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STUDIES ON *IN-VITRO* ANTICANCER AND ANTIOXIDANT PROPERTIES FROM MARINE BACTERIAL PIGMENT ISOLATED FROM THE COASTAL AREA OF MARAKANAM (TN)

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Keywords:

MB_4 strain, Yellow pigment, DPPH activity, Anti-cancer properties, Raman spectroscopy, Marine water

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ABSTRACT: Marine bacteria have the potentiality to produce diverse bioactive molecules such as pigment. Therefore, it needs to exploit and identifying a novel type of pigment from marine bacteria for Industrial applications. This study aimed to investigate the marine bacterial pigment against antioxidant and anti-cancer properties; the marine bacteria producing pigment were isolated from water samples collected at the coastal of Marakkanam (TN), India. The isolates were screened out based on the growth characteristics and performance of different media and the strain designated as MB_4, which was taken as further studies. The strain MB_4 characterized by SEM analysis showed that coccoid cell morphology, nonsporulating, Gram-positive with yellow pigmentation and positive for MR-VP, catalase, lipase, acetoin production, and hemolysis. The cells were able to tolerate 10 percent NaCl concentration and ability to grown pH 9. The MB_4 strain was shown a higher wave-number (1395.77) cm^{-1} against Raman Intensity to identify pigment production. The methanolic extracted pigment was produced at a maximum peak at 260 nm. The yellow-pigmented crude extract checked for anti-cancer properties using the colon cancer cell line (HCT_{15}), the cell viability has been reduced after treatment of the extract (25-500 µg ml^{-1}) and also exhibits IC_{50} value of 255.58 ± 43.51 mg ml^{-1} antioxidant DPPH radical scavenging activity. Due to their yellow pigment productions which have antioxidant activity and anti-cancer properties, this could be a novel pigment-producing strain for biomedical and industrial applications.

INTRODUCTION: Synthetic pigments made up of heavy metals and petroleum compounds are reported to be carcinogenic, allergic, induce hyperactivity, toxic, and organ damage, which are unsafe for both environment and human health [1]. Due to their low cost, increased stability, and wide range of spectra, the synthetic pigments are widely used [2].

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In recent years, natural pigments are highly demanding for the use of colouring agents in foods, fabrics, feed, printing ink, and cosmetics, which is nonpolluted, eco-friendly, and less cost.

Naturally occurring colourants are safe to use produced by microflora and fauna, which are nontoxic, noncarcinogenic and easily degradable [3]. Generally, microbial pigments are the micro-organisms that produce colour, which is more attractive nowadays due to easy methods available for cultivation; pigments are highly stable and year-round availability [4]. An alternative source for natural pigment produces from bacteria, fungi, and microalgae [5-9].

Bacteria have great potential to produce various bioproducts among the microorganisms, and one of the physiological characters produced by the bacteria is pigment production [10]. Biomedical and pharmaceutical industries utilize marine natural products, and the bioactive compounds are produced by marine bacteria [11]. Microorganisms are a promising source of natural pigments like carotenoids, flavins, chlorophyll, quinines, and prodigiosin are the pigments produced by coloured microorganisms found in the different environmental niche. In survey [12], yellow (31.3 %), orange (15.2%), brown (9.9%), and red or pink (5.4%) were found to be microorganisms recovered from marine sources. Various concentrations of minerals, a wide range of temperature, and it should be the ability to tolerate different pH is the good qualities of pigment producers [5]. Marine organisms produce a variety of metabolites that are treated against antitumor, antioxidant, and antimicrobial activities.

In recent years, Raman spectroscopy is a popular analytical tool applied for the study of microorganisms, especially pigments and biomolecules, through qualitative and quantitative analysis. Strong Raman signals were exhibits when applied to the microbial pigments for the understanding of different pigments, even pure cultures as well as extracted pigments [13, 14]. It has been used to monitor different types of pigments present in the microorganisms directly through pure cultures and environmental samples [15]. Antioxidants may reduce the risk of diseases, particularly heart and cancer, in the health sector. It can absorb free radicals that may oxidize lipid or DNA, proteins, nucleic acids and cause degenerative disease [16]. Many other findings reported that the β-carotene and other carotenoid extracted pigments have antioxidant activities [17]. Antioxidant able to donate a hydrogen atom reduces DPPH as a result of colour loss which determines the scavenging capacity of a molecule [18]. The carotenoid pigment producer *Planococcus* sp. TRC1 was showed appreciable antioxidant activity leading to pharmaceutical and food applications [19].

The activation, viability, and proliferation of the cells were measured quantitatively in calorimetric for MTT Assay [20]. The dehydrogenase enzymes associated with the endoplasmatic reticulum and mitochondria in the living cell convert MTT into a purple-blue formation which is water-insoluble. The viable cell number is directly proportionate to product formation and inversely proportional to cytotoxicity [21]. In this study, we are investigating the pigmented bacteria isolated from marine water samples to evaluate the antioxidant and anti-cancer properties of the extracted pigment.

MATERIALS AND METHODS:

Collection of MARINE WATER SAMPLE: Samples collected from the marine water surface at different sites along with the coastal areas of Marakanam (TN), India. The marine water samples from the sea surface were collected by using Teflon plates dipped into water, lifted horizontally, and scrapped off the adhering surface film till the procedure repeated the total volume of 30 to 50 ml water sample was collected. The samples were stored at 4°C until the isolation was carried out within 24 h [22]. The salinity of the collected marine water samples was determined by using the protocol reported by [23].

Isolation of Yellow-Pigmented Bacterial Isolates from Marine Water: Isolation of pigment-producing colonies from collected seawater samples using selective Zobell marine agar medium [24]. The plates were incubated at 37°C for 48 h, the colonies showing yellow, red, orange, and brown pigmentation subcultured for purification.

Screening, Morphological and Biochemical Characterisation of the Isolates: The bacterial isolate was plated on Zobell marine agar, Luria Bertani agar and Tryptic soy agar incubated at 37°C for three days. By using standard Microbiological techniques, the different cultural characteristics, cell morphological parameters, and Gram's reaction were studied to the bacterial cells.

Scanning Electron Microscopic (SEM) Analysis for MB4 Isolate: The yellow-pigmented bacterial culture isolate was grown on Luria Bertani broth were centrifuged, phosphate buffer saline (pH 7.0) used to wash for thrice to remove salts, glutaraldehyde (2%) solution was fixed with sample and allowed for alcoholic dehydration at 6-12 h. The dehydrated sample was prepared and analysed on SEM [25].

Biochemical Characterisation of the Pigmented Isolate: The biochemical characteristics of the yellow-pigmented bacterial MB_4 isolate.

Kovac's Oxidase Test: By using a sterile toothpick, a well-isolated colony of MB_4 was picked and thoroughly rubs into an area of the moist test disc impregnated with oxidase reagent. After 30 sec, the inoculated area was observed for colour change. A bluish-purple colour indicated a positive reaction [26].

Catalase Test: Cultures grown on MB_4 slants for 24 to 48 h flooded with 0.5 ml of 3 per cent hydrogen peroxide. Rapid effervescence shows a positive result for catalase activity [27].

Methyl Red-Voges Proskauer Test (MR-VP test): Methyl Red-Voges Proskauer tests were used to differentiate acid producers from those producing a neutral product, acetoin. The isolate was inoculated in 5 ml MRVP tubes and incubated at 35 °C for 48 h. Methyl red positive tubes were observed by the change of the colour of the media from yellow to red. Voges-Proskauer test was recorded positive by the development of red colour due to the addition of Barritt's reagent-I and Barritt's reagent-II [28].

Lipase Activity: The isolate MB_4 was inoculated on egg-yolk agar and incubated at 37°C for 48 h. The positive reaction of the lipase activity indicated that the development of opalescent precipitates [29].

Urease Test: It was performed on 5 ml urea broth in test tubes containing phenol red (pH 6.8) as the pH indicator. The urea broth tubes inoculated with isolates were incubated for 24 h. The positive tubes were developed on red colour [30].

Assay of Phosphatase Activity: The loopful of bacterial growth of strain was collected and deposited on the surface of the tangible medium containing the phosphatase substrate (para-nitrophenyl phosphate) to a final concentration of 1 mg ml^{-1}. The plates were then incubated at 37°C as described by [31].

Coagulase and DNAse Production: It was detected by the method using ethylenediamine-tetraacetic acid (EDTA) treated coagulase plasma by the formation of a clot after 1, 2, 4 or 24 h recorded as positive. During the investigation, 0-1 % DNA (BDH) was added to this medium to enhance the detection of DNase [32].

Physiological Characterisation of the MB_4 Isolate: The physiological characteristics such as pH and NaCl tolerance were determined. The effect of pH was determined by the preparation of nutrient broth with incremental pH values ranging from 4 to 14. Strain MB_4 was inoculated and incubated for 48 h at 37°C, the growth in the culture broth read at 620 nm. The pigments were extracted and observed for maximum pigment production [33]. The inoculation of MB_4 also examined the effect of NaCl tolerance in nutrient broth in different concentrations ranging from 6 to 12% of NaCl. The flasks were incubated at 37°C for 48 h, and the results were observed for growth and pigment production.

Extraction of pigments from bacterial isolates: The yellow-pigmented MB_4 isolate was grown in LB broth for seven days kept in a rotary shaker at 160 rpm at 37°C. The broth was centrifuged at 8,000 rpm for 15 min, and the cells were harvested and poured off the supernatant. The cell pellet was rewashed with sterile distilled water centrifuged. The pellets were resuspended with 5 ml methanol and sonicate the mixture until all visible pigments were extracted. The solvent mixtures were centrifuged at 4,000 rpm for 15 min; the pigment supernatant was separated and filtered through a Whatman no. 1 filter paper and analyzed by scanning the absorbance in the wavelength region from 300-700 nm using a UV-VIS spectrophotometer [34].

Raman Spectroscopy Analysis: The MB_4 strain was grown on LB agar medium after 48 h of incubation and the pigmented pure culture to determine the strong Raman signalling for Raman spectroscopy [15].

Determination of DPPH Scavenging Assay: [35] was reported the method of determination of DPPH radical scavenging activity. An aliquot of 0.5 ml of the extracted pigment sample solution in methanol was mixed with 2.5 ml of 0.5 mM methanolic solution of DPPH. The sample mixture was shaken vigorously and incubated for 30 min in the dark at room temperature. The absorbance was measured

at 517 nm using a UV spectrophotometer. Ascorbic acid was used as a positive control. DPPH free radical scavenging ability (%) was calculated by using the formula.

% of inhibition = absorbance of control – absorbance of sample/absorbance of control ×100

Cell Culture and MTT Assay: The MB_4 pigmented extract was carried out with MTT assay. The HCT_{15} colon cancer cell line was plated separately using 96 well plates with the concentration of 1×10^4cells/well in DMEM media with 10% fetal bovine serum and 1X Antibiotic Antimycotic Solution in a CO_2 incubator at 37°C with 5% CO_2. The 200 µl of 1X PBS cells were washed, and then the cells were treated with various concentrations of crude extract of the pigmented compound in serum-free media and incubated for 24 h. At the end of the treatment period, the medium was aspirated from cells. Prepare 0.5 mg ml^{-1} MTT mixed in 1X PBS and incubated at 37°C for 4 h using CO_2 incubator. After the incubation period, the medium containing MTT was discarded from the cells and washed using 200 µl of PBS. The formed crystals were dissolved with 100 µl of DMSO and thoroughly mixed. The formazan dye turns to purple-blue colour. The colour intensity was measured at 570 nm using a microplate reader [36].

Statistical Analysis: Statistical analysis was performed with Mean and Standard Deviation (SD) in excel, and all analyses were carried out in triplicates.

RESULTS AND DISCUSSION:

Isolation of Pigmented Bacteria from Marine Water: The bacteria producing pigment were isolated from the marine water sample by using Zobell Marine Agar media.

TABLE 1: SCREENING OF PIGMENT-PRODUCING BACTERIAL ISOLATES

Location	Total no. of isolates	Pigment producing strains
Marakanam coastal region (12.1899° N, 79.9249° E)	MB_1 MB_2 MB_3 MB_4 MMB_5 MMB_8 MBS_7 MBS_8 SB_2 SB_{10}	MB_2, MB_4, MMB_8 & SB_2

Ten isolated strains were purified and named *viz*., MB_1, MB_2, MB_3, MB_4, MMB_5, MMB_8, MBS_7, MBS_8, SB_2 and SB_{10}; the results were shown in **Table 1**. Based on the growth performance and pigment production, the strain MB_4 was screened for further studies.

Morphological Characters of Bacterial Isolates: Pigment-producing bacteria are isolated from marine samples, which are ubiquitous [37]. A result of **Fig. 1**, the isolated colony was round, smooth with yellow-pigmented, non-motile, non-sporulating, and gram-positive bacteria. [38] were reported that the carotenoid-producing microbes were isolated from the extreme environmental niche. The sixty marine species of yellow (19), orange (5), pink or salmon colour (5), brown (5) and red (1) were described by [39]. The marine organism isolated in the present study was yellow-colored. All bacterial pigmented isolates need not be carotenogenic [40]. Some pigmented bacteria from marine are *Alteromonas* (yellow, violet) [41-43], *Flavobacterium* (yellow) [44], *Deleya*, *Marinomonas*, *Pseudomonas* and *Shewanella* (yellow) [45], *Erythrobacter* (yellow) [46], *Pseudoal-teromonas* (purple, red, or yellow pigments), *Xanthomonas* (yellow) [47], *Exiguobacterium* (yellow, orange) [48, 34,] and *Bacteroidetes* (yellow, orange, pink or red) [49] were reported to possess antagonistic activities. The SEM image of **Fig. 2** was clearly shown that the isolate belongs to Cocci with irregularly arranged cells.

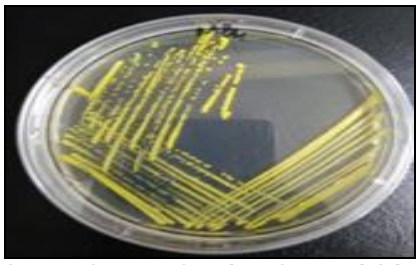

FIG. 1: GROWTH OF MB_4 STRAIN ON LB AGAR MEDIUM

Biochemical Reaction of the Strain: The yellow-pigmented bacterium from deep-sea sediment was oxidase, DNase, methyl red, urease negative, and catalase, Voges–Proskauer positive exhibited by *Croceicoccus marinus* gen. nov., sp. nov., [50]. The present investigation of the MB_4 strain was shown in **Table 2**, which is positive for MR-VP, catalase, lipase, acetoin production, and hemolysis and negative for oxidase, coagulase, urease, and phosphatase.

TABLE 2: BIOCHEMICAL CHARACTERS OF THE PIGMENTED STRAIN

S. no.	Biochemical reaction	MB_4 strain
1	Oxidase	-
2	Catalase	+
3	Coagulase	-
4	Acetoin production	+
5	Phosphatase	-
6	Hemolysis	+
7	Urease	-
8	MR-VP	+
9	Lipase	+

Positive (+), Negative (-)

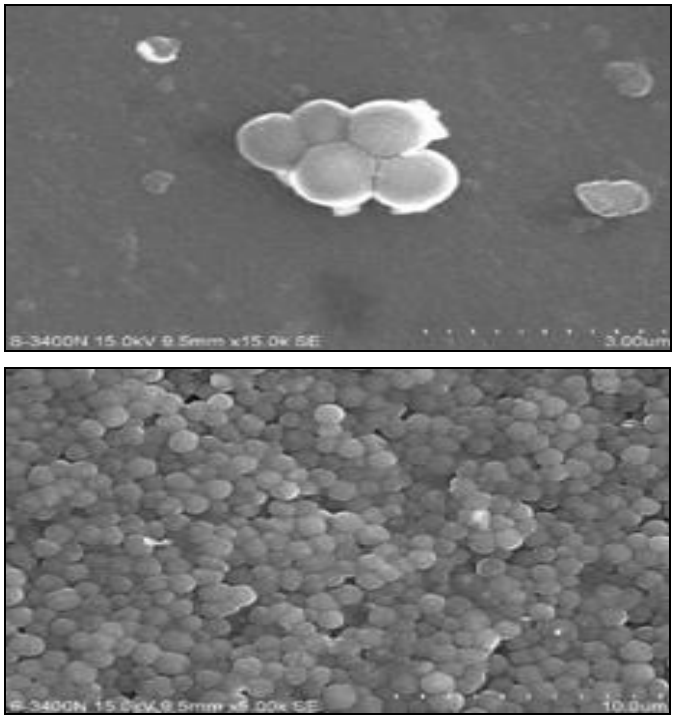

FIG. 2: SEM IMAGES OF PIGMENTED MARINE BACTERIAL MB_4 STRAIN

Physiological Characterisation of the Isolates: Studies were reported the yellow-pigmented *Polaribacter butkevichii* sp. nov. were grown in pH between 7.6 and 8.2 isolated from marine water samples [51]. The MB_4 strain exhibits maximum growth at pH nine as compared to pH from 4 to 14 **Fig. 3**. The extreme halophiles were unable to grow in the presence of NaCl less than 12%, and it can also be able to grow in saturated NaCl, including halobacteria and *Halocooci*. The isolate MB_4 was the ability to tolerate salt concentration of 10% NaCl which was shown in **Fig. 4**. It has been reported that the growth was exhibited up to 8.5% NaCl concentration by *P. balearica* strain [52]. The KMM 1447T yellow-orange pigmented strain isolated from marine ascidian was the ability to grow in 8% NaCl [53].

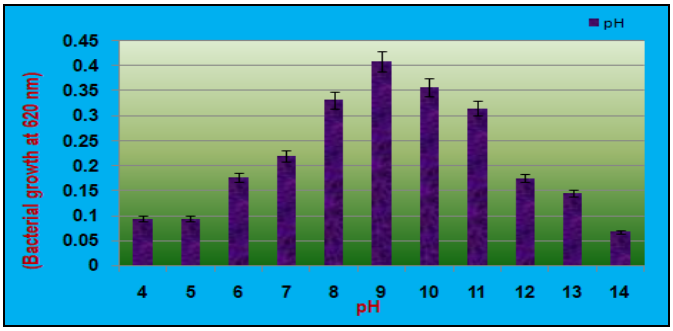

FIG. 3: PIGMENTED MB_4 STRAIN WAS GROWN AT DIFFERENT pH

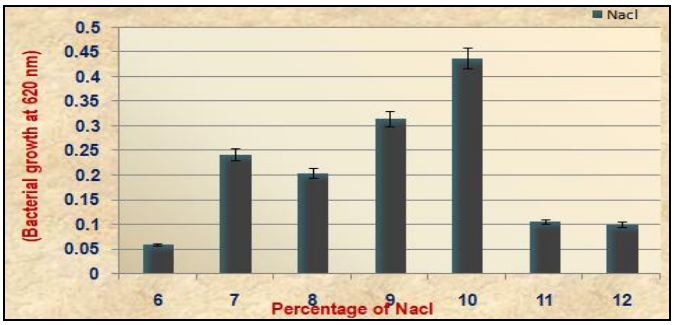

FIG. 4: THE BACTERIAL PIGMENTED MB_4 STRAIN WAS GROWN AT DIFFERENT NACL CONCENTRATION

Extraction of Yellow Pigment from the Strain MB4: The isolated autotrophic cell cultures were extracted yellow coloured pigments using methanol solvent results much efficient in the quantification was better than acetone [54]. The yellow pigments were separated from cell pellets by methanol extraction from the bacterial broth **Fig. 5**. Based on the absorption spectrum, the characterisation of crude extracted yellow pigment exhibited a maximum at 260 nm **Fig. 6**. As compared to water, PBS or acetone extraction, pigments recovered even better by using methanol extraction [34, 55, 56]. The lemon yellow colour pigment is extracted by methanol from *Halomonas aquamarina* MB598, also reported by [2].

FIG. 5: PELLETS ARE SEPARATED FROM THE PIGMENTED MB_4 STRAIN

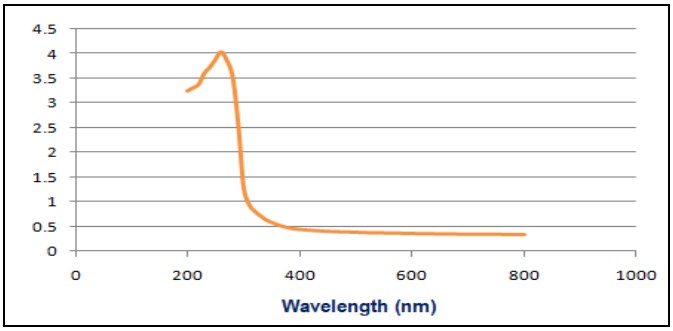

FIG. 6: METHANOLIC EXTRACTION OF YELLOW-PIGMENTED BACTERIAL ISOLATES

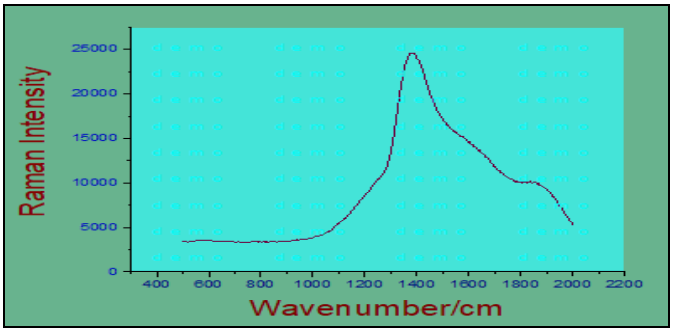

FIG. 7: RAMAN SPECTROSCOPY ANALYSIS OF PIGMENTED MB_4 BACTERIAL ISOLATE

Raman Spectroscopy Analysis for Pigmented Strain: Raman spectroscopy, one of the highly sophisticated instruments applied in Microbiology research in recent years for the characterisation of microbial pigments. The pure cultured MB_4 strain was analyzed by using Raman spectroscopy for pigment production, and the strain was showed that maximum wavenumber (1395.77) cm^{-1} against Raman intensity **Fig. 7**. The excitation of electronic absorption of the spectrum which produces a specific enhancement of certain Raman intensity (bands) correspondingly the portion of the molecule electronic transition occurs indicates moving of atoms in the chromophore when vibration takes place [57, 58].

Antioxidant Activity of Pigment Extract: Natural products like pigments, frequently used to evaluate antioxidant activity, the ascorbic acid, glutathione, cysteine, and tocopherols, were used as reducing agents which are decolorizing involving in DPPH radicals [59].

The reducing activity of free radical owing to antioxidant depending on the reduction of one electron which exhibits scavenging of DPPH; as a result development of antioxidant properties due to reducing power [60]. The anticarcinogenic and antioxidants properties are present in carotenoid pigments [61].

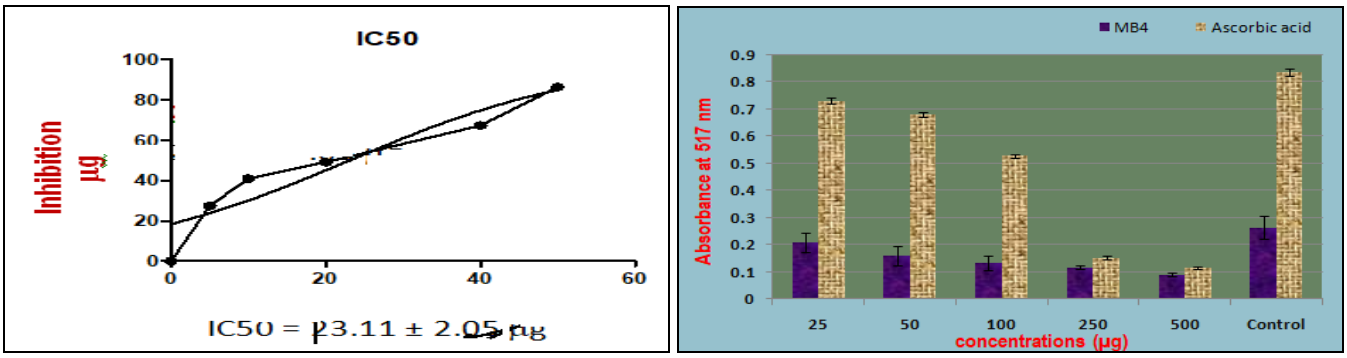

FIG. 8A: GROWTH PERFORMANCE OF A PIGMENTED EXTRACT OF MB_4 AND ASCORBIC ACID

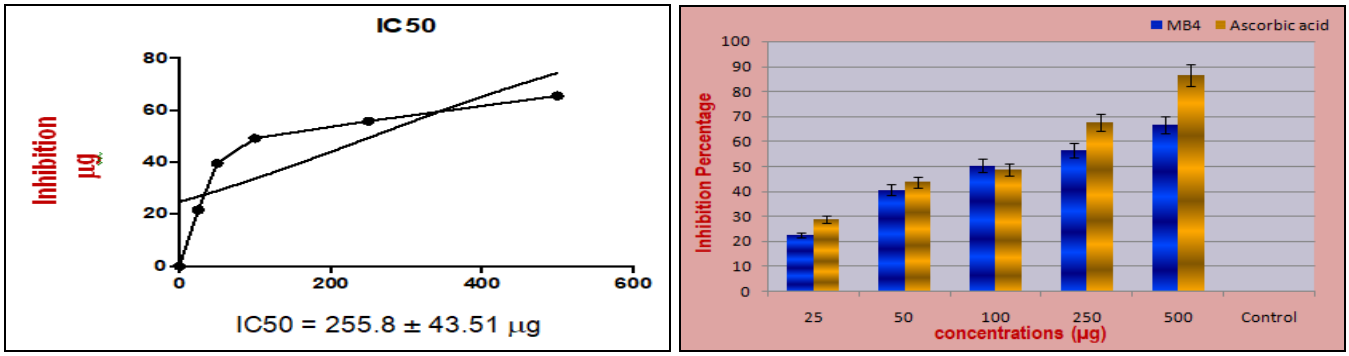

FIG. 8B: SCAVENGING ACTIVITY OF MB_4 PIGMENTED EXTRACT AND ASCORBIC ACID

In-vitro, the potential of antioxidant activity of yellow-pigmented crude extract (YPCE) was scavenged due to free radicals in 500 µg ml^{-1} concentrations, and the percentage of inhibition for

DPPH showed 68.30 percent **Fig. 8A** with IC_{50} 255.58 ± 43.51 among different concentrations from 25 to 500 µg ml^{-1} **Fig. 8B**.

The carotenoid pigment is the biological compound available for human beings that have properties of ulcer prevention [62]. The DPPH free radical scavenging activity was shown in different concentrations by using the methanol extract [59]. The pigment production by halophilic bacteria and their relation to radical scavenging property was reported by 63. The yellow pigment was extracted from *Kocuria flava* SIF3 discovered to have DPPH radical scavenging activity with an IC_{50} value of 1.25 mg ml^{-1} *in-vitro* antioxidant assay [64].

Pigment Extract Evaluated for Anti-cancer Activity: Although advanced techniques developed in the treatment, prevention, and diagnosis of the disease. Still, one of the most serious human health problems in the world is cancer despite their understanding of its biology, which causes severe to mankind [65]. Microbial pigments possess anti-cancer activity, and prodigiosin pigment produces cytotoxicity on U937 leukemia cells extracted from *Pseudoalteromonas* sp. 1020R [66]. The present investigation was performed on a potential crude extract of yellow pigment against colon cancer cell line (HCT_{15}), which showed that the different dose concentrations of the methanolic fraction of MB_4 isolated from seawater could largely inhibit cell proliferation at a concentration of 500 µg ml^{-1} **Fig. 9A** and **Fig. 9B** and no cytotoxicity was detected against the standard untreated cell. It was found to be reported similar studies by [67].

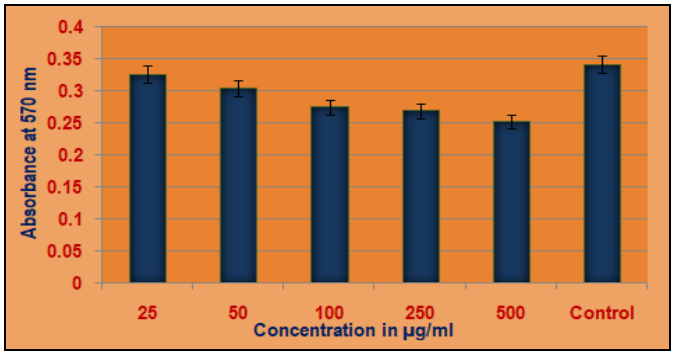

FIG. 9A: CELL PROLIFERATION OF CRUDE EXTRACT OF MB_4 STRAIN ON COLON CANCER (HCT15) CELL LINE

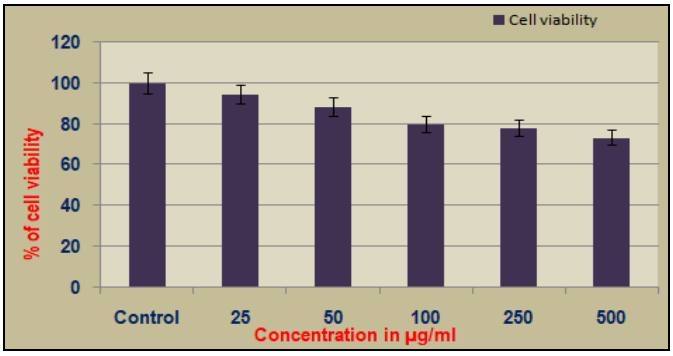

FIG. 9B: CELL VIABILITY OF MB_4 PIGMENTED EXTRACT ON COLON CANCER CELL LINE (HCT15)

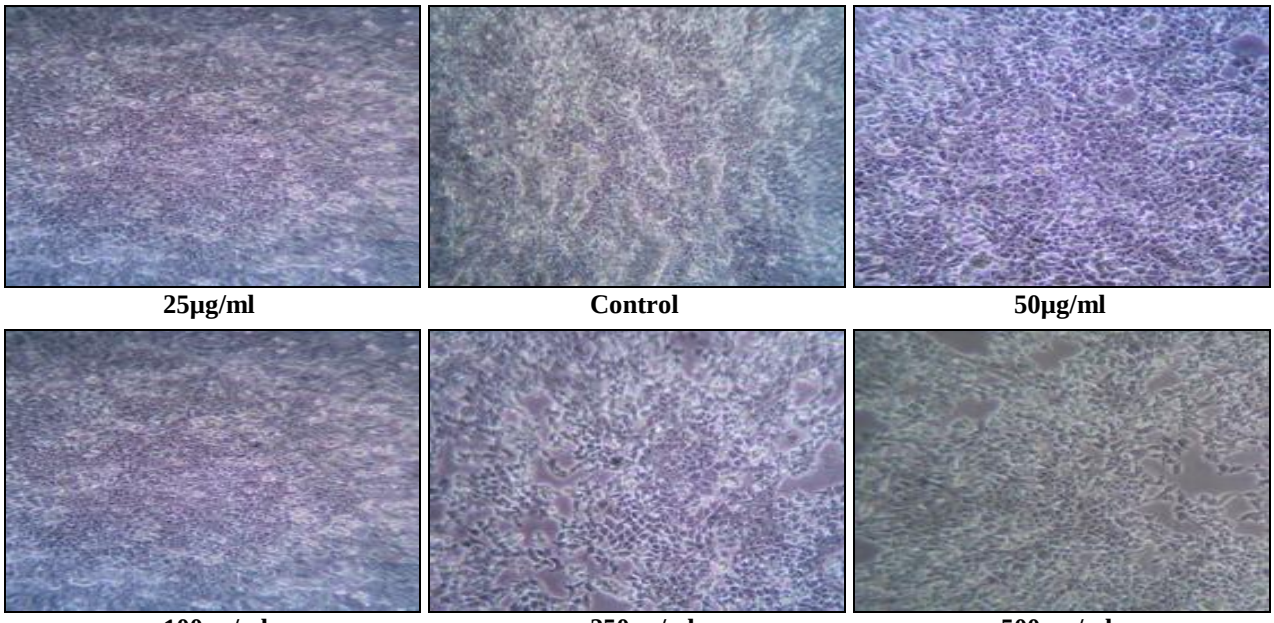

FIG. 10: EFFECT OF MB_4 STRAIN CRUDE EXTRACT ON COLON CANCER CELL LINE

Microbial pigments possess antimicrobial, anti-inflammatory, anti-cancer, and antioxidant activities, which also act as colouring for various industries like food processing and cosmetics [4]. The

extracted crude pigment MB_4 was checked against the colon cancer cell line (HCT_{15}) for cytotoxic activity **Fig. 10**.

Many studies had reported that the inhibition of cell cycle and apoptosis induced by microbial pigments [68, 69]. It inferred that the crude pigment extract of marine MB_4 shown significant antioxidant and potential anti-cancer activity against (HCT_{15}) cell lines of colon cancer. The cytotoxic activity against cervical cancer cells (HeLa) and HepG2 were demonstrated in yellow pigment from *Streptomyces griseoaurantiacus* [70]. The potential breast cancer cell lines and lung cancer cells have experimented with pigment carotenoid extracted from *Kocuria* sp. QWT-12 [71]. The pigment was found to exhibited inhibitory action against the growth of human cancer cell lines, which develops anti-cancer drugs from *Salinicoccus* sp. [72]. The novel compound from yellowish pigment produced by *Rhodococcus maris* reduced the risk of breast cancer [73]. The *Natrialba* sp. M6 produce carotenoid pigments that were effective against anti-cancer and antiviral activities [74].

CONCLUSION: Bacterial pigments for different therapeutics are an area of recent research interest. The present study attempts to validate the anti-cancer potential of bacterial pigment using colon cancer cell line (HCT_{15}) cells and antioxidant properties. We used *in-vitro* cultured cells (HCT_{15}) to determine the anti-cancer activity of extracted pigments from MB_4 strain by using MTT assay for measuring the cell viability, and our results depict a dose-dependent by increasing concentration of the extract which decreases in cell viability. The DPPH radical scavenging activity revealed an increased concentration of the extract with an IC_{50} value of 255.58 ± 43.51 mg ml^{-1}. The anti-cancer and antioxidant activity were induced by the extracted marine bacterial pigments. The presence study envisages the antioxidant and anti-cancer potential of bacterial pigments isolated from marine water, which can find applications in therapeutic treatments. The organisms were isolated from marine water, and yellow color pigment-producing bacteria were used for pigment extraction. Biochemical identification studies have confirmed the MB_4 organism to be gram-positive, Cocci with a distinct yellow color, positive for MR-VP, catalase, lipase, acetoin production, and hemolysis which can tolerate 10 percent NaCl and it can also grow pH at 9.

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DECLARATION OF INTEREST: The authors declare no conflict of interest exists.

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