In Vitro Anticancer Activity of Staphyloxanthin Pigment Extracted from *Staphylococcus gallinarum* KX912244, a Gut Microbe of *Bombyx mori*

Delicia Avilla Barretto¹ · Shyam Kumar Vootla¹

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Abstract The present study reports the in vitro biological nature of the pigment produced by *Staphylococcus gallinarum* KX912244, isolated as the gut microflora bacterium of the insect *Bombyx mori*. The purified pigment was characterized as Staphyloxanthin based on bio-physical characterization techniques like Fourier transform infrared spectroscopy, high performance liquid chromatography, Proton nuclear magnetic resonance spectroscopy (¹H NMR), Liquid chromatography-Mass spectroscopy and Gas chromatography-Mass spectroscopy. The Staphyloxanthin pigment presented considerable biological properties including in vitro antimicrobial activity against pathogens *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*; in vitro antioxidant activity by % DPPH free radical scavenging activity showing IC₅₀ value of 54.22 µg/mL; DNA damage protection activity against reactive oxygen species and anticancer activity evaluated by cytotoxicity assay against 4 different cancer cell lines like the Dalton's lymphoma ascites with IC₅₀ value 6.20 ± 0.02 µg/mL, Ehrlich ascites carcinoma having IC₅₀ value 6.48 ± 0.15 µg/mL, Adenocarcinomic human alveolar basal epithelial cells (A549 Lung carcinoma) bearing IC₅₀ value 7.23 ± 0.11 µg/mL and Mus mucus skin melanoma (B16F10) showing IC₅₀ value 6.58 ± 0.38 µg/mL and less cytotoxicity towards non-cancerous human fibroblast cell lines (NIH3T3) with IC₅₀ value of 52.24 µg/

mL. The present study results suggest that Staphyloxanthin acts as a potential therapeutic agent especially due to its anticancer property.

Keywords Cancer · Staphyloxanthin · *Staphylococcus gallinarum* · *Bombyx mori* · Free radicals

Introduction

Cancer is a major health issue in almost every part of the world. There is a need for development of new effective anticancer agents as the cancer treatment methods like chemotherapy and radiation therapy pose several side effects [1]. Hence, presently, natural products have been explored and investigated for the development of potent anticancer agents bearing potent antioxidant activity and minimum toxicity to the host cell [2]. The antioxidant compounds are predicted to decrease mutagenesis and thus carcinogenesis through decrease of oxidative DNA damage and by decrease of oxidant stimulated cell division [3]. The reactive oxygen species (ROS) like hydrogen peroxide (H₂O₂), superoxide anion (O₂[−]), hydroxyl radicals (OH[−]) and nitric oxide (NO₂) as well as their biological metabolites generated during normal aerobic metabolism and the electrically charged oxygen molecule trying to take off electrons from other molecules causes damage to the DNA that may become irreversible and lead to cancer [4]. ROS induces strand break base modification in DNA and DNA–protein cross links. These oxidants also contribute to aging and degenerative diseases such as atherosclerosis and cancer through oxidation of DNA, proteins and lipids [5]. Antioxidants can directly scavenge ROS and/or inhibit cell proliferation thus decreasing oxidative stress induced carcinogenesis [6].

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✉ Shyam Kumar Vootla
vootlashyam@gmail.com; vootlashyam@kud.ac.in

¹ Department of Biotechnology and Microbiology, Karnataka University, Dharwad 580003, Karnataka, India

Biological pigments are the colored substances found in plants, animals and microorganisms that carry particular color, analogous to their structure [7]. Microbial pigments are the biological pigments produced by microbial cells. In many circumstances, the microbial pigments are known to cause diseases by interfering with host immune mechanisms or by expressing pro-inflammatory or cytotoxic properties [8]. But, the breakthrough in the microbial pigments research is due to the carotenoid pigments performing diverse important biological roles in various living forms [9]. Bacterial carotenoid pigments provide promising route for applied biomedical research attributing to the wide range of bioactive properties [10]. Carotenoid pigments carry antioxidant property that helps fight the reactive oxygen species that are destructive to cellular molecules like DNA, proteins and lipids [11, 12]. Carotenoids also play photoprotectant role by contributing to protection against the damage caused by UV radiation [13, 14]. Naturally occurring carotenoids, like zeaxanthin, astaxanthin, neoxanthin, fucoxanthin, capsanthin, crocetin, β -cryptoxanthin, lycopene, α -carotene and β -carotene, lutein and phytoene are also known to have significant anticancer nature [15]. Preclinical trials of certain carotenoid pigments from microbial sources have shown potent in vivo and in vitro antitumor effects that suggests potential therapeutic roles for the carotenoid compounds [16].

Staphyloxanthin is a type of apocarotenoid triterpenoid pigment produced by many strains of *Staphylococcus* spp. Apocarotenoids are the organic compounds occurring in living organisms derived by oxidative cleavage of carotenoids [17]. Alike carotenoids, apocarotenoids are also found to be biologically active molecules bearing potent anticancer activities and antioxidative compounds [18]. The Staphyloxanthin pigment is one of the crucial virulence factors of *S. aureus* [8]. The triterpenoid pigment provides integrity to the cell membrane of the bacteria [19]. It has shown to enhance bacterial survival in unfavourable environments especially during infections [20, 21]. Staphyloxanthin is the membrane protein known for its antioxidant activity as it plays a powerful part in resistance to ROS such as O_2^- , H_2O_2 and $HOCl$ produced by host neutrophils [22–25]. Being a membrane carotenoid the strains are known to exert pathogenicity when the pigment is intact the cell but the pigment on its own is a strong antioxidant. Owing to the antioxidant nature of the Staphyloxanthin pigment combating ROS, the present study is centered on the anticancer evaluation of the Staphyloxanthin carotenoid pigment produced by the bacteria.

In this study, the bacteria producing Staphyloxanthin pigment is extracted from the resident microflora bacterium isolated from the midgut of the healthy Lepidopteran larva *Bombyx mori* (*B. mori*). The microflora present in the

insect gut has mostly shown to present symbiotic association with the insect. The resident microbial flora in insects benefits the insect by producing essential compounds like vitamins, digesting and metabolizing food, nutrient absorption, etc. [26], providing resistance to pathogens, pheromones production and immunity [27]. The commensal flora are found to play crucial part in survival of the insects by protecting their insect hosts against the natural enemies and pathogens through varied mechanisms that include colonial resistance, production of toxins and insects immune system activation to fight the invader organism [28]. The positive interaction between the gut microflora and the host has led to the increasing demand on the use of probiotic microbes for the health of human beings in order to treat or prevent diseases [29]. The probiotic microorganisms obtained from the gut and/or their secondary metabolites are known to evade infections, increase immunity, treat gut dysbiosis, fight cancer, etc. [30]. Proportionately, this host-microbe association throws light on a variety of secondary metabolites that can be derived from these microorganisms and used for a variety of purpose either for humans or for the host itself. Thus, the gut microflora and their secondary metabolites have turn out to be a huge reservoir of bioactive natural products which play beneficial part as therapeutic agents [31]. Likewise, a large number of natural products have been isolated and studied from many insect-associated microbes exhibiting biological activities like antimicrobial, anticancer, insecticidal, etc. [32–36]. Our results indicate that the Staphyloxanthin pigment isolated from the strain *Staphylococcus gallinarum* KX912244 exerts strong anticancer, antioxidant, antimicrobial and DNA damage protection activity that may be possibly due to the potent antioxidant nature of the pigment.

Materials and Methods

Chemicals

Nutrient agar and broth media and gram staining reagents were purchased from Himedia Laboratories, India. Methanol, hydrogen peroxide, ascorbic acid, ferric chloride, EtBr, agarose electrophoretic running buffer and gel loading dye were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd., India. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich chemicals Pvt. Ltd., India. pBR322 plasmid DNA was purchased from Chromous Biotech, India and agarose was bought from Lonza Pvt. Ltd, India.

Silkworm Rearing and Isolation of Gut Microflora

The silkworm, *B. mori* strain (silkworm race CSR₂ X CSR₄, Bivoltine hybrids) was used in this study. The silkworm larvae were reared on fresh mulberry leaves in the laboratory at optimum environmental conditions of 26 °C and 65% RH. Healthy silkworm was dissected to collect the midgut under sterile conditions and was washed in sterile distilled water to get rid of the food bolus and other contaminants. Sterile bacteriological saline was pipetted into the midgut and the fluid was inoculated onto the sterile nutrient agar plates. The plates were incubated at 37 °C for 24 h in order to isolate the gut microflora.

Screening of Potent Yellow Pigmented Bacteria

The colonies were screened for the intense yellow pigment production. The most intense yellow pigmented colony was isolated and sub-cultured. As the isolated colony also showed antimicrobial nature on a crowded plate technique, the colony was further subjected to secondary screening by giant colony method to confirm the capability of antimicrobial nature of the bacteria as described by Casida [37].

Identification of the Strain

Morphological Characterization

The potent isolated strain Bm 5 was subcultured and maintained on nutrient agar medium (Himedia). The strain was checked for its gram nature and morphological characteristics.

16S rRNA Gene Sequencing and Phylogenetic Analyses

Molecular identification of the isolated bacterial strain was carried out at National Collection of Industrial Microorganisms (NCIM)- National Chemical Laboratory (NCL), Pune using bacterial 16 s rRNA gene sequencing [38]. The full gene sequence of strain was compared automatically using the BLAST against the sequences of bacteria available in databanks <http://www.ncbi.nlm.nih.gov> and EzTaxondatabases [39]. A phylogenetic tree was constructed using the neighbor-joining method [40] using MEGA version 6.0.

Nucleotide Sequence Accession Number

The 16S rRNA gene sequence of the bacterial strain Bm 5 identified in this research has been deposited in National Center for Biotechnology Information (NCBI) GenBank under the accession number KX912244.

Pigment Production and Characterization

Pigment Production and Extraction

The subcultured bacterial colony was grown on milk salt agar medium at 37 °C for 4–5 days to enhance the pigment production. The bright pigmented colony was then mass cultured in nutrient broth medium and incubated at 37 °C for 7–8 days. The broth was then centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the cell pellet was used for the pigment extraction. The harvested cell pellet was re-suspended in distilled water. The pigment was then extracted with 100% methanol by repeated centrifugation until the cell pellet becomes colorless. The pigment extracted in methanol was dried by rota-evaporation [41]. The dried yellow pigment was then resuspended in 100% methanol. The methanol soluble pigment was run on the Thin layer chromatography (TLC) silica gel using 75% butanol, ethanol, water (4:1:1 v/v). The fluorescent yellow spot detected on the preparative TLC plate was scrapped off and dissolved in 100% methanol and centrifuged for 5 min at 12,000 g to remove the silica gel and to extract the pure pigment. This pure pigment was further used for characterization and for evaluating its biological activities.

Characterization of the Extracted Pigment

The pigment was characterized by biophysical and mass spectrometric techniques according to Velez [42].

Fourier Transform Infra Red Spectroscopy The FTIR spectrum was generated using KBr pellets by Nicolet 170SX FT-IR spectrometer. This method helps to identify the functional groups of the molecule. The FTIR chromatogram was scanned at 400–4000 cm⁻¹.

High Performance Liquid Chromatography HPLC was carried out using C18 column, 250 nm.

¹H NMR The ¹H NMR was obtained by JEOL at 25 °C in 5 mm NMR tubes. Operating parameters were: freq 399.78 MHz, field strength: 9.38 (400 MHz), resolution: 0.45 Hz and acquisition time: 2.18 s.

LC–MS and GC–MS Liquid chromatography–Mass spectrometry (LCMS; taken in negative mode M-H⁺) was recorded through LCMS 2010, Shimadzu, Japan and Gas chromatography–Mass spectrometry (GCMS) analysis was done through QP2010S, Shimadzu, Japan.

Biological Activities of the Pigment

Antioxidant Activity by DPPH Radical Scavenging Activity

The antioxidant activity of the pigment was measured on the basis of DPPH (1, 1-diphenyl 2-picrylhydrazyl) free radical scavenging activity as described by Brand-Williams et al. [43]. 2 mL of DPPH (0.1 mM in methanol) solution was mixed with 1 mL of different concentrations (20–100 µg/mL) of the pigment. Ascorbic acid (100 µg/mL) was used as the reference standard. 1 mL distilled water and 2 mL DPPH solution was used as the control. The reaction mixture was incubated at room temperature for 30 min in the dark. The absorbance was recorded at 517 nm spectrophotometrically.

The antioxidant activity of the compounds was calculated based on the percentage of DPPH radical scavenged as the following equation:

$$\text{Scavenging effect(\%)} = \frac{[\text{control absorbance} - \text{sample absorbance}]}{\text{Control absorbance}} \times 100$$

DNA Damage Protection Activity

The DNA damage protective study of the pigment was evaluated by agarose gel electrophoresis using the *E. coli* pBR322 plasmid as a target DNA [44]. A mixture of 10 µL of the pigment (100 µg/µL), and plasmid DNA (1:1) was incubated at room temperature for 10 min. 10 µL of Fenton's reagent (30 mM H₂O₂, 50 µM ascorbic acid and 80 µM of FeCl₃) was then added to the mixture. It was made up to 20 µL with sterile distilled water and incubated for 1 h at 37 °C. The DNA was analysed by 1% agarose gel electrophoresis using ethidium bromide staining and the extent of DNA cleavage was visualized and imaged using Gel documentation unit (Molecular Imager Geldoc gel-XR imaging system, Biorad, India). The mixture of plasmid DNA and Fenton's reagent untreated with the extract was used as a negative control.

In Vitro Anticancer Activity by Cytotoxicity Assays

The cytotoxic effect of the extracted Staphyloxanthin molecule was evaluated against Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC), Adenocarcinomic human alveolar basal epithelial cells (A549 Lung carcinoma) and Mus mucus skin melanoma (B16F10) and non-cancerous human fibroblast cell line (NIH3T3) cells in vitro using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [45]. The cells were cultured in DMEM medium and were treated using

increasing concentrations of the pigment (0, 10, 20, 50 and 100 µg/mL) for various time intervals (0–48 h), while the controls received only DMSO and each experiment was repeated a minimum of 3 independent times and used for further experiments. Briefly, MTT (Sigma-aldrich, USA) was dissolved in PBS (Phosphate buffered saline) of pH 7.5 to prepare 5 mg/mL of stock solution and filtered through 0.22 µm syringe filter to sterilize and to remove the small amount of insoluble residues. To the 100 µL of cell suspension in 96 well culture plates 10 µL of MTT was added and incubated for 48 h in a humidified incubator at 37 °C. After the incubation period, 100 µL of DMSO was added to each well and mixed thoroughly to dissolve the insoluble blue formazan crystals. The plates were read on the micro-ELISA reader (Robonics, India) at 570 nm. The readings were recorded and the inhibitory concentration-50 (IC₅₀) values of the extracted Staphyloxanthin was calculated.

Statistical Analysis

Statistical analysis was performed using SPSS software, version 20.0. Experiments were carried out in triplicate and the data are shown as mean ± standard deviation (SD). Turkey's multiple comparison test was used to evaluate significant differences between the standard ascorbic acid and the pigment. Correlation analysis was achieved using Pearson's correlation analysis using the value $P = 0.01$.

Antimicrobial Activity

The antimicrobial activity of the extracted pigment was performed by agar well diffusion method. Lag phase cultures of gram positive bacteria *Staphylococcus aureus*, gram negative bacteria *Escherichia coli* and yeast *Candida albicans* were used as the test microorganisms. 100 µL of the test organisms were swab inoculated onto the sterile nutrient agar for bacteria and potato dextrose agar for the fungus. 4 wells were bored into the agar plates using a sterile cork borer (6 mm diameter). Different concentration (25, 50, 75 and 100 µg/mL in methanol) of the pigment were added into the corresponding wells. The plates were incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for yeast followed by observing and measuring the antimicrobial activity of the pigment by measuring the zones of inhibition in millimeters using the Hi media antibiotic zone scale [46].

Results and Discussion

Isolation of *B. mori* Gut Microflora

In contrast to the diverse favorable environmental conditions insect gut provides for microbial colonization, the gut

bacteria conceivably present many useful services to their hosts [47]. The silkworm *B. mori* midgut showed a diverse group of cultivable bacterial load when grown on nutrient agar media by crowded plate method.

Screening of Potent Pigmented Bacteria

Among the bacterial colonies cultured on nutrient agar plates, the most intense yellow pigmented bacterial colony Bm 5 was isolated and sub-cultured for further research (Fig. 1). The isolated colony of bacteria also showed the property of antimicrobial producing microbes as the bacterial colony was seen as a single isolated colony on a bacterial overcrowded media repelling most microbes surrounding it which is a peculiar feature of the crowded plate technique for detection and isolation of antimicrobial compound producers [37]. Thus the isolated colony was then further evaluated for the antimicrobial activity by secondary screening technique like the giant colony method which indicated that the bacterial strain Bm 5 showed antimicrobial property against many other bacteria (Fig. 2).

Identification of the Bacterial Strain

The strain Bm 5 was observed to produce yellow colored pigmented colony on nutrient agar. On gram staining it appeared as gram positive cocci in clusters as shown in Fig. 3. The strain showed positive for catalase biochemical test and changed the media color of mannitol salt agar which is more likely the characteristics of the *Staphylococcus* genus [48]. The genus *Staphylococcus* is known to be a predominant genera among the gut microbiota of healthy silkworm but is best known for its pathogenicity during opportunistic infections [49]. The molecular and

Fig. 1 Isolation of microflora of silkworm midgut by crowded plate technique

Fig. 2 Secondary screening of antimicrobial property of the isolated bacterial colony by giant colony technique

Fig. 3 Gram positive cocci in clusters on gram staining

phylogenetic analysis showed close relatedness with *Staphylococcus gallinarum* and thus the strain was identified as *Staphylococcus gallinarum* the phylogenetic analysis of which is given in Fig. 4. *Staphylococcus gallinarum* has been cultured and identified as normal gut microflora of many insect species among the *Staphylococcus* genera [50]. Atteyat et al. [51] has reported the role of *Staphylococcus gallinarum* as a biological control agent isolated from an insect *Bemisia tabaci* owing to produce an antibiotic molecule gallidermin.

Pigment Production and Characterization

Pigment production and extraction of *Staphylococcus gallinarum* showed enhanced pigmentation when cultured on milk salt agar medium (Supplementary file Fig. S1 A). Milk salt agar medium is mostly used for selective isolation

Fig. 4 Phylogenetic tree representing evolutionary relationship of *Staphylococcus gallinarum* DSBM5.1 (GenBank accession no: KX912244) with other closely related *Staphylococcus* strains

and cultivation of *Staphylococci* as it contains high salt concentration of 6.5% avoiding the growth of other unwanted microbes as *Staphylococci* can tolerate high salinity [52] and also enhances pigmentation [48]. The intensified pigmented bacterial colony was then mass cultured in nutrient broth (Supplementary file Fig. S1 B) and the pigment was extracted in methanol and air dried. The pigment was then purified by preparative TLC method and the Supplementary file Fig. S1 C shows the pure pigment.

Characterization of the Extracted Pigment

FTIR Analysis of the Extracted Pigment

The FTIR data presented large number of functional groups like the alcohols, phenols, alkanes, esters, saturated aliphatic, aromatics, carboxylic acids, ethers (Table 1 and Fig. 5). The functional groups observed are due to the presence of moieties like β -D-glucopyranose, methyltracosane, fatty acid ester, triol, etc. in the Staphyloxanthin structure.

HPLC Analysis of the Extracted Pigment

The HPLC chromatogram of the extracted pigment showed a retention time of 0.645 as shown in Fig. 6 which is the

characteristic of certain pigments as shown by Varzakas and Kiokias [53].

^1H NMR Analysis of the Extracted Pigment

The ^1H spectra of the extracted pigment were recorded in the CDCl_3 solvent which are in good agreement with proposed structure of the Staphyloxanthin molecule as shown in Fig. 7. The -OH protons of C_{18} , C_{19} and C_{20} were resonated at 4.97, 4.83 and 4.38 ppm respectively whereas $\text{C}_{17}\text{-H}$, $\text{C}_{21}\text{-H}$ and $\text{C}_{25}\text{-H}$ were resonated at 3.65, 6.45 and 7.28 ppm respectively and the protons were resonated in the expected region. The structure elucidation by ^1H NMR confirms that the pigment is Staphyloxanthin that was in correlation with the similar findings by Pelz et al. [54] and Marshall and Wilmoth [55].

LC-MS and GC-MS Analysis of the Extracted Pigment

The major mass spectrum (MS) peak of 819 was characterized as Staphyloxanthin based on LC-MS analysis in negative mode (Fig. 8). LC-MS scan showed mass peak of 819 $[\text{M-H}]^+$ which corresponds to the molecular weight of Staphyloxanthin ($\text{C}_{51}\text{H}_{78}\text{O}_8$) [54], a C_{30} a membrane-bound triterpenoid carotenoid of *Staphylococcus* spp. [55]. The molecular weight Staphyloxanthin $[\text{M} + \text{H}]^+$ as 819.3

Table 1 FTIR data for the extracted Staphyloxanthin pigment

Frequency (cm ⁻¹)	Bond	Functional groups
3443.29	OH-stretch, H-bonded	Alcohols, phenols
2924.75, 2854.23	C–H stretch	Alkanes
1748.16	C=O stretch	Esters, saturated aliphatic
1636.40, 1458.62	C–C stretch (in ring)	Aromatics
1268.36, 1168.27, 1109.04, 1018.02	C–O stretch	Alcohols, carboxylic acids, esters, ethers

Fig. 5 FTIR chromatogram of extracted Staphyloxanthin pigment **Fig. 6** HPLC chromatogram of the extracted Staphyloxanthin pigment

has previously been predicted by Pelz et al. [54]. Apart from the MS peak mass fragmentation (m/z) peaks were also evaluated for the Staphyloxanthin molecule. Mass fragmentation of Staphyloxanthin elucidated using Chem-Draw Ultra 8.0 software showed m/z peaks at 415 $[M-H]^+$ and 432 $[M-H]^+$ that is due to the molecules like 4,4-Diaponeurosporene-aldehyde and 4,4-Diaponeurosporenoate molecules [55]. The mass fragmentation (m/z) pattern of Staphyloxanthin when studied using Chem-Draw Ultra 8.0 software illustrated 3 fragments of

Staphyloxanthin that also matches well with the GC–MS peaks (Fig. 9, supplementary file Fig. S2).

Biological Activities of the Pigment

The carotenoid pigments are best known for a variety of biological activities that make them centre of many research especially as antioxidant, antiproliferative, anti-cancer, antimicrobial, DNA damage protection, etc.

Fig. 7 ^1H NMR spectrum of the extracted Staphyloxanthin pigment

Fig. 8 LC-MS chromatogram showing mass peak and m/z values for the extracted Staphyloxanthin pigment

Antioxidant Activity

Saphyloxanthin has shown considerable DPPH free radical scavenging activity (%) with IC_{50} value 54.22 $\mu\text{g/mL}$ when compared to the standard ascorbic acid with IC_{50} value of 35.54 $\mu\text{g/mL}$ as shown in Table 2 and Fig. 10. The standard deviation values are significant at 0.001 level by

biostatistics analysis with SPSS 20.0 software. Clauditz et al. [23] has demonstrated that the Staphyloxanthin molecule scavenges free radicals with its conjugated double bonds. Hence, it's obvious that the Staphyloxanthin carotenoid play the most potent role as antioxidant. Almost all carotenoids typically bear antioxidative activity [56]. The antioxidant activities of carotenoids have been

Fig. 9 GC-MS chromatogram showing Staphyloxanthin m/z value

Table 2 DPPH free radical scavenging activity of the extracted Staphyloxanthin pigment

Concentration in $\mu\text{g/mL}$	Radical scavenging activity (%)	
	Standard ascorbic acid**	Staphyloxanthin pigment**
20	41.37 $\pm$ 1.19	30.82 $\pm$ 1.60
40	56.28 $\pm$ 1.55	36.89 $\pm$ 1.45
60	76.35 $\pm$ 1.79	61.32 $\pm$ 1.77
80	82.19 $\pm$ 1.92	67.96 $\pm$ 1.59
100	91.76 $\pm$ 1.58	81.02 $\pm$ 1.59
IC ₅₀ $\mu\text{g/mL}$	35.54	54.22

Values are expressed as mean $\pm$ SD of n = 3 in each group

**Correlation is significant at the 0.01 level (Pearson's 2-tailed)

Fig. 10 Graph showing antioxidant activity of Staphyloxanthin pigment

examined quite often as it is produced in almost all living forms. A typical carotenoid pigment possess a carbon backbone with alternating single and double bonds. These alternating bonds make the carotenoid molecule a strong antioxidant by absorbing excess energy from ROS [57]. Kim et al. [58] investigated the radical scavenging activity of C₃₀ carotenoids, using the DPPH free radical method that proved the antioxidant activity of these carotenoids. Liu et al. [59] has reported the pigment Staphyloxanthin as a strong antioxidant imparting virulence character for the Staphylococci allowing them to evade the neutrophil killing.

DNA Damage Protective Activity

The Staphyloxanthin pigment depicted significant DNA damage protective activity (Fig. 11). The agarose gel picture showed supercoiled and open circular bands for the control the pBR322 plasmid DNA. The cleavage of the DNA was seen for the pBR322 DNA as shearing of the plasmid DNA on reaction with the Fenton's reagent that produces ROS creating stress and damage to the DNA

Fig. 11 DNA damage protection activity of the extracted Staphyloxanthin pigment. Lane 1: pBR322 DNA. Lane 2: pBR322 DNA + Fenton's reagent. Lane 3: pBR322 DNA + Fenton's reagent + Staphyloxanthin pigment

(lane 2). But the Staphyloxanthin treated DNA when reacted with the Fenton's reagent avoids DNA damage and thus the DNA appears intact without any shearing of the DNA (lane 3). The study by Clauditz et al. [23] has demonstrated that the Staphyloxanthin molecule is useful in fighting the ROS produced during the oxidative burst, thus protecting the biomolecules like nucleic acids, proteins and lipids. This throws light on the DNA damage protective nature of the pigment. Our findings have also proved that the Staphyloxanthin molecule presents significant DNA damage protection against ROS produced. Devasagayam et al. [60], has reported the capability of natural oxidants like carotenoids to protect the plasmid DNA pBR322 against ROS. Matos et al. [61] have investigated on carotenoids involved in protection against oxidative damage caused in prostate cancer. Antioxidants and their protective role against DNA damage has also been demonstrated by Nisha and Deshwal [62]. ROS like hydroxyl free radicals are known to cause damage to the cellular DNA in humans and can make the DNA damaged cell a cancerous one [63]. Hence, antioxidants also are useful in combating cancer (Table 3).

In Vitro Anticancer Activity

The cytotoxicity and cell proliferation assays carried out to evaluate the anticancer activity of Staphyloxanthin pigment has shown considerable in vitro anticancer activity against standard 4 different cancer cell lines Dalton's lymphoma ascites (DLA) with IC_{50} value 6.20 ± 0.02 $\mu\text{g/mL}$, Ehrlich ascites carcinoma (EAC) possessing IC_{50} value

6.48 ± 0.15 $\mu\text{g/mL}$, Adenocarcinomic human alveolar basal epithelial cells (A549 Lung carcinoma) with IC_{50} value 7.23 ± 0.11 $\mu\text{g/mL}$ and Mus mucus skin melanoma (B16F10) indicating IC_{50} value 6.58 ± 0.38 $\mu\text{g/mL}$ and less cytotoxicity towards non-cancerous human fibroblast cell lines (NIH3T3) with IC_{50} value of 52.24 $\mu\text{g/mL}$. This confirms that the pigment is a potential anticancer agent that can destroy the cancer cells in very low concentration without harming the normal cells and is biocompatible. The anticancer nature of the pigment maybe due to the antioxidant nature of the molecule as the research by Nisha and Deshwal [62] highlights that the antioxidant nature of several carotenoid molecules is the key factor to fight cancer by avoiding the damage of DNA and other biological molecules like proteins and lipids. A review by Sharoni et al. [18] describes that the apocarotenoids may acts as anticancerous agents and thus can be useful in the prevention of cancer and other degenerative diseases. Several studies have reported the role of microbial pigments especially the Prodigiosin pigment, from *Serratia marcescens* to induce apoptosis and cell cycle inhibition of cancer cells [64, 65]. Prodigiosin has shown antiproliferative and cytotoxic effects when investigated for cytotoxicity against 60 human tumor cell lines of colon, renal, lung, ovarian, leukaemia and brain cancer melanoma [66]. But no study has been reported on Staphyloxanthin as an anticancer agent till date.

Antimicrobial Activity

Apart from the anticancer nature of the pigment, it indicated antimicrobial activity examined against gram positive bacteria *Staphylococcus aureus*, gram negative bacteria *Escherichia coli* and yeast *Candida albicans* as shown in Fig. 12 and Table 4. The maximum inhibition was seen for *Escherichia coli* followed by *Staphylococcus aureus* and *Candida albicans*. The Minimum inhibitory concentration (MIC) was recorded as 25 $\mu\text{g/mL}$ for *Escherichia coli* and 50 $\mu\text{g/mL}$ for *Staphylococcus aureus* and *Candida albicans*. A study by Kirti et al. [67] has reviewed the antimicrobial property of carotenoid pigments produced by different microbes. Li et al. [35] has demonstrated the antifungal activity of pigments produced by *Photobacterium luminescens* a gut symbiont of the nematode

Table 3 Anticancer activity of the extracted Staphyloxanthin pigment

IC_{50} value $\mu\text{g/mL}$	DLA (Lymphoma)	EAC (Carcinoma)	A549 (Lung Carcinoma)	B16F10 (Melanoma)	NIH3T3 Fibroblast
	6.20 ± 0.02	6.48 ± 0.15	7.23 ± 0.11	6.58 ± 0.38	52.24 ± 0.12

Values are expressed as mean $\pm$ SD of $n = 3$ in each group

**Correlation is significant at the 0.01 level (Pearson's 2-tailed)

Fig. 12 Antimicrobial activity of the extracted Staphyloxanthin pigment against pathogens A) *E. coli*, B) *S. aureus* and C) *C. albicans*

Table 4 Antimicrobial activity of the extracted Staphyloxanthin pigment

Concentration in µg/mL	Zone of inhibition in mm		
	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>
25	–	10	–
50	10	11	10
75	12	13	11
100	13	15	12

Heterorhabditis. Antimicrobial activity of the carotenoids isolated from yeast has been previously reported by Manimala et al. [68] and Umadevi and Krishnaveni [69] also reported antibacterial activity of a pigment isolated from the bacterial strain *Micrococcus luteus*. Antimicrobial activity of *Pseudomonas aeruginosa* produced pigment Pyocyanin was reported by El-Shouny et al. [70]. As the C₃₀ Staphyloxanthin carotenoid pigment extracted in this study has very low MIC value it indicates that it can be used as antimicrobial agent against pathogenic microbial forms.

Conclusion

Cancer, a major cause of death, is a salient free radical disease. The free radicals are known to cause DNA damage and hence play an important role in carcinogenesis. The antioxidants are known to decrease carcinogenesis by scavenging ROS and thus reducing the oxidative DNA damage. Insect-associated microbes have recently been used as a potent source of natural products with biological activity. Pederin, an anticancer agent has been explored from an insect symbiotic bacteria. Likewise, in this study, we have reported the C₃₀ triterpenoid carotenoid pigment molecule named Staphyloxanthin produced by a microflora bacteria *Staphylococcus gallinarum* isolated from the insect *Bombyx mori* gut. Staphyloxanthin is a strong antioxidant produced by mostly the pathogenic *S. aureus* strains evading ROS, hence this carotenoid bearing the antioxidant property was able to scavenge free radicals.

The pigment showed considerable in vitro anticancer activity against 4 different cancer cell lines, but very less cytotoxicity towards normal non-cancerous cell lines, thus making this pigment a potent anticancer agent. Apart from the antioxidant and anticancer nature, this pigment showed good antimicrobial activity against bacteria and fungi thus making it a good therapeutic agent.

To the best of our knowledge, this study is the first report on anticancer property of Staphyloxanthin pigment with regard to its antioxidant nature and also on antimicrobial nature of the pigment. The molecule Staphyloxanthin is best known for its virulence nature when produced intracellularly, but the antioxidant nature of the pigment was neglected in light of the potential use in therapy. Hence, our study has successfully proved the anticancer nature of the pigment that paves way for the molecule to be used as a therapeutic agent.

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