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ANTIBIOFILM AND IN VITRO CYTOTOXIC ACTIVITY OF PIGMENT EXTRACTED FROM MARINE BACTERIA *Kocuria flava* (AP2)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Natural pigments can be obtained from various sources like plant, microorganisms, and marine algae because synthetic pigments have some toxicity issues and are carcinogenic to both products and workers. Therefore, it is thought that bacterial pigment is a crucial metabolic product beneficial to bacteria and may possess certain biological features like antioxidant, antibacterial, and anticancer activity. Therefore, the present study aimed to assess the antibiofilm and anticancer activities of crude pigment extract from a marine bacterial isolate. The Antibiofilm assay was studied by the 96-well plate method. The pigment from *K. flava* (AP2) has the ability to inhibit biofilm formation of *Staphylococcus aureus, Streptococcus pyogenes, Serratia marcescens, Enterobacter sp, Enterococcus faecalis, and Escherichia coli.* The optical density at 570 nm wavelength on a plate reader to quantify the overall bacterial growth in each well. Among the six strains, moderate biofilm inhibition were observed at 2.5 µg/ml. In-vitro anticancer activity of bacterial pigment extract was evaluated by MTT assay against human colon cancer (HT-29). As a result of the pigments' strong cytotoxic action, they could be employed as biological agents in medicine.

Keywords: Marine bacteria; antibiofilm; colon cancer; MTT assay.

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1. INTRODUCTION

"Microorganisms represent naturally derived pigments such as carotenoids, melanins, flavins, monascins, violacein, and indigo" [1]. "There are numerous natural and synthetic pigments available. Due to the toxic, carcinogenic, and teratogenic properties of synthetically derived pigments, interest in microbial sources has increased as a safe alternative" [2-6].

It was revealed in the early 1970s that bacteria have the ability to communicate with one another, allowing bacteria colonies to regulate collective behavior. It enables a colony to act as a single organism. This type of cell-to-cell communication is known as "quorum sensing" [Nealson et al., 1969]. Some examples are movement growth rates, biofilm creation, and the generation of protective chemicals [7]. These chemical signals are important for infection establishment and can act as a switch to a pathogenic state [8]. Biofilms are film-like structures generated on biotic and abiotic surfaces by the aggregation of bacterial cells. These naturally occurring biofilms pose a significant risk to humans [9], accounting for 80% of bacterial infections [10]. According to Jeyachandran et al., [11], "they found that the extracted pigments could successfully suggest that shrimp carotenoids are a notable quorum-sensing and biofilm inhibitor against many clinical bacterial strains".

Cancer has been identified as a hazard as a result of increased usage of carcinogens, lifestyle changes, and greater life expectancy [12]. Cancer develops in people's bodies in a slow and dynamic manner [13]. Many anticancer chemicals are natural products or their derivatives, most of which are generated by microbes [14,15]. Microorganisms and plants are the primary suppliers of natural pigments [16,17]. These pigments are not only employed in business, but they also have antibacterial, antiviral, antioxidant, and anticancer properties [18,19]. Recent anticancer medication research has concentrated on newer and more effective chemotherapeutic medicines with fewer hazardous side effects. Research on bacterial pigment as an anticancer drug has been conducted on various forms of cancer.

2. MATERIALS AND METHODS

Bacterial strains were procured from Rajah Muthiah Medical college Hospital (RMMCH), Department of microbiology laboratory, Annamalai University, Chidambaram, Cuddalore district. Pathogens like *Staphylococcus aureus, Streptococcus pyogenes, Serratia marcescens, Enterobacter Sp Enterococcus faecalis, Escherichia coli.* cultures were routinely in MacConkey agar plate maintain at 37°C for the further use.

2.1 Isolation and Identification of Pigment Producing Bacteria

"The pigment producing bacterium was isolated from marine soil. The soil sample was inoculated in Zobell agar plates and kept for incubation at 37°c for 48 hr. The colonies showing pigment production were selected and purified. The yellowish Single colonies were sub cultured for every 30 days and kept under refrigeration at 4°C" [20]. A small yellow bacterial colony was chosen, and an identified phylogenetic tree was created using a neighbor-joining technique after the nucleotide sequences of the strain were compared to known sequences in the NCBI database.

2.2 Preparation of Inoculum and Extraction of Pigments

"The pure bacterial culture samples were transferred to 1000 ml of enrichment media in a super seal bottle and incubated on rotary shaker for 10 days. Extraction of the pigment was done by the following method.100 ml of bacterial cultures were centrifuged at 6000 rpm for 10 min. The harvested cells were resuspended in acetone and methanol (7:3 ratio) three times repeated by centrifugation at 10000 rpm until the cell debris turned colorless. The crude pigment extract was concentrated and used for biofilm and anticancer activity" [21].

2.3 Antibiofilm Assay of Bacterial Pigment

The antibiofilm experiment was performed to assess the effectiveness of metabolic extracts in preventing biofilm formation [22]. The 96 wells of flat-bottom polystyrene titer plates were used for the biofilm inhibition assay. After adding 10 lit µL of an overnight pathogenic bacterial culture into the wells containing 100 µL of Muller Hinton broth (MHB), the various concentration of crude pigment extract of (1.2,2.5,10 and 20 µg) was added to the wells, and further incubated at 37 °C for 24 hours. Following incubation, the well's contents were taken out and washed with 0.2 mL of phosphate buffer saline (PBS), pH 7.2, to eliminate any free-floating bacteria. Crystal violet (0.1% w/v) was used to stain the sessile bacteria's adhesion after sodium acetate (2%) was used to fix it. The deionized water wash removed the bulk of the stain and stored it for drying. Additionally, dried plates were cleaned in 95% ethanol before measuring the optical density at 570 nm with a microtitre plate reader.

2.4 Anticancer Activity of Bacterial Pigment

2.4.1 Cell culture

The Human colon cancer (HT-29) cells were procured from the National Centre for Cell Sciences (NCCS), Pune, India. The selected cancer cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 2mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100µg) were adjusted to 1mL/L. The cells were maintained at 37°C with 5% CO_2 in a humidified CO_2 atmosphere [23].

2.4.2 Evaluation of cytotoxicity activity of bacterial pigment

The inhibitory concentration (IC_{50}) value was evaluated using an MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The cells were grown (1×10^4 cells/well) in a 96-well plate for 48 h in to 80% confluence. The medium was replaced with fresh medium containing serially diluted sample, and the cells were further incubated for 48h. The culture medium was removed, and 100µL of the MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37°C for 4 h. After removal of the supernatant, 50µL of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multi well plate reader (Thermo Multiskan EX, USA). The OD value was used to calculate the percentage of viability using the following formula.

% of viability = OD value of experimental sample/OD value of experimental control ×100

3. RESULTS

3.1 Isolation and Extraction of Pigment-producing Bacteria

The pigment producing bacteria was isolated from marine soil and identified as bacteria *Kocuria flava* (AP2) as 16S rRNA sequences with a Gene bank accession number (ON651728) in Fig. 1, Fig. 2 shows that this isolate produces yellow pigment in the colony after 48 hrs of incubation on agar. The isolated strain was grown in zobell marine broth for 10 days of incubation, resulting in extensive growth of pigment producing bacteria. Fig. 3 shows that for the extraction of pigment producing bacteria, solvents like acetone and methanol (7:3 ratio) were used, so that cells get lysed and intracellular pigment can be extracted.

3.2 Antibiofilm Assay of Bacterial Pigment

The ability of crude pigment to inhibited bacterial biofilm formation Fig. 4 represents the optical densities read for each of the samples tested and their controls. The results clearly showed that all of the pathogens could form biofilms on the bottom of the tissue culture plates. It is also clear that the crude pigment extract affects pathogens' ability to form biofilms. The crude pigment extract was highly effective in inhibiting the ability of all *Staphylococcus aureus, Streptococcus pyogenes, Serratia marcescens, Enterobacter sp, Enterococcus faecalis,* and *Escherichia coli* to form biofilms. All of the other pathogens in this 2.5 µg/ml study demonstrated moderate antibiofilm activity.

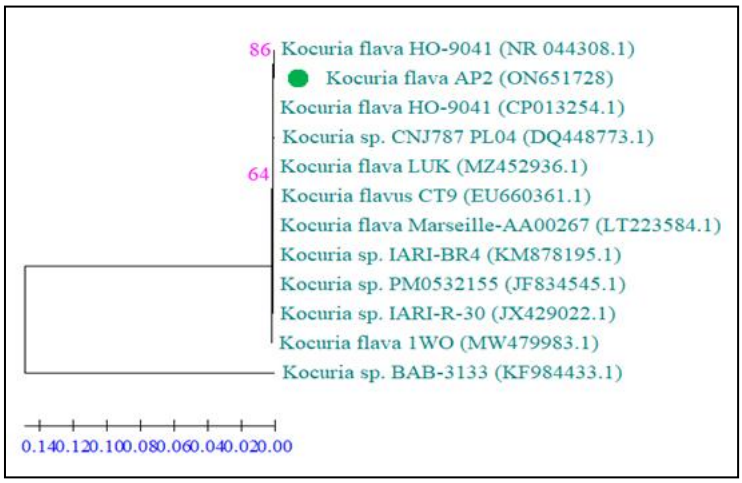

Fig. 1. Phylogenetic tree

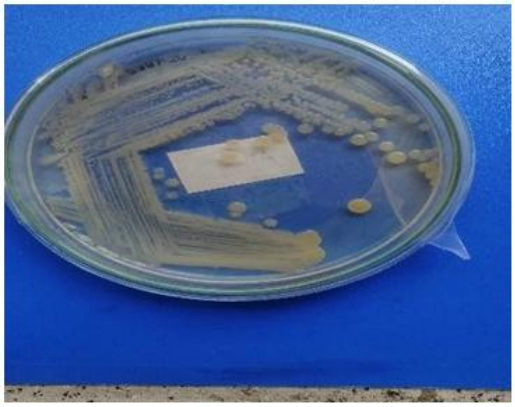

Fig. 2. Isolation of pigment producing bacteria

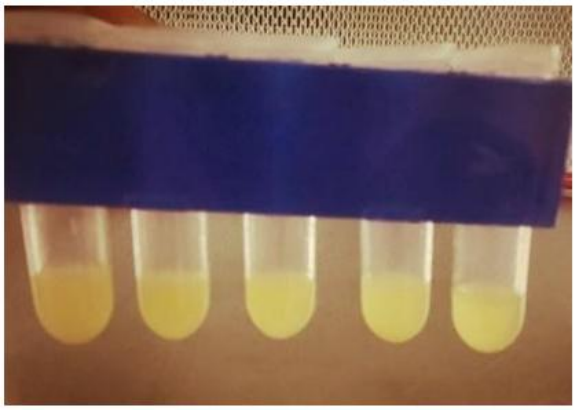

Fig. 3. Extraction of pigment producing bacteria

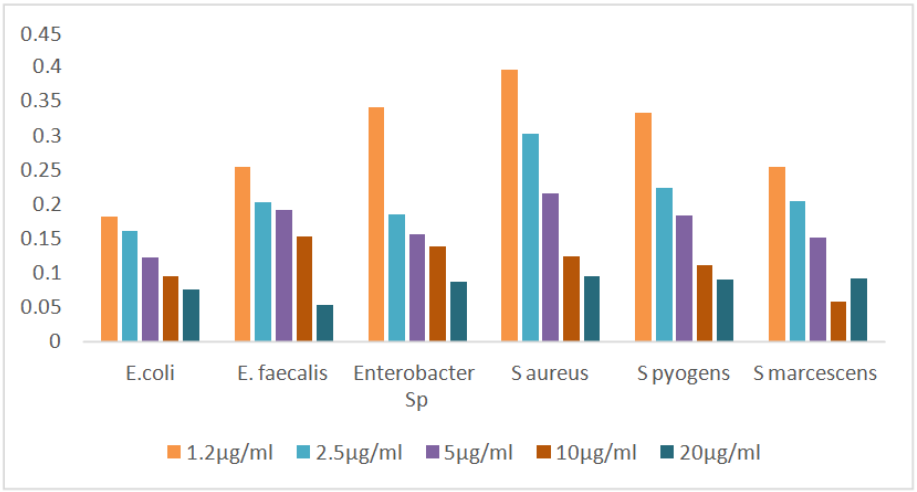

Fig. 4. Antibiofilm inhibitory assay

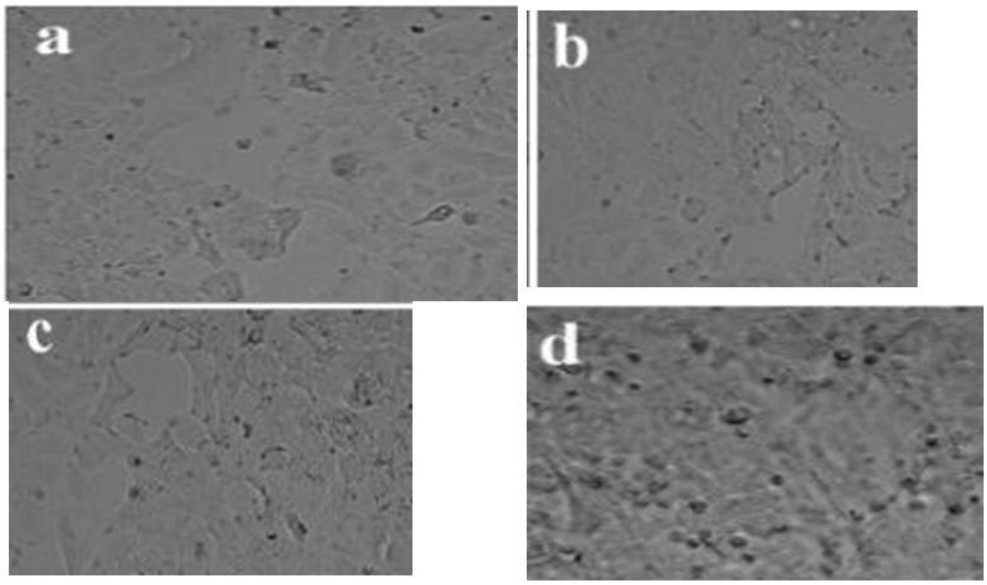

Fig. 5. Bacterial biofilm formation

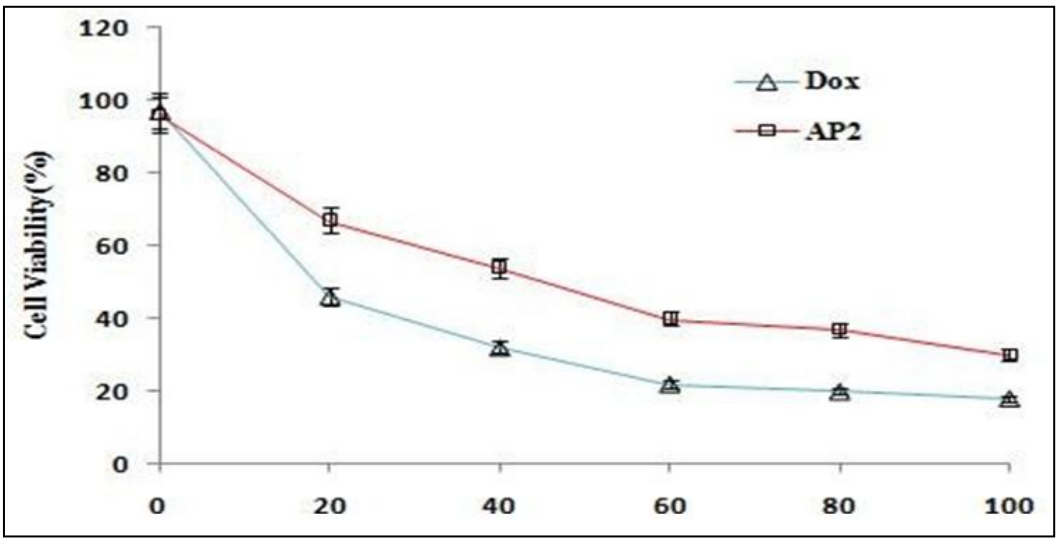

Fig. 6. Effect of cell viability in different concentration

3.3 Anticancer Activity of Bacterial Pigment

Cytotoxic activity of the pigments against human colon cancer (HT-29) cell lines was investigated by MTT assay and OD values at 620 nm were taken for different concentrations Fig. 6 shows that the standard Doxorubicin showed the highest decreases in viability, and untreated cells showed 100% viability. The inhibitory effect of pigment treated cells was observed after 24 h of inhibition. The maximum cell growth inhibition was observed at a concentration of 100 µg/ml for the colon cancer (HT-29) cell line. A decrease in the proliferation of pigment extract treated cells was observed when compared to the untreated control.

4. DISCUSSION

Researchers are interested in marine microorganisms because they may synthesise new compounds with unique biological characteristics [24]. Each year, more and more microbial species are isolated from the vast oceanic regions. Considering the importance of pigment isolated from marine bacteria, in this study, the antibiofilm and anticancer activities of pigment extracted from *Kocuria flava* (AP2) were investigated. Similarly the genus *Kocuria,* seventeen species have been described so far [25] and are found to produce pigments like ethinenone, echinenone, beta-carotene, lycopene, canthaxanthin, alfa-carotene, etc [26].

According to Kirishna et al. [27], pigments are often organic in nature and can be effectively extracted using organic solvents. The extraction of pigment-producing microorganisms has been done using a variety of techniques, such as ethanol addition during centrifugation and filtration to lyse the cell and allow the extraction of internal pigment [28]. So that the solvent is vital in the extraction of pigment from an isolated bacterial strain.

"Bacterial growth in biofilm is more resistant to antimicrobial agents and chemical biocides, necessitating extensive research to discover new compounds with antibiofilm and antibacterial properties" [29,30]. Bin et al. [31] investigated whether *A. auricula* melanin inhibited QS-regulated biofilm formation in *E. coli* K-12, *P. aeruginosa* PAO1, and *P. fluorescens* P-3 without interfering with growth. The inhibitory action on biofilm-producing bacterial strains has already been reported in many bioactive compounds from marine resources. "The methanol extract of the pigment was highly effective in preventing the biofilm-forming ability of all *S. saprophyticus*, *A. baumannii*, *E. cloacae*, *K. pneumoniae* isolates, *P. mirabilis* isolates, and *E. coli* isolates" [32]. The pigment was discovered to be a promising antibiofilm compound due to its ability to inhibit biofilm formation at low concentrations [33-35].

The potential of bacteria-derived pigments to kill cancer cells was demonstrated, also supporting earlier research [36]. Numerous studies on the anticancer properties of bacterial pigments like prodigiosin, violacein, astaxanthin, pyocyanin, and beta-carotene have been published [37]. Using the MTT assay, Koyyati et al., [38] investigated the cytotoxic activity of the pigments against HeLa cell lines, DU145 cell lines, MCF-7 cell lines, and Miapaca-2 cell lines. Among the four cell lines tested, the Miapaca-2 cell line had the most cytotoxic activity. Various studies

have shown that bacterial pigments have the potential to be effective anticancer medicines [39]. In this study, it was revealed that the pigment extracted from *Kocuria flava* (AP2) has cytotoxic effects on a colon cancer (HT-29) cell line.

5. CONCLUSION

Marine microorganisms have demonstrated the benefits and potential clinical applications of pigmented secondary metabolites in the treatment of a variety of diseases. So, in the present study, crude pigment was found to exhibit inhibitory action against the growth of antibiofilm and human colon cancer (HT-29) cell lines. Therefore, bacterial pigments can be used as biological agents in many industries, including the pharmaceutical and medical industries. Its potential strain for future use in nanoparticle synthesis is to improve the pigment's stability and shelf life.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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