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ISOLATION AND IDENTIFICATION OF POTENTIAL ANTI-CANCER PIGMENT-PRODUCING BACTERIA FROM THE GUT OF FISH DEVARIO AEQUIPINNATUS

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ABSTRACT
Cancer is a major public health problem in the world and it is the second leading cause of mortality worldwide. One in 4 deaths in the world is due to cancer. According to WHO Cancer is a generic term for a large group of diseases that can affect any part of the body. Currently, there are several conventional anti-cancer treatments, including surgery, chemotherapy, and radiotherapy. But these treatments are costly and have harmful side effects. Therefore, the exploration of newer and better anti-cancer agents with low side effects is essential to rectify this deleterious situation.
In the present study pigment producing bacteria from the gut of *Devario aequipinnatus* was isolated and identified. The pigment was characterized for possible anti-cancer property. The purification of pink pigment produced by the bacterial isolate was carried out using solvent extraction method post solvent optimization. Characterization of the pigment was done by GC- MS analysis and cytotoxicity profiling by MTT assay and anti-cancer property was evaluated via Acridine orange-ethidium bromide (AO/EB) staining. The overall completed study revealed that the pink pigment from *Massilia haematophila* CCUG 38318(T), isolated from *Devario aequipinnatus* fish gut has potential anti-cancerous property.

Keywords: Anti-Cancerous Property, *Devario Aequipinnatus*, Pigment Producing Bacteria, GC-MS Analysis.

Introduction

Cancer is a major public health problem in the world and it is the second leading cause of mortality worldwide. One in 4 deaths in the world is due to cancer. According to WHO Cancer is a generic term for a large group of diseases that can affect any part of the body (Taylor et al. 2007). It is estimated that there are nearly 2 to 2.5 million cancer cases at any given point of time in India. Over 7 lakhs cases and 3 lakhs Deaths occur annually due to cancer (Chaudhry et al., 2002).
Currently, there are several conventional anti-cancer treatments, including surgery, chemotherapy, and radiotherapy. But these treatments are costly and have harmful side effects (Elfahri *et al.*, 2016). Therefore, the exploration of newer and better anti-cancer agents with low side effects is essential to rectify this deleterious situation (Wang et al., 2014; Kahouli *et al.*, 2015). Presently, natural products comprise a large portion of current-day pharmaceutical agents, most notably in the area of

cancer therapy. Therefore, with a myriad of organisms yet to be explored and new technologies employed, biologically active compounds obtained from natural products will certainly continue to offer vast opportunities as sources of new anticancer therapeutic leads (Baindara et al., 2020).
A number of used anticancer therapeutics originate from natural sources, such as irinotecan, vincristine, etoposide and paclitaxel from plants, actinomycin D and mitomycin C from bacteria as well as marine-derived bleomycin (Wali et al.,2019). Many studies reported the anticancer ability of bacterial pigments with strong cytotoxic effect in a time-dependent manner (Lin et al., 2005). The demand for new drug expands with the increased cases of cancer reporting. Bacterial pigments or metabolites, isolated from novel ecosystem can be potential alternative source for new drugs. Various studies reported the screening of pigments isolated from various soil bacteria (Numan et al., 2018). In the present study pigment producing bacteria from the gut of *Devario aequipinnatus* was isolated and identified. The pigment was characterized for possible anti-cancer property.

Materials & Methods

Ezcount ^{TM} MTT cell assay kit, Nutrient agar, and Starch agar from Hi-Media. Isopropyl alcohol**,** Chloroform, H_2O_2, Iodine, Indole Kovacs reagent, potassium hydroxide, Saffranine, Crystal violet was from Merck chemicals Pvt. Ltd, India. All reagents were of analytical reagent grade.

Collection of Sample

Collection of *Devario aequipinnatus* fishes were performed according to the protocol of (Kannan et al., 2016). Live *Devario aequipinnatus* fishes were collected from a pond in Ponmundam, Malappuram, Kerala, India [Lat 10.96733°, Long 75.939433°] during the month of April 2021. The collected samples were transported in sterilized polythene bags containing habitat water. The fish samples were identified using standard Reference manuals (Zacharias et al 2013).

Isolation of Bacteria from Fish Gut

After 12 h of starvation, Gastrointestinal (GI) tract of *Devario aequipinnatus*, was dissected under sterilized conditions. Further, it was homogenized using sterile distilled and serially diluted. 0.1ml was spread over nutrient agar plate and incubated at room temperature for 24h and observed for any colonies.

Characterization of Bacterial Strain

The isolated colonies were subjected to identification by various morphological, physiological and biochemical analysis.

Colony morphology: Colony morphology was analysed for difference in their shape, size, colour and texture. The observations were recorded.

Staining: Gram staining was performed to distinguish between Gram positive and Gram-negative groups.

Biochemical analysis: Different biochemical tests like Catalase test, Amylase test, indol test, KOH test was performed according to the protocol of Facklam and Elliott, 1995, Hemraj et al. 2013, Mac Faddin, 2000 respectively for for identification of bacteria.

Identification of Bacterial Isolate by 16s rRNA Sequencing

The identification of isolates was carried out at the sequencing Facility of National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune according to their standard protocol.

Production and Extraction of Pigment

The extraction of the intracellular pigments from the broth was carried out using a modified solvent extraction procedure by Bisht et al., 2020. In brief, pigment-producing bacterial strain was cultured in Nutrient broth at 30°C for 7 days with continuous shaking at 140 rpm. The optimization of best solvent for extracting intra-cellular pigment was done using different polar solvents viz. Ethanol, methanol and non-polar solvents viz. Diethyl ether, and chloroform. 15 ml of 7 days old culture was centrifuged at 7800 rpm at 30 °C for 15 min and the pellet obtained was dissolved in 5 ml of each of these solvents. This was further centrifuged at 4 °C at 7800 rpm for 15 min to extract the pigment from cell debris, and purified using Chloroform.

Characterization of Pigment

The Characterization of Pigment was carried out by GC -MS analysis in an Instrument Model of 7890 A GC with 5975C with triple axis detector and Column – DB 5MS 30 m x 0.250mm Diameter x 0.25 Micro Meter Thickness. 1mL of syringe filtered sample was injected and analysed. Helium gas (99.9995%) was used as the carrier gas at a flow rate of 1 mL/min. The analysis was performed in the EI (electron impact) mode with 70 eV of ionization energy. The injector temperature was maintained at 280°C (constant). The column oven temperature program was set as in table 1. The compounds were identified after comparing the spectral configurations obtained with that of available mass spectral database (NIST -08 SPECTRAL DATA)

Table 1: The column oven temperature program for GC-MS

Oven	Rate °C/min	Value °C/min	Hold Time
Initial	-	50	10
Ramp 1	10	100	0
Ramp 2	7	150	0
Ramp3	5	280	15

Determination of Cell Cytotoxicity by MTT Assay

Determination of cell cytotoxicity was done according to the kit protocol of Ezcount ^{TM} MTT cell assay. Briefly, 100µl of 1×10^6 cells/ml in RPMI-1640 w/o phenol red was seeded in a 96 well plate and incubated at 37˚C with 5% CO_2 for 2 hrs. Non adherent cells were removed by washing with PBS and new media containing varying concentrations of bacterial pigment extract from 0.04 µg/ml to 40.0 µg/ml was added and incubated at the same conditions for a time period of 24 hrs. MTT reagent at a final concentration of 10% of total volume was added and the plate was wrapped in aluminium foil and incubated at 37˚C with 5% CO_2 for 4 hrs. The culture medium was aspirated off and solubilizing solution was added and mixed properly to dissolve the formazan crystals (Abhimannue et al., 2016). Absorbance was read on Thermo scientific Varioskan ELISA plate reader at 570 nm with a reference wavelength at 650 nm. Cell viability percentage was calculated as $[(A_T\text{-}A_B/A_C\text{-}A_B)^*100]$, where A_T is the absorbance of test, A_B, absorbance of blank and A_C is the absorbance of control.

Apoptotic profiling by Acridine orange- Ethidium bromide dual staining

AO/EB staining was performed on MCF-7 cells according to the protocol of Ribble et al., 2005 (Ribble et al., 2005). 100µl of 1×10^6 cells/ml in RPMI-1640 was seeded in a 96 well plate and incubated at 37˚C with 5% CO_2 till it attained confluency. Bacterial pigment extract at a concentration of 4.0 µg/ml, was added and incubated at the same conditions for a time period of 24 hrs. AO/EB solution was added to the wells and the nuclei were immediately visualized in the green filter of Inverted fluorescence microscope, Olympus IX51. Cells were counted to determine the percentage of apoptotic cells. The untreated cells were observed as controls and atleast 300 cells were counted in each sample.

Result & Discussion

Collection of Sample & Isolation of Bacteria from Fish Gut

The fish species, namely *Devario aequipinnatus* was collected from a pond in, Ponmundam, Malappuram, Kerala, India [Lat 10.96733°, Long 75.939433°]. The length and weight of the collected fishes were recorded and fishes were found to have an average body weight of 4.543g.

Fig 1: Collected fishes - *Devario aequipinnatus*

In the laboratory, the gut of the fish was surgically removed under aseptic condition. Serially diluted gut homogenate solution was spread over nutrient agar plate and incubated at room temperature for 24h. Microbial colonies were separated using streaking methods and fifteen bacterial strains were isolated and subjected to analysis and identification.

Characterization of Bacterial Strain

Out of 15 colonies isolated, only one strain showed a pink colour pigment production. This colony was isolated and streaked in agar plates and were incubated at 37°C overnight. The superior strain was selected based on its ability to produce pigment. The strain was sub-cultured in pure plates to avoid further contamination and was designated as BIO/SMC/DAG2/2021.

Fig 2: Pure culture of BIO/SMC/DAG2/2021

Among bacteria, pigment production is highly variable, although usually present in Actinobacteria. Several genera, such as *Streptomyces, Nocardia*, *Thermomonospora*, *Microbispora*, *Streptosporangium*, *Rhodococcus,* and *Kitasatospora* produce a wide variety of pigments. While the main application of these pigments is in the textile, food, and cosmetic

industries, pigments like melanin, quinones, violaceins, and indigoidines have been reported as good antimicrobial agents. Besides, pigments can be used as antioxidants, bioindicators, and anticancer agents; thus, their potential is becoming an important field of biomedical application (Celedon et al., 2021).

Morphological characterization of pigment producing bacteria

The isolated bacteria were identified and characterized with the help of morphological characteristics (Table 2) and their genus-level identification was done using Bergey's Manual of Determinative Bacteriology.

Table 2: Morphological and physiological characteristics of pigment-producing bacterial isolates.

Sl no:	Bacterial isolate	Morphological parameters
1	Pigment colour	Pink
2	Gram's reaction	Gram negative
3	Cell shape	Rod shape
4	Texture	Convex
5	Margin	Entire
6	Colony	Circular
7	Motility	Negative

Biochemical characterization of pigment producing bacteria

The isolated bacteria were identified and characterized with the help of biochemical reactions (Tables 3) and their genus-level identification was done using Bergey's Manual of Determinative Bacteriology

Table 3: Biochemical reactions of pigment-producing isolates

Sl no:	Biochemical test	Response
1	Indole test	Negative
2	Catalase test	Positive
3	KOH test	Positive
4	Amylase test	Positive

Identification of bacterial isolate by 16s rRNA sequencing

Identification of the bacterial isolate under study was done by 16s rRNA Sequencing at NCCS, Pune. The BLAST result of the query sequence revealed the isolate to be 99.70% similar to *Massilia haematophila* CCUG 38318(T). Identification report was generated using EzBioCloud Database and the confidence in identification is limited by both the availability and the Extent of homology shown by the ~1200 bp sequence of the sample with its closest neighbour in the Data base,it was designated as *Massilia haematophila* CCUG 38318(T).

Sequence Text (in FASTA format font: courier new 10) :>B_MAY_22_008

CGGCATGCTTTACACATGCAAGTCGAACGGCAGCACGGGCTTCGGCCTGGTGGCGAGT
GGCGAACGGGTGAGTAATACATCGGAACGTACCCAGAAGTGGGGGATAACGTAGCGA
AAGTTACGCTAATACCGCATACGTTCTACGGAAGAAAGTGGGGGATCGCAAGACCTCA
TGCTTTTGGAGCGGCCGATGTCTGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGC
GACGATCAGTAGCTGGTCTGAGAGGACGACCAGCCACACTGGAACTGAGACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCA
GCAATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTTGTCAGGGAAGA
AACGGCGGAGGCTAATATCCTTTGCTAATGACGGTACCTGAAGAATAAGCACCGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGG

GCGTAAAGCGTGCGCAGGCGGTTTTGTAAGTCTGACGTGAAAGCCCCGGGCTCAACCT
GGGAATTGCGTTGGAGACTGCAAGGCTTGAATCTGGCAGAGGGGGGTAGAATTCCACG
TGTAGCAGTGAAATGCGTAGAGATGTGGAGGAACACCGATGGCGAAGGCAGCCCCCTG
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AGCTAACGCGTGAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAA
AAACCTTACCTACCCTTGACATGTCAGGAATCCTCGAGAGATCGGGGAGTGCCCGAAA
GGGAGCCTGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTGTCATTAGTTGCTACGCAAGAGCACTCTAATGA
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTA
TGGGTAGGGCTTCACACGTCATACAATGGTACATACAGAGGGCCGCCAACCCGCGAGG
GGGAGCTAATCCCAGAAAGTGTATCGTAGTCCGGATCGTAGTCTGCAACTCGACTACGT
GAAGTTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGT
CTTGTACACACCGCCCGT

CHARACTERIZATION OF PIGMENT

GC-MS Chromatogram of the chloroform extracted pigment showed different peaks (Fig: 3) on a graph with relative abundance on Y axis and retention time on X axis. The highest peak observed at 60 min was further subjected to mass spectrum and identified. The GC–MS analysis of crude pigment indicated the presence of a variety of components as indicated in Table 4.

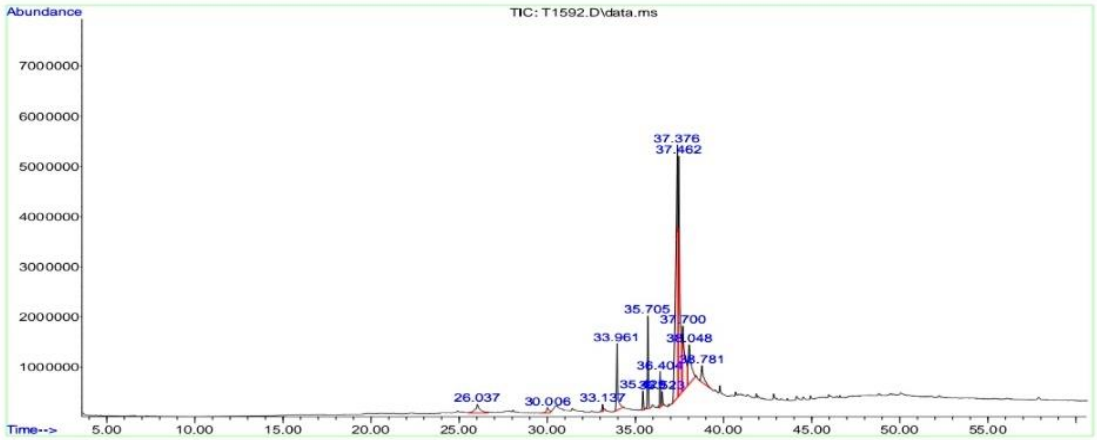
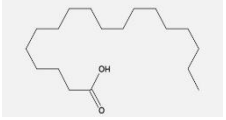
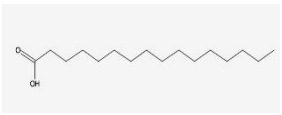
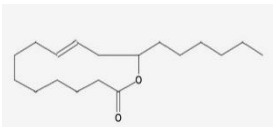
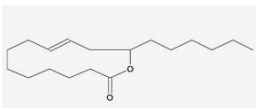
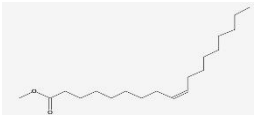
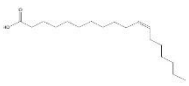
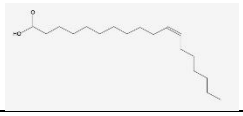
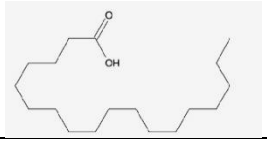
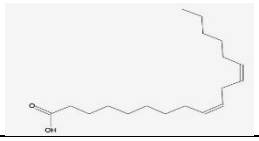
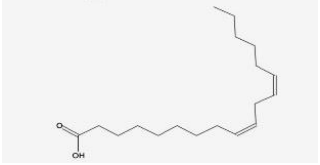


Fig 3: Total Ion Chromatogram of bacterial pigment extract

Table 4: Components identified in bacterial pigment extract

Sl No:	Rt (Min)	Name	Mol. Weight	Molecular Formula	Structure
1	26.065 min	Dodecanoic acid	200	C12H24O2	
2	30.041 min	Tetradecanoic acid	228	C14H28O2	

3	33.142 min	Hexadecanoic acid, methyl ester	270	C17H34O2	
4	33.958 min	: n-Hexadecanoic acid	256	C16H32O2	
5	(35.427 min	13-Hexyloxacyclotridec-10-en-2-one	280	C18H32O2	
6	(35.699 min	13-Hexyloxacyclotridec-10-en-2-one	280	C18H32O2	
7	(36.396 min	9-Octadecenoic acid (Z)-, methyl ester	296	C19H36O2	
8	36.523 min	9-Octadecenoic acid (Z)-, methyl ester	296	C19H36O2	
9	37.364 min	cis-Vaccenic acid	282	C18H34O2	
10	37.458 min	cis-Vaccenic acid	282	C18H34O2	
11	37.704 min	Octadecanoic acid	284	C18H36O2	
12	(38.044 min	9,12-Octadecadienoic acid (Z,Z)	280	C18H32O2	
13	38.774 min	9,12-Octadecadienoic acid (Z,Z)	280	C18H32O2	

The GC–MS analysis of crude pigment indicated the presence of a variety of complex components. Among them, the main two dominating compounds were identified as cis-Vaccenic acid (37.364min) and cis- 13-Octadecenoic acid (37.364 min) known for its antibacterial activity and hypolipidemic effects in rats respectively. Further evaluation was carried out to check the cytotoxicity profiling of the pigment by MTT analysis where it was showed not to be cytotoxic against HeLacells studied for the analysis. Hence, it could be used for further utilization if found, positive for biological activity.

EVALUATION OF BIOACTIVE PROPERTIES OF BACTERIAL PIGMENTS

Effect of bacterial pigment extract on viability of cells

The percentage of cell viability on HeLa was determined by MTT assay. The results presented in Fig: 4 indicate that cells were more than 95% viableat 24 hrs. The result indicates that the bacterial pigment isolated is not cytotoxic to the cells under study.

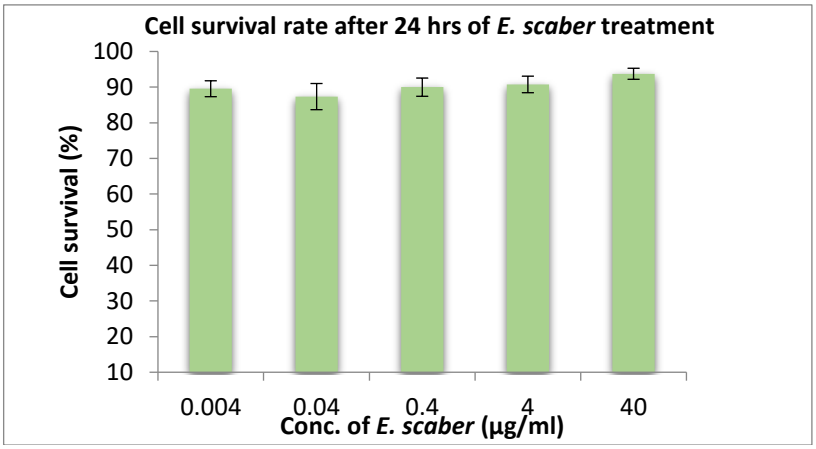


Fig4.21: The cell survival rate (in percentage) of HeLaupon treatment with bacterial pigmentincubated for a period of 24 hrs.Values are expressed as mean ± SD, from the assays done in triplicate.

4.5.1. Acridine orange- ethidium bromide (AO/EB) staining

Acridine orange is a vital dye and can stain live cells uniform green. But Ethidium Bromide could stain only dead cells that had lost membrane integrity. Late apoptotic cells, upon staining appear orange, but with deep dots in nuclei that are indicative of chromatin condensation. Necrotic cells too appear orange, but, with no nuclear morphology (Liu and Jiao, 2019). Upon treatment with bacterial pigment extract (4 µg/ml concentration), a very low apoptotic index of and 6.200 ± 0.371 percentages was obtained.

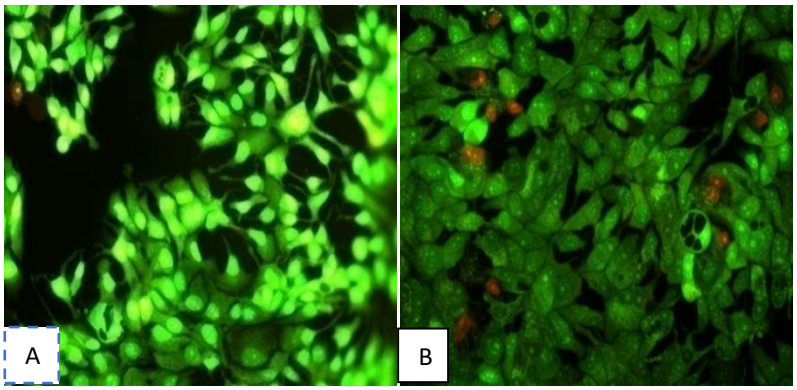


Fig 5: AO/EB dual staining of HeLa cell lines upon treatment with bacterial pigment extract. A) Represents HeLa cells without any treatment serving as the control; B) HeLa cell treated with bacterial pigment extract

In Acridine orange - Ethidium Bromide staining with bacterial pigment extract (4 µg/ml concentration), a very low apoptotic index of and 6.200 ± 0.371 percentages was obtained.This needs to be further evaluated with higher concentration.

Conclusion

In this study live *Devario aequipinnatus* fish sample were collected and dissected the fish gut. Pigmented producing bacteria was isolated and maintained for further morphological and biochemical characterization. Subsequently, the bacterial isolates were subjected to 16s rRNA Sequencing. The purification of pink pigment produced by the bacterial isolate was carried out using solvent extraction method post solvent optimization. Characterizationof the pigment was done by GC- MS analysis. Cytotoxicity profiling was done by MTT assay and anti-cancer property was evaluated via Acridine orange- ethidium bromide (AO/EB) staining.

The overall completed study revealed that the pink pigment from *Massilia haematophila* CCUG 38318(T), isolated from *Devario aequipinnatus* fish gut has potential anti-cancerous property.From the GC-MS studies revealed that the pigment contains thirteen components with various functions.The percentage of cell viability on HeLa was determined by MTT assay. The results indicate that cells were more than 95% viable at 24 hrs. The Bacterial pigment treated with Acridine orange- ethidium bromide (AO/EB) staining, and determined that a very apoptotic index of and 6.200 ± 0.371 percentages was obtained. These results offer justification for further research to evaluate the structural features of the pigment molecule and its effect on specific cancer cell lines.It inhibits the growth and proliferation of HeLa cells in vitro through induction of apoptosis. Similarly, various other assays like annexin-propidium iodide or DNA fragmentation assay needs to be performed to confirm the anti-cancerous property. Also, the component identified in GC-MS analysis needs to be subjected to molecular docking too to obtain further biological activities in terms of anti-cancerous property. The present data provides a preliminary observation on the pigments extracted from the gut microbiome of *Devario aequipinnatus.*

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