemble those of cholesterol biosynthesis and blocking staphyloxanthin biosynthesis is therefore a potentially attractive therapeutic target. Mishra *et al.*, 2011, on the other hand, elucidated that carotenoid pigment of *Staphylococcus aureus* provides integrity to its cell membrane (CM) and limit oxidative host defense mechanism. Membrane pigments have also been hypothesized to be a virulence factor in *Staphylococcus aureus* potentially by detoxifying reactive

oxygen species produced by phagocytes, such antioxidant potential of carotenoid pigment may be related to their specific orientation, location, and organization within the membrane (Widonska *et al.*, 2009).

Lennette *et al.*, 1985; Clauditz *et al.*, 2006 elucidated that over 90% of *Staphylococcus aureus* strains isolated from human infections are pigmented staphyloxanthin producing STX as a triterpenoid membran-bound carotenoid which plays a role in the environmental fitness *S. aureus* loss of pigmentation translates to a significant decrease in *Staphylococcus aureus* virulence in murin skin abscess or systemic infection mode (Liu *et al.*, 2009).

Some biochemical properties of extracted and partially purified pigments of *Staphylococcus aureus* and *Staphylococcus capitis* had been studied such as conductivity and UV-spectra. High conductivity for both pigments had been registered 469.25 for *Staphylococcus aureus* and 391.25 for *Staphylococcus capitis*. As well as a significant peak at (478 nm, 466 nm) as shown in Fig (6,7) had been determined respectively for both staphyloxanthin and carotenoid pigment. Other researchers also determined the absorption spectra of purified staphyloxanthin such as Pelz *et al.*, 2005, that obtained a peak between 463-490 nm, while Liu *et al.*, 2005 revealed triple-peak spectral profile of carotenoid pigment at 440-462-461nm wave length Clauditz *et al.*, 2006 also elucidated that the absorption maxima of purified staphyloxanthin show a significant peak at 478nm and this absorption had been decreased with the oxidation of purified staphyloxanthin by hydroxyl radicals generated in a Fenton reaction Lan *et al.*, 2010 also measured the extracted carotenoid pigment optical density at 465 nm. Sahin 2011 indicated that the absorption spectra can accelerate the identification and characterization of extracted and partial purified pigments from *S. aureus* at 468nm when used with other distinctive biochemical tests. Antibacterial activity against a limited number of bacterial species had been also done. The highest antibacterial activity recorded against *Staphylococcus epidermidis*, *Acinetobacter baumannii* and *Proteus mirabilis* while the lowest activity was shown against *Shigella dysenteriae* and *Pseudomonas aeruginosa* Table (1), but no antibacterial effect had been shown by partially purified carotenoid pigment.

The conclusion of this research indicated that staphylococcal pigments are very important virulence factor due to its ability to resist the reactive oxygen species but it also acts as an antioxidant factor better than antibacterial agent against other bacterial isolates.

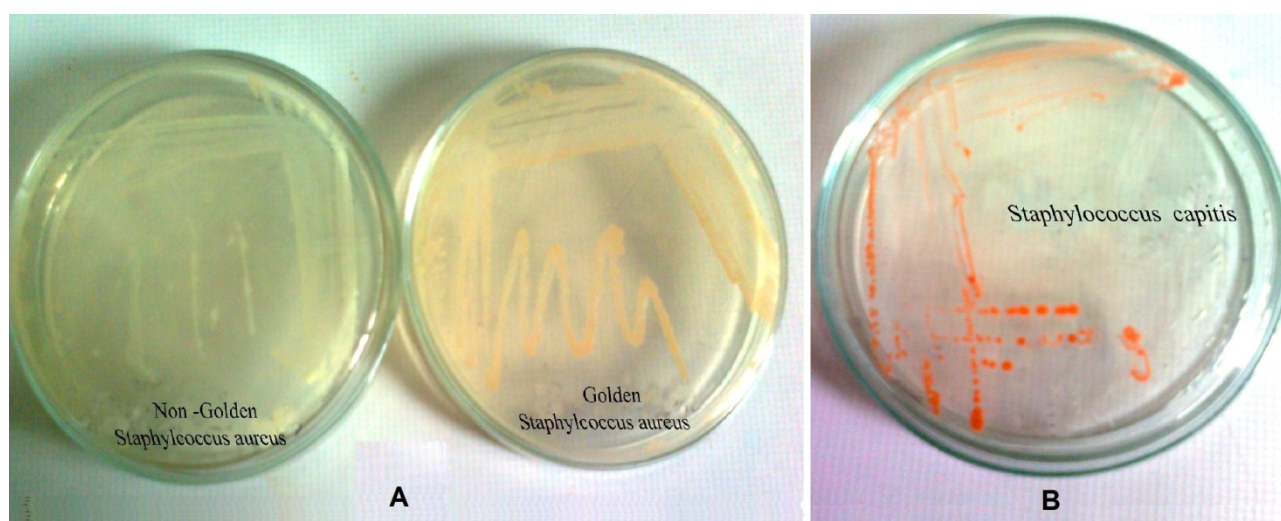


Fig. 2 : A: *Staphylococcus aureus* and B: *Staphylococcus capitis* on nutrient agar

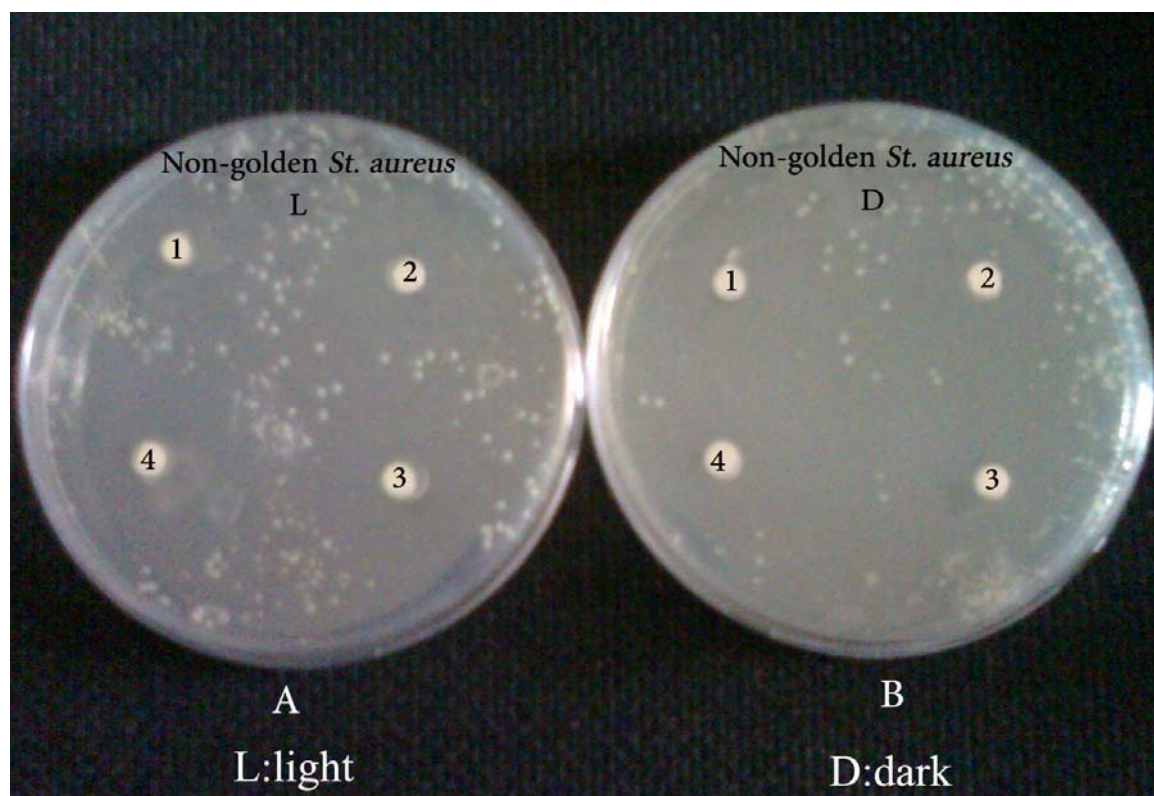


Fig. 3: Survival ability of *Staphylococcus aureus* non- producing staphyloxanthin cultured in light (A) and dark (B) condition with different concentration of H_2O_2 : 1= (0.6%), 2= (0.9%), 3= (1.2%), 4= (1.5%)

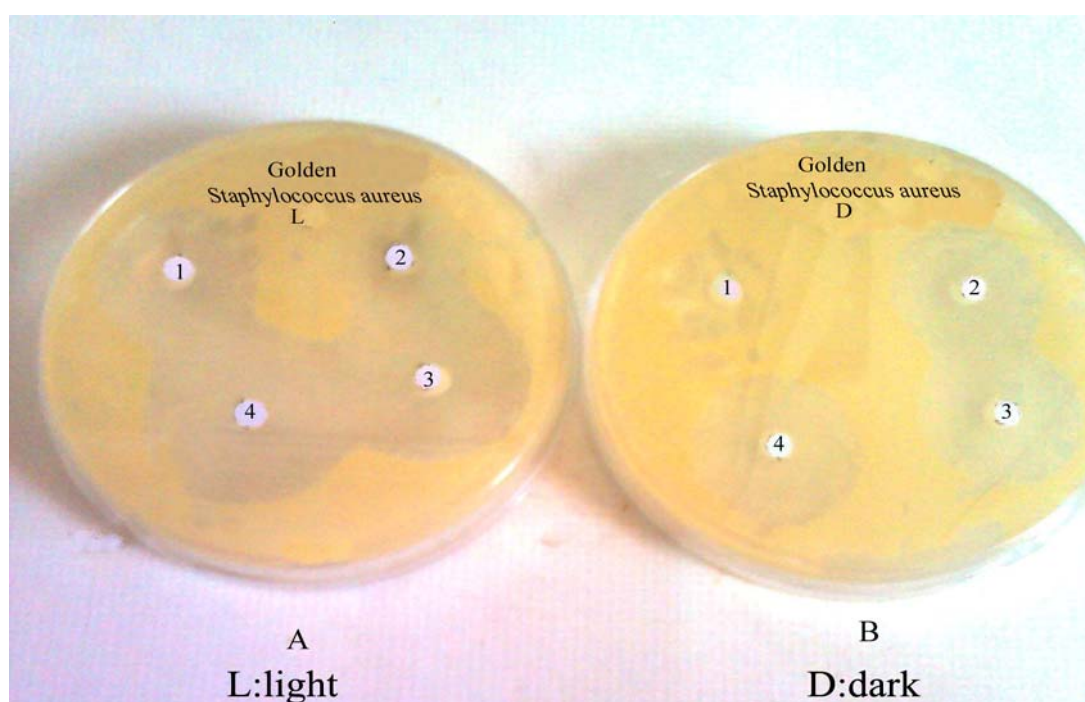


Fig. 4: Survival ability of *Staphylococcus aureus* producing staphyloxanthin cultured in light(A) and dark(B) condition with different concentration of H_2O_2 : 1= (0.6%), 2= (0.9%), 3= (1.2%), 4= (1.5%)

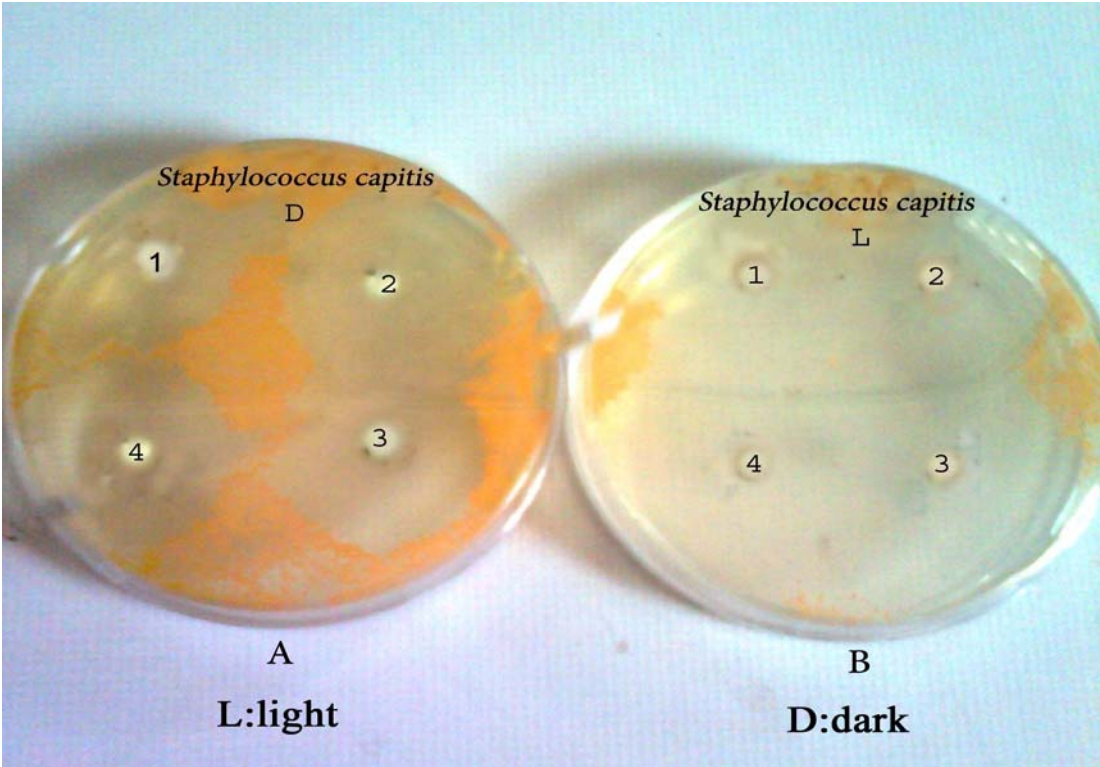


Fig. 5: Survival ability of *Staphylococcus capitis* producing pigment cultured in dark (A) and light (B) condition with different concentration of H_2O_2 : 1= (0.6%), 2= (0.9%), 3= (1.2%), 4= (1.5%)

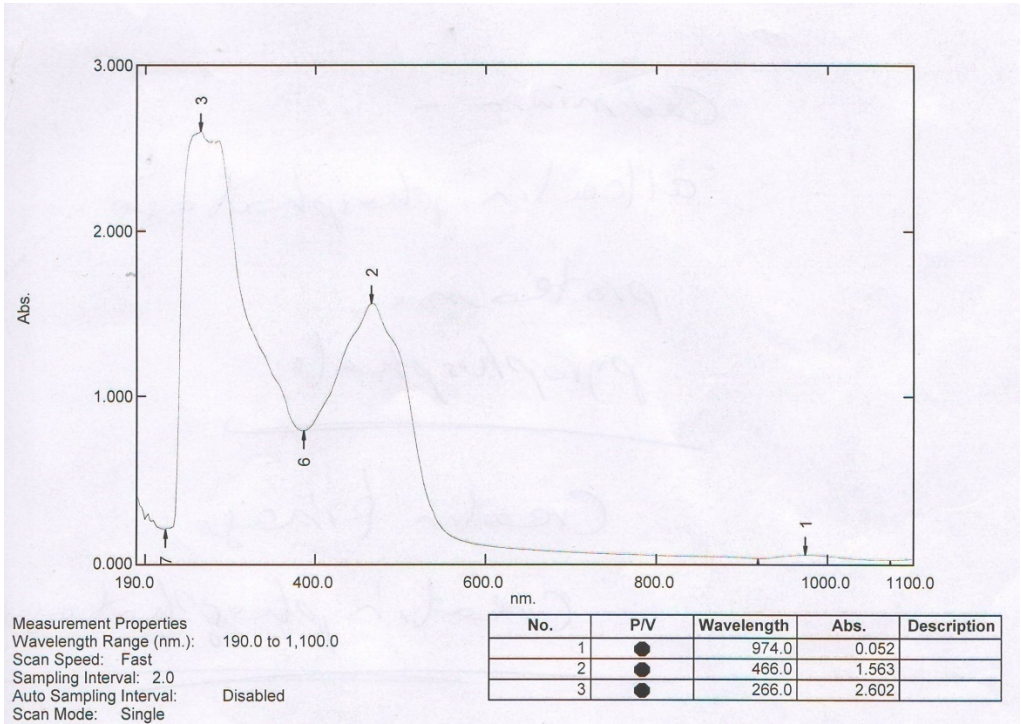


Fig. 6: UV-spectra of Extracted staphyloxanthin from *Staphylococcus aureus*. Peak 2 at 466 nm elucidate staphyloxanthin spectrum(UV-1601)

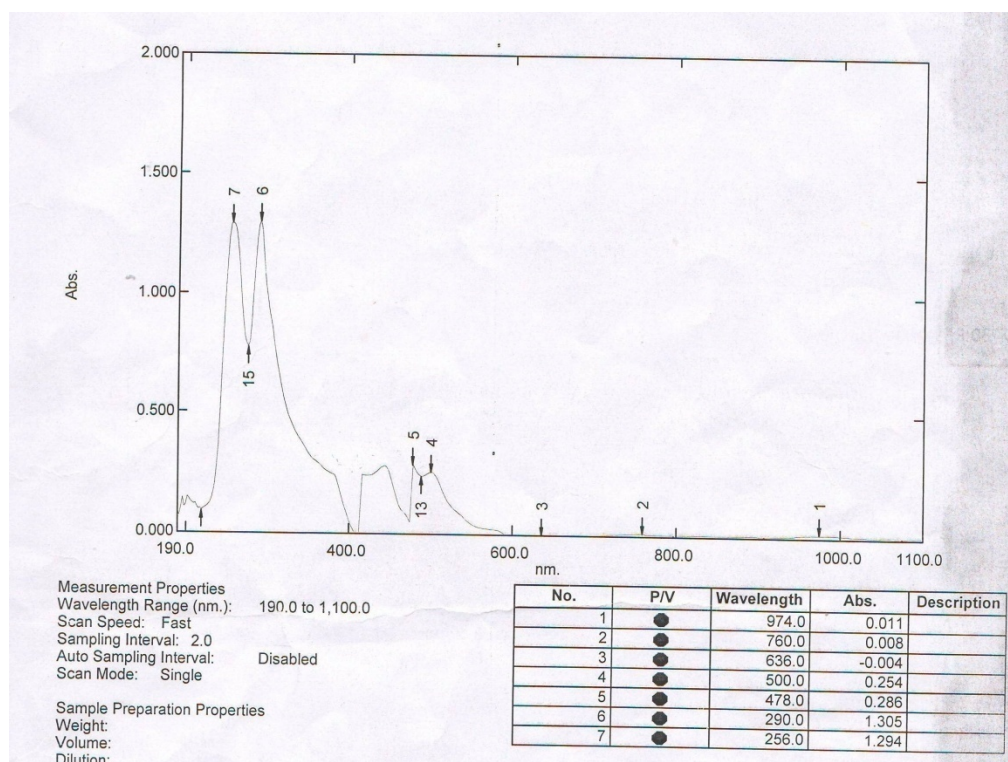


Fig. 7: UV-spectra of Extracted Carotenoid pigment from *Staphylococcus capitis* Peak 5 at 478 nm elucidate the pigment spectrum (UV-1601)

Table 1: Antibacterial activity of staphyloxanthin against different bacterial species

Bacterial species	Antibacterial activity (diameter of zone inhibition in mm.)	
	Staphyloxanthin Con. disc (400 mg/ml)	Imipemen (10 mcg)
<i>Acinetobacter baumannii</i>	12	37
<i>Escherichia coli</i>	9	24
<i>Proteus mirabilis</i>	12	24
<i>klebsiella pneumoniae</i>	10	23
<i>Shigella dysenteriae</i>	7	23
<i>Salmonella enteritidis</i>	8	23
<i>Pseudomonas aeruginosa</i>	7	33
<i>Bacillus subtilis</i>	8	23
<i>Staphylococcus epidermidis</i>	13	30
<i>Acinetobacter baumannii</i>	12	37

REFERENCES

- Clauditz, A.; Resch, A.; Wieland, K.P.; Peschel, A.; Götz. (2006). Staphyloxanthin plays a role in fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infect. Immun.* **74**, 4950-4953.
- Chung, P.Y.; Navaratnam, P.; Chung, L.Y. (2011). Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. *Annals Clin. Microbi. and Antimicrobial.* **5**(10), 25-28.

- El-Agamey, A. (2004). Carotenoid radical chemistry and antioxidant/ pro- oxidant properties. *Arch Biochem Biophys.* **430**, 37-48.
- Fang, F.C. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol.* **2**, 820-832.
- Lan, L.; Cheng, A.; Dunman, P.M.; Missiakas, D.; He, C. (2010). Golden pigment reduction and virulence gene expression are affected by metabolism in *Staphylococcus aureus*. *American Society for Microbiology.* **192**(12), 3068- 3077.
- Lenette, E.H.; Balows, A.; Hausler, W.J.; Shadomy, H.J. (1985). Manual of *American Society for Microbiology* clinical microbiology, 4th ed. Washington, DC, p. 146.
- Lui, C.; Lui, G.Y.; Song, Y.; Yin, F.; Hensier, M.E.; Jeng, W.; Nizet, V.; Wang, A.H.J.; oldfield, E. (2008). Acholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. *Science.* **319**(5868), 1391-1394.
- Liu, G. Y.; Essex, A.; Buchanan, J.T.; Datta, V.; Hoffman, H.M.; Bastian, J.F.; Fierer, J.; Nizet, V. (2005). *Staphylococcus aureus* golden impairs neutrophil killing and promotes virulence through its antioxidant activity. *JEM.* **202**(2), 209-215.
- Mishra, N.N.; Liu, G.; Yeaman, M.R.; Neast, C.C.; Proctor, R.A.; Mckinnella, J.; Bayer, S.A. (2011). Carotenoid-alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *American Soci. for Microbi.* **55**(2), 531-563.
- Plez, A.; Wieland, K.P.; Putzbach, K.P.; Hentschel, P.; Albert, K.; Cötze, F. (2005). Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. *The J. Biological Chem.* **280**, 32493-32498.
- Ryan, K.J.; Ray, C.G (2004). Sherris Medical Microbiology (4th ed.). McGraw Hill.
- Sahin, N. (2011). Significance of absorption spectra for chemotaxonomic characterization of pigmented bacteria. *Turkey J. Bio.* **35**, 167-175.
- Schmidt, K.; Connor, A.; Britton, G. (1994). Analysis of pigments carotenoids and related polyenes. In Goodfellow M, O Donnell, AG. Eds. Chemical methods in prokaryotic systematic. Johan Willy and Sons. Ltd, 403-461.
- Widomska, J.A.; Gugala, K.; Latowski, D.; Gruszecki, W.L.; Strzalka, K. (2009). Calorimetric studies of the effect of cis-carotenoids on the thermotropic phase behavior of phosphatidylcholine bilayers. *Biophys. Chem.* **140**, 108- 114.