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Original article

STUDY THE IMPACT OF PURIFIED STAPHYLOXANTHIN EXTRACTED FROM STAPHYLOCOCCUS AUREUS ON CAL-51 CANCER CELL LINE

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Abstract

Background. Breast cancer is a disease characterized by the abnormal growth of breast cells, which can grow uncontrollably and form tumors. If left untreated, these tumors can spread throughout the body and become life-threatening.

The CAL51 cell line was first isolated from a malignant pleural effusion of a woman with metastatic breast cancer. These cells grow in continuous culture and exhibit the morphological and ultrastructural features of epithelial cells of mammary origin. They are tumorigenic in nude mice and form colonies in soft agar. Estrogen receptors are not detected in these cells.

This study aimed to investigate the cytotoxicity of purified staphyloxanthin on a specific breast cancer model, the CAL51 cell line, and to demonstrate the pigment's effect as a potential treatment for this type of cancer. The study also sought to determine the ideal concentration of staphyloxanthin required to inhibit tumor cell growth. In addition, it evaluated the effect of the pigment on a normal, non-malignant cell line (REF cells).

Materials and methods. A total of 106 specimens were collected from hospitals in Baghdad. Patients of different age groups, both male and female, were included in the study, which spanned from October to April 2023. All bacterial isolates were

examined for their microscopic, biochemical, and cultural characteristics. The results were further confirmed using the Vitek2 system.

Staphyloxanthin was extracted using ethyl acetate and acetone as solvents and purified through column chromatography. Four concentrations of the staphyloxanthin pigment, incubated for 24 hours, were used in the trial treatment of the CAL-51 tumor cell line and the normal REF cell line in vitro.

Results. A total of 53 bacterial isolates were collected, with 50% identified as *Staphylococcus aureus*. The results indicated that the primary source of *S. aureus* infection was the nasal cavity (from patients and medical staff), followed by wound ulcers and burns, which accounted for 38.7% of the infections. These sites were identified as the most common sources of bacterial infection.

In addition, the results showed that bacterial infections in urine and blood samples accounted for 22% and 26%, respectively, while 21% of the isolates were obtained from eye samples. Other sources of bacterial isolates were found at lower levels.

The ability of the bacteria to produce the pigment was tested, with the TID12 strain showing the highest production value of 1.87.

The extracted pigment concentration was 170.997 U/cell. In the CAL-51 tumor cell line, the maximum inhibition rate was 83% at a concentration of 1000 μg/ml, followed by 76% at 500 μg/ml, 73% at 250 μg/ml, and 70% at 125 μg/ml.

In contrast, for the normal REF cell line, tested at the maximum time point of 72 hours, the inhibition rate was significantly lower: 28% at 1000 μg/ml, 25% at 500 μg/ml, 22% at 250 μg/ml, and 20% at 125 μg/ml.

Conclusion. The purified staphyloxanthin pigment demonstrated a cytotoxic effect on the CAL-51 cell line at varying concentrations. However, it exhibited little to no effect on the normal REF cell line.

Keywords: staphyloxanthin pigment; CAL-51; REF; TID12; breast cancer; cell line

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Introduction

The objective of this work was to examine the cytotoxic effects of bacterial staphyloxanthin on the normal cell line (REF) and the breast cancer cell line CAL-51. In addition, the study aimed to evaluate the safety of using DMSO as a solvent on both cancerous and normal cell lines.

Staphylococcus aureus is a well-known human pathogen responsible for a variety of clinical symptoms [1]. It is a Gram-positive bacterium with a cocci

shape that forms “grape-like” clusters. The term “aureus,” meaning golden or yellow, refers to the color of its colonies. When cultured, *S. aureus* can withstand up to 10% NaCl and grow within a temperature range of 18°C to 40°C. It is often classified as a facultative anaerobe. Standard identification tests include mannitol fermentation, coagulase, and catalase tests [2].

Literature review

Staphyloxanthin is a carotenoid pigment produced by certain strains of *Staphylococcus aureus*. Carotenoids are physiologically active substances with strong antioxidant and anticancer properties [3]. Membrane-bound carotenoids, such as staphyloxanthin, are composed of triterpenoid carotenoids. These differ from the C40 carotenoids found in other organisms by their unique C30 chain [4].

To develop effective anticancer drugs with strong antioxidant activity and minimal toxicity to host cells, researchers have focused extensively on natural compounds [5]. Antioxidant compounds are believed to reduce mutagenesis and carcinogenesis by decreasing oxidative DNA damage and oxidative stress-induced cell proliferation [6].

Preclinical studies have demonstrated significant in vivo and in vitro anticancer effects of carotenoid pigments derived from microorganisms, suggesting their potential therapeutic applications [7]. This study focuses on evaluating the anticancer effects of the staphyloxanthin carotenoid pigment produced by bacteria to assess its potential for treating tumors.

CAL-51 is a widely used breast cancer cell line replicated by various research organizations over many years [8]. It is considered a reliable model for global research on breast tumors, including studies on cancer therapies [9]. The CAL-51 cell line was derived from a malignant pleural effusion of a woman with metastatic breast cancer. These cells display the morphological, ultrastructural, and immunohistochemical characteristics of mammary epithelial cells. They grow in continuous culture, are tumorigenic in nude mice, and form colonies in soft agar. However, they do not exhibit estrogen receptors [10].

Previous studies have shown that certain natural products derived from plants exhibit significant cytotoxicity against CAL-51 triple-negative breast cancer cells, often achieving lower IC50 values [11].

Materials and methods

Materials

A total of 109 distinct pathogenic specimens were collected from patients in hospitals located in Baghdad. The patients varied in age and gender, and

the specimens were obtained between December 2022 and March 2023. The samples included blood, urine, eye secretions, and specimens from burns and wounds.

Assessment the ability of isolates to produce staphyloxanthin

To assess staphyloxanthin formation, a tube containing 10 ml of prepared brain heart broth was inoculated with 100 µl of bacterial isolate for each sample. The isolates were cultured at 37°C for 18 hours to achieve a concentration of 1.5×10^8 CFU/ml, as determined by comparison with the McFarland standard. Subsequently, 100 µl of each isolate was added to skim milk agar, which was incubated at 37°C for two days, followed by an additional two days at 20°C.

A positive result was indicated by the growth's appearance with yellow or orange pigmentation [12]. The pigment extraction from the selected isolate (TID12) was performed using ethanol and ethyl acetate solvents, based on the method described in [13].

The extraction technique involved removing mature bacterial cells from the milk agar plates and rinsing the agar surfaces with double-distilled water. The harvested cells were centrifuged at 6000 rpm for 15 minutes. The resulting pellet was suspended in 10 ml of ethanol solvent, while the supernatant was discarded. The extract was incubated at 40°C for 20 minutes, and the supernatant was concentrated under vacuum to isolate the pigment.

For further extraction steps, 1.7 M aqueous NaCl solution (1:1 v/v) and ethyl acetate were added, followed by vortex mixing. The crude extract was collected under vacuum, and the total pigment volume was quantified [12].

Staphyloxanthin assay

According to the procedure described by [14], the extracted pigment was quantified using the method outlined in [15]: The carotenoid content was measured in units per cell using the following equation:

$$\text{Carotenoid measurement (unit/cell)} = (V \times A / 0.175 \times W),$$

where: $\boldsymbol{A}$ is refers to the absorbance of the pigment dilution measured at 450 nm, $\boldsymbol{V}$ is the final volume of the extracted pigment (ml), $\boldsymbol{W}$ is the total weight of the dried pigment powder (g), and **0.175** is the extraction coefficient for carotenoid pigments.

The purification steps of pigment using column chromatography

After completing the extraction steps for the TID12 pigment (selected as the highly productive isolate), purification was performed using a column chromatography procedure. Silica gel (Merck, 20 g) was used, with ethanol serving as the dissolving agent. The crude extract was applied to a chromatography column with dimensions of 1.5 × 60 cm.

Fractions were eluted using ethyl acetate and subsequently dried through evaporation. To protect the light-sensitive pigments, all purification steps were carried out in complete darkness. The absorbance of the separated fractions was measured at 450 nm.

The purified fractions were stored at -20°C. To measure pigment absorption, the peak fraction of staphyloxanthin was combined [15].

Treatment steps of cancer cells by the purified pigment

As described in [16], after examining plates containing freshly prepared cells under an inverted microscope to ensure proper maturation, the following steps were carried out:

1. Preparation of Pigment Concentrations: To obtain pigment concentrations of 125, 250, 500, and 1000 µg/ml, sterile test tubes were used for serial one-half dilutions. All pigment dilutions were prepared immediately before the experiment. Staphyloxanthin doses were prepared by dissolving 0.01 g of purified pigment in 0.4 ml of DMSO solvent, followed by the addition of 10 ml of serum-free RPMI medium. This mixture was considered the initial 1000 µg/ml concentration. Serial dilutions were then prepared by transferring 5 ml from the 1000 µg/ml solution into subsequent tubes to achieve the desired concentrations.
2. Application to Culture Wells: After removing the transparent adhesive film, the culture media in each Falcon well was carefully removed. Then, 0.2 ml of each pigment concentration was added to approximately three wells per concentration. One designated column of wells served as a negative control, to which 0.2 ml of serum-free RPMI medium was added instead. All additions were performed as quickly as possible to maintain consistency, and the plates were resealed with adhesive film and gently swirled to ensure even distribution.
3. Incubation: All plates were incubated at 37°C for 24 hours, except for the Rat Embryo Fibroblast (REF) plate, which was incubated for 72 hours.

Staining steps of tissue culture plates

To determine the cytotoxic effect of staphyloxanthin on tumor cells, crystal violet staining was used, as described in [17]. The procedure was as follows:

1. Pigment Exposure and Staining: After a 24-hour period of pigment exposure on tumor cells, the contents of each well were emptied. Crystal violet solution (0.1 ml) was added to each well, and the plates were incubated for 30 minutes at 37°C.
2. Rinsing and Drying: After incubation, the crystal violet solution was removed, and any excess stain was gently washed away using water. This was

followed by rinsing with distilled water. The plates were then turned over and left to dry at room temperature.

3. Optical Density Measurement: The optical density (OD) of the crystal violet-stained cells was measured using an ELISA microplate spectrophotometer at 492 nm.
4. Inhibition Rate Calculation: The percentage inhibition rate (IR) was calculated based on the formula provided in [13].
5. $$Inhibition\ ratio\% = \frac{(\text{Optical density of control} - \text{Optical density of test})}{(\text{Optical density of control})} * 100\%$$

Statistical analysis was conducted on the optical density data to determine the pigment concentration required to reduce the viability of each cell line.

Result and discussion

Cultural characteristics

The colony morphology of the *S. aureus* isolates grown on blood agar base media, mannitol agar, and selective media revealed distinct cultural characteristics. These isolates appeared smooth, raised, glistening, and translucent, with varied pigmentation. Consistent with [18], the isolates exhibited *S. aureus* features, including cocci forms arranged in grape-like clusters, Gram-positive staining, and non-motile behavior.

Figure 1. Extraction of staphyloxanthin by using ethyl acetate and ethanol.

The ability of *S. aureus* isolates to produce staphyloxanthin was assessed by culturing the isolates on skim milk agar. Out of 52 isolates, only ten were found to produce staphyloxanthin. Among these, isolate TID12, obtained from a burn infection, demonstrated the highest productivity, with a measured value of 1.87.

The extraction of staphyloxanthin using ethyl acetate and ethanol yielded a highly concentrated product, with an absorbance of 0.628 at 450 nm. Figure 1 shows that the staphyloxanthin concentration produced by the selected isolate was 172.989 U/cell.

Staphyloxanthin treatment of cancer cell line

The CAL-51 cell line was treated with four concentrations of staphyloxanthin pigment over a 24-hour exposure period. The inhibition ratio was statistically significant ($p < 0.05$), as shown in Table 1 and Figure 2. The highest inhibition rate observed was 83% at a concentration of 1000 µg/ml, followed by 76% at 500 µg/ml, 73% at 250 µg/ml, and 70% at 125 µg/ml. In addition, the results indicated that the solvent DMSO had minimal or no impact on the tumor cells (Table 1).

Table 1.

Inhibition rate of CAL-51 tumor line when exposed at 24 hrs with the pigment.

Concentration ug/ml	Inhibitory concentration of pigment %	Inhibitory Concentration of the solvent %	X^2 :P /values
1000 Mg/ml	83%	22%	0.000
500 Mg/ml	76%	16%	0.000
250 Mg/ml	73%	13%	0.000125
125 Mg/ml	70%	6%	0.000

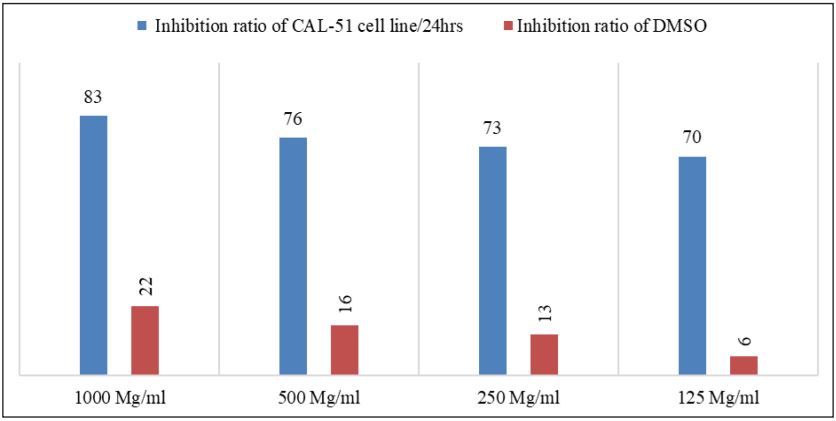

Figure 2. The inhibition rate of CAL-51 when exposed to pigment at 24 hours.

Effect of staphyloxanthin on normal (REF) cell line

The fourth concentration of staphyloxanthin pigment was applied to the REF normal cell line during a 72-hour exposure period. According to Table 2 and Figure 3, the inhibition ratio was found to be non-significant ($p > 0.05$). The inhibition rates were 25% at a concentration of 1000 µg/ml, 22% at 500 µg/ml, 19% at 250 µg/ml, and 17% at 125 µg/ml.

In addition, the data demonstrate that the solvent DMSO had no impact or only a minor effect on normal cells. For the four concentrations (1000 µg/ml to 125 µg/ml), the inhibition rates caused by DMSO alone were 12%, 8%, 7%, and 4%, respectively.

Table 2.

The inhibition rate to REF normal line when exposed to pigment at 72 hours.

Concentration Mg/ml	Inhibitory Concentration rate of pigment%	Inhibitory Concentration of DMSO solvent %	X^2 test:P value
1000 Mg/ml	25%	12%	0.67
500 Mg/ml	22%	8%	0.33
250 Mg/ml	19%	7%	0.18
125 Mg/ml	17%	4%	0.22

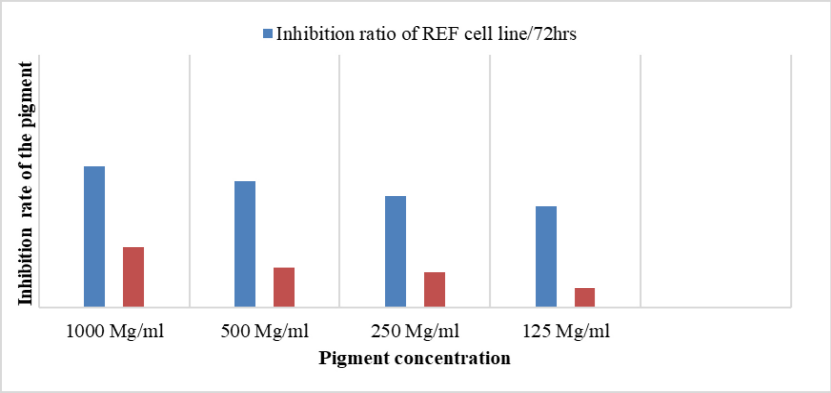

Figure 3. The Inhibition rate REF normal line when exposed to pigment at maximum time (72 hrs).

Discussion

The results of pigment production align with those reported in [19], which revealed that the total quantity of staphyloxanthin produced was 154.948 U/cell. Cytotoxicity assays were conducted to assess the pigment's anticancer effect, showing significant in vitro activity against various cancer cell lines.

Several studies have demonstrated the potent cytotoxic effects of staphyloxanthin. For instance, the crude form of staphyloxanthin extract exhibited significant cytotoxic activity, with an IC50 value of 841.3 μg/ml on the MCF7 breast cancer cell line [20]. Similarly, studies on lycopene treatment revealed a reduction in cell proliferation, with higher concentrations of carotenoid extracts leading to decreased viability. Lycopene was shown to inhibit the viability of the HepG2 liver cancer cell line by approximately 30% [21].

Another study [22] demonstrated that prodigiosin, a related pigment, exhibits its anticancer effects by inducing apoptosis in human choriocarcinoma (JEG3) and prostate cancer (PC3) cell lines, even at low concentrations. Furthermore, research has highlighted the broad in vitro anticancer activity of carotenoid pigments against four different cancer cell lines: Ehrlich ascites carcinoma (EAC), Dalton's lymphoma ascites (DLA), melanoma (B16F10), and adenocarcinomic human alveolar basal epithelial cells (A549 lung carcinoma). Interestingly, these pigments showed minimal cytotoxicity against non-cancerous human fibroblast cell lines (NIH3T3), with an IC50 value of approximately 52.24 μg/ml [23]. This suggests that staphyloxanthin could be a promising anticancer agent, capable of selectively targeting tumor cells while sparing normal cells like NIH3T3 and REF cell lines.

The anticancer properties of staphyloxanthin are likely linked to its antioxidant activity, attributed to its apocarotenoid structure, as discussed in [24]. This structure helps protect DNA and other biological molecules, such as lipids and proteins, from damage, which is critical in combating tumors. A study by [25] further emphasized that apocarotenoids, including staphyloxanthin, are strong antitumor agents and should be considered in cancer prevention programs and treatments for other severe diseases.

Moreover, studies have shown that staphyloxanthin has no adverse effects on REF cell lines, even after prolonged exposure of up to 72 hours, suggesting that the pigment does not induce physiological or structural changes in normal cells [26]. Another study [27] confirmed that the pigment lacks genotoxicity in tested ordinary cell lines, supporting its potential use in pharmaceutical development and future cancer treatment strategies.

Conclusion

The TID12 strain of *Staphylococcus aureus*, isolated from a burn infection, demonstrated a high pigment yield of 1.87. This strain's resilience to environmental stress allows it to colonize burns, attach to the site, replicate, and produce pigments in substantial quantities. The crude form of staphyloxanthin

pigment showed significant cytotoxic activity against the CAL-51 breast cancer cell line at various concentrations, ranging from 1000 µg/ml to 125 µg/ml. Higher concentrations exhibited greater cytotoxic effects compared to lower doses. Selecting a more precise diluted dose could help identify the optimal concentration required for effective treatment of cancer cells. The choice of these doses (1000, 500, 250, and 125 µg/ml) reflects natural antibiotic dosing regimens commonly used in medical practice.

The pigment exhibited no significant effect, or only a slight effect, on the normal REF cell line when compared to its impact on the CAL-51 cell line. This selective effect supports the potential of staphyloxanthin as a safe and effective treatment for cancer cells without harming normal cells.

In addition, the study demonstrated that the DMSO solvent had a negligible and non-significant effect on both cell lines (CAL-51 and REF). This finding highlights the suitability of DMSO as a solvent for the pigment in experimental therapeutic trials. Moreover, DMSO can be used to dissolve the pigment in later stages of purification. Future studies should focus on modifying the purification process to eliminate the use of classical solvents like ethanol and explore alternative solvents that enhance the extraction and stability of the pigment.

Conflict of interest information. All authors declare no conflict of interest.

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