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ANTIBACTERIAL, ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF BACTERIAL CAROTENOIDS ISOLATED FROM *RHODOPSEUDOMONAS PALUSTRIS* KRPR01 AND KRPR02

Rama Koyyati, Karunakar Rao Kudle and Pratap Rudra Manthur Padigya *

Department of Biochemistry, University College of Science, Osmania University, Hyderabad - 500007, Telangana, India.

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Correspondence to Author:

Pratap Rudra Manthur Padigya

Professor,
Department of Biochemistry,
University College of Science,
Osmania University, Hyderabad -
500007, Telangana, India.

E-mail: mpprataprudra@gmail.com

ABSTRACT: The microbial pigments have more applications than synthetic pigments and are easily biodegradable and safe to use. Among all bacteria, the anoxygenic phototrophic purple non-sulfur bacteria have more applications and can synthesize different pigments. In this present study, bacterial carotenoids were isolated from the two novel strains of *Rhodospseudomonas palustris* and evaluated its applications. The antibacterial activity was studied by zone inhibition method. The pigment A1 and A2 showed more antibacterial activity against gram-negative and gram-positive bacteria. *In-vitro* antioxidant activity of bacterial pigments and bacterial extracts were evaluated by DPPH assay. The pigment A2 and Rp. KRPR02 showed more radical scavenging activity. The percentage inhibition of DPPH radical for pigment A2 (82.74 ± 4.49) and bacterial extract of Rp. KRPR02 bacteria (83.33 ± 5.46) is almost nearer to the percentage inhibition of DPPH radical for standard (ascorbic acid) which is 96.41 ± 1.81 . *In-vitro* cytotoxic activity was studied by MTT assay and found that the pigments showed cytotoxic activity against the HeLa cell line, DU145 cell line, MCF7 cell line and Miapaca -2 cell line. The pigments were found to have significant antibacterial, antioxidant and cytotoxic activity, thus can be used as potential biological agents in medicine.

INTRODUCTION: Pigments are colorants which are produced from plants, animals, microorganisms and can also be synthesized by chemicals synthetically. The pigments have many applications in industries¹ (food, textile, paper, cosmetic, plastic, paint) agriculture, biology^{2,3,4} (antibiotics, antimicrobial agents, anticancer agents) etc. Now a day's pigments produced from the living organisms gained more importance as the synthetic pigments are toxic and show harmful effects like carcinogenicity⁵, mutagenicity⁶, teratogenicity, genotoxicity⁷, cytotoxicity, neurotoxicity and have harmful impact on the ecosystem.

The microbial productions of pigments are in many ways superior to the pigments produced from the plants and animal sources due to several reasons such as their rapid growth rate, easy downstream processing, cost-effectiveness, independent of season and geographical conditions, controllable, more stable and safe to use^{8,9}. Microbial pigments can be produced from bacteria, algae, fungi and protozoa.

Pigments produced from microbes possessing different shades of colors like yellow, purple, pink, orange, bluish red, red pigments etc. Microorganisms produce various pigments like carotenoids, bacteriochlorophylls a & b, melanins, flavonoids, quinines, prodigiosins, phenazines and more specifically monascins, violacein or indigo¹⁰. They possess various biological activities like antioxidant, antimicrobial, anticancer, anti-inflammatory, antiproliferative, anti-obesity and are used as bio-indicators^{11,12}.

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They also act as coloring agents in various industries like paint, plastic, cosmetics, textile, paper and pharmacy. The microbial pigments are also used as feed supplements in aquaculture^{13, 14}. Anoxygenic phototrophic purple non-sulfur bacteria are gram-negative bacteria which are the major group of phototrophic bacteria that convert light energy into chemical energy by an oxygenic photosynthesis with the presence of photosynthetic pigments. These pigments appear in cell suspension in various colors such as brown, orange, red¹⁵, pink, beige, purple¹⁶ and these pigments have characteristic absorption spectra. Photosynthetic pigments^{17, 18} (bacteriochlorophyll *a* or *b* and carotenoids) are located in the cytoplasmic membrane and internal membrane systems.

So far, researchers isolated the bacteria such as *Rhodospirillum rubrum*^{19, 20, 21, 22}, *Rhodobacter sphaeroides*²³, *Rhodopseudomonas palustris*²⁴, *Rhodopseudomonas faecalis*, *Rhodovulum* sp, *Rhodopseudomonas spheroids*^{25, 26}, *Rubrivivax gelatinosus*, *Rhodomicrobium vannielii* and mutant strains of *Rhodopseudomonas capsulate*^{27, 28} of the purple non-sulfur anoxygenic phototrophic bacteria which produced the pigments.

This research aimed to isolate the pigment-producing an oxygenic phototrophic bacteria from industrial sugar effluent and evaluate the antimicrobial, antioxidant and anticancer activity of bacterial pigments.

MATERIAL AND METHODS:

Isolation and Growth of Bacterial Strains: The pigment producing bacterium was isolated from industrial sugar effluent collected from Nizam sugar industry, Nizamabad, Telangana, India in 2013. The water sample was inoculated in modified Biebl and Pfenning media and incubated for 10 days in anaerobic conditions at 3000 lux. Single colonies were subcultured for every 30 days and kept under refrigeration at 4 °C.

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 anaerobically for 10 days under light at 3000 lux. Extraction of the pigment was done by the following method. 100 ml aliquots of bacterial cultures were centrifuged at

6000xg for 10 min. The harvested cells were resuspended in acetone and methanol separately by repeated centrifugation at 10000xg until the cell debris turned colorless.

Antibacterial Activity of Bacterial Pigments:

The gram-negative bacteria and gram-positive bacteria were grown overnight in Luria Bertani (LB) medium and spun at 6,000 rpm for 5 min. The pellet was washed with saline and resuspended in LB medium. The antibacterial activity was tested using the zone inhibition method. One hundred microliters of the suspended bacterial culture were spread plated uniformly on LB agar plates. On the solid media 2 mm, sterilized discs were placed and 20 µl of pigment extracts and bacterial cultures were added to the respective disc. The plates were incubated at 37 °C for 24 h in an orbital shaker. The antibacterial activity was assayed by measuring the diameter of the inhibition zone (mm) formed around the disc. Ampicillin (1mg/ml) was used as a positive control.

Antioxidant Activity of Bacterial Pigments:

The radical scavenging activity of bacterial pigments was estimated using the method of DPPH assay. A solution of DPPH (2,2-diphenyl-1-picrylhydrazyl), 5 mg in 100 ml methanol, was prepared and 3.0 ml of this solution was mixed with 10 ml of bacterial pigments/bacterial extracts. The reaction mixture was shaken vigorously and left in the dark at room temperature for 15 min. The absorbance was measured at 517 nm with ascorbic acid as standard. The following equation was used for calculating percentage inhibition of DPPH.

$$\text{DPPH \% inhibition} = \frac{[(\text{Abs control} - \text{Abs sample}) / (\text{Abs control})] \times 100}{}$$

Abs control is the absorbance of DPPH radical + methanol; Abs sample is the absorbance of DPPH radical + bacterial pigments / bacterial extract / standard.

Cytotoxic Activity of Bacterial Pigments:

a) Cell Lines Maintenance and Growth: The cytotoxicity potential of bacterial pigments was studied against HeLa cell lines, MCF-7 cancer cell lines, Miapaca-2 cancer cell lines and DU145 cancer cells which were purchased from NCCS (National center for cell sciences), Pune, India. All 4 cell lines were sub-cultured and were maintained

at 37 °C at 5% CO₂ in a CO₂ incubator. Cultures were examined for every 24 h under an inverted microscope to assess the degree of confluency and to confirm the absence of any microbial contamination.

b) Evaluation of the Cytotoxic Activity of the Bacterial Pigments by (MTT) Test: An *in-vitro* study of the cytotoxicity effect of bacterial pigments was assessed by MTT (3- (4, 5-dimethyl thiazolyl-2)-2, 5- diphenyltetrazolium bromide) assay. Cell lines were subcultured and 250 µl of media (containing 10000 cells) were transferred into 96 well plates and incubated for 24 h. The bacterial pigments and bacterial extracts were added at 50 µl and then the final volume was made to 200 µl with the media and incubated for 4 h. After incubation 20 µl of MTT reagent (6 mg/ml in PBS) was added to each well-containing media and incubated for 3 h at 37 °C under an atmosphere of 5% CO₂ until a purple precipitate was observed. Media was removed without disturbing the cells and 200 µl DMSO (MTT solvent) was added to dissolve the purple precipitate. Absorbance was read at 570 nm with a reference filter of 660 nm. Percentage viability and cytotoxicity were calculated.

$$\% \text{ Viability} = \Delta A_{570} (\text{treated}) / \Delta A_{570} (\text{control}) \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \% \text{ Viability}$$

RESULTS AND DISCUSSION: The pigment-producing bacteria were isolated from industrial sugar effluent and were identified as novel strains of anoxygenic phototrophic bacteria *Rhodospirillum rubrum* (KRPR01 and KRPR02) with Gene bank accession numbers KM200829²⁹ and KM200830³⁰. By solvent extraction method four pigments (one yellow, two orange and one green) were isolated from two novel strains of bacteria by using acetone and methanol as a solvent **Fig. 1.**

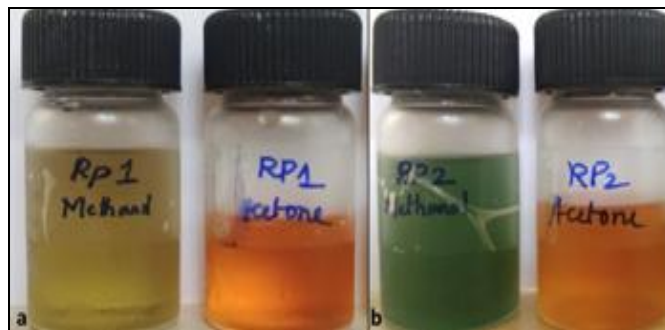


FIG. 1: PIGMENTS ISOLATED FROM RHODOSPILRILLUM RUBRUM KRPR01 AND KRPR02

Antibacterial Activity of Bacterial Pigments and Bacterial Extract: Antibacterial activity of bacterial pigments and bacterial extracts against gram-negative (*Escherichia coli* and *Klebsiella pneumonia*) and gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria was revealed and zone of inhibition was measured.

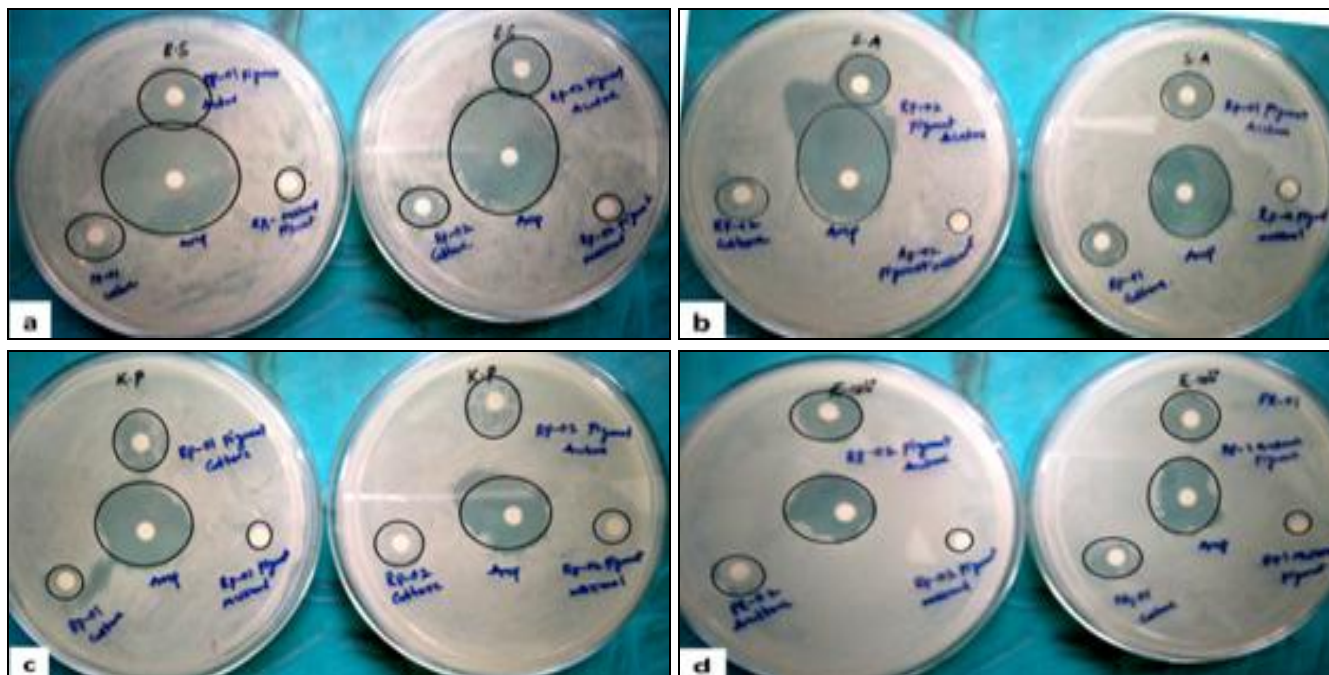


FIG. 2: ANTIBACTERIAL ACTIVITIES OF PIGMENTS AND BACTERIAL EXTRACTS

The results indicated that bacterial pigments isolated from two novel strains of *Rhodopseudomonas palustris* showed effective antibacterial activity against gram-negative and gram-positive bacteria which is compared with ampicillin (standard) **Fig. 2**. Comparing to pigments extracted by methanol (M1 and M2), the pigments extracted by acetone (A1 and A2) were showing more antibacterial activity **Table 1**.

TABLE 1: TABLE SHOWING THE ZONE OF INHIBITION (mm) OF PIGMENTS AND BACTERIAL EXTRACT AGAINST GRAM POSITIVE AND GRAM-NEGATIVE BACTERIA

Sample	<i>B. subtilis</i>	<i>S. aureus</i>	<i>K. pneumonia</i>	<i>E. coli</i>
A1	9.4	10.0	8.6	9.5
M1	1.0	1.2	0	0
KRPR01	4.8	6.3	3.0	4.6
Ampicillin	11.9	12.6	10.4	8.6
A2	10.2	8.5	9.8	9.4
M2	0	0	1.8	0
KRPR02	6.0	5.2	6.3	5.2
Ampicillin	12.8	12.1	8.6	7.3

Antioxidant Activity of Bacterial Pigments and Bacterial Extract by DPPH Assay: The radical-scavenging activity of bacterial pigments and bacterial extracts was estimated by comparing the percentage inhibition of formation of DPPH radicals with that of Ascorbic acid.

The pigment isolated by acetone (A2) and bacterial extract (KRPR02) of Rp. KRPR02 showed the highest antioxidant activity when compared with bacterial extract (KRPR01), pigments isolated from Rp. KRPR01 (A1 and M1) and pigment isolated from Rp. KRPR02 by methanol (M2) **Table 2**.

TABLE 2: DPPH RADICAL SCAVENGING ACTIVITIES OF PIGMENTS AND BACTERIAL EXTRACTS

Sample	OD value at 517 nm (Mean ± SD)	% inhibition of DPPH
A1	0.34 ± 0.01	39.29 ± 1.79
M1	0.35 ± 0.02	38.69 ± 3.72
KRPR01	0.42 ± 0.02	23.81 ± 3.72
A2	0.1 ± 0.03	82.74 ± 4.49
M2	0.37 ± 0.02	33.93 ± 3.57
KRPR02	0.10 ± 0.03	83.33 ± 5.46
Ascorbic acid	0.08 ± 0.01	96.41 ± 1.81
DPPH (Blank)	0.56 ± 0.01	-

Radical scavenging activity of pigment A2 and bacterial extract KRPR02 is almost equal to the standard ascorbic acid **Fig. 3**.

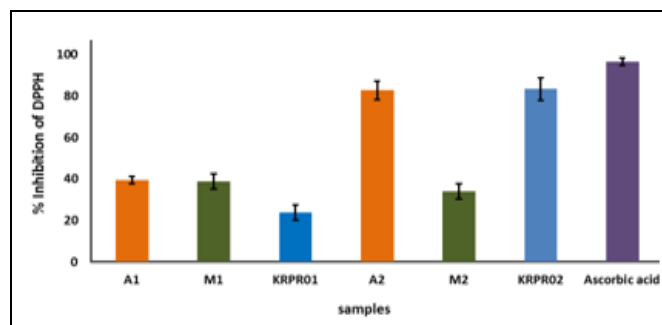


FIG. 3: % INHIBITION OF DPPH RADICAL SCAVENGING ACTIVITY OF PIGMENTS AND BACTERIAL EXTRACTS

Cytotoxic Activity of Bacterial Pigments: The cytotoxic activity of the pigments against HeLa cell line, DU145 cell line, MCF-7 cell line and Miapaca-2 cell line was investigated by MTT assay and the OD values at 570 nm were taken for different concentrations (2 µl, 4 µl and 6 µl). The standard Doxorubicin showed the highest decrease in the viability and untreated cells showed 100% viability **Fig. 4**.

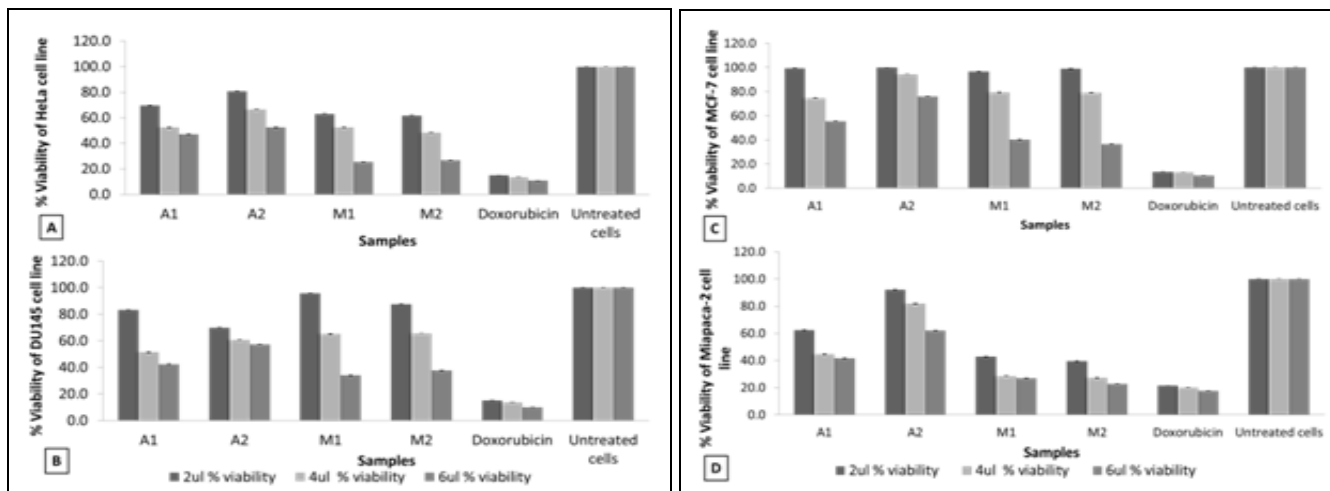


FIG. 4: % VIABILITY OF PIGMENTS AGAINST, A. HELA CELL LINES, B. DU145 CELL LINE, C. MCF7 CELL LINE, AND D. MIAPACA -2 CELL LINE

Comparing to the pigments A1 and A2 pigments M1 and M2 showed more cytotoxic activity in all four cancer cell lines, which is compared with

standard Doxorubicin. Among the 4 cell lines, the bacterial pigments showed more cytotoxic activity against the Miapaca-2 cell line as shown in **Fig. 5**.

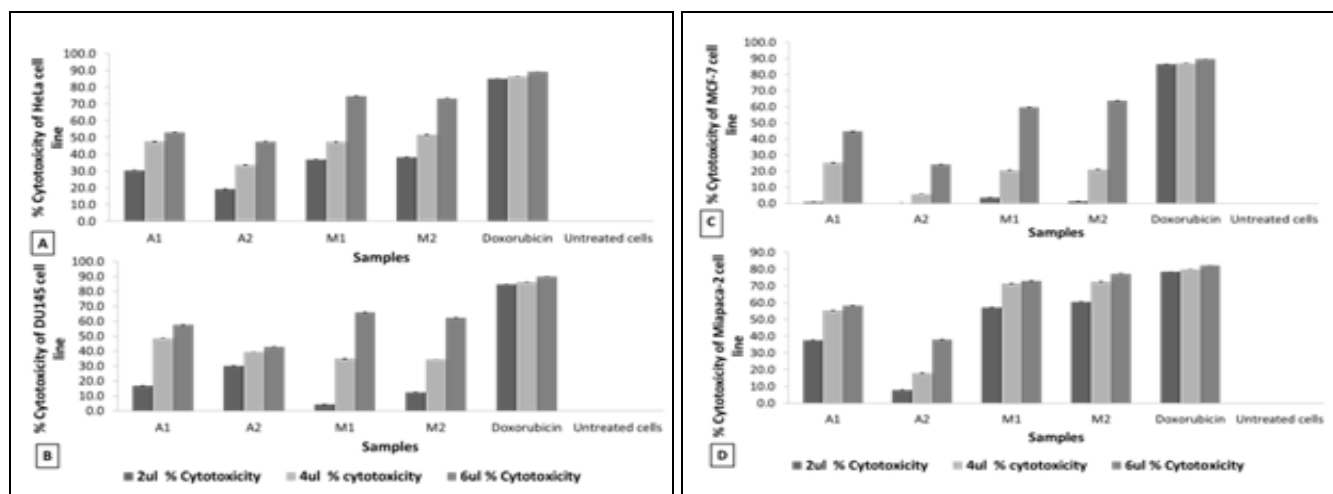


FIG. 5: % CYTOTOXICITY OF PIGMENTS AGAINST, A. HELA CELL LINES, B. DU145 CELL LINE, C. MCF7 CELL LINE, AND D. MIAPACA -2 CELL LINE

CONCLUSION: The bacteria's isolated from the industrial sugar effluent were identified as novel strains of *Rhodopseudomonas palustris*. The pigment A1 and A2 showed more antibacterial activity against gram-negative and gram-positive bacteria. The pigment A2 and Rp. KRPR02 showed more radical scavenging activity when compared to pigments A1, M1, M2, and Rp. KRPR01 bacteria. The pigments showed cytotoxic activity against four cell lines. The bacterial pigments showed significant antibacterial, antioxidant and cytotoxic activity; hence the bacterial pigments can be used as biological agents in various fields such as medicine, pharmaceutical industries etc.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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