Melanin pigment producing marine actinobacterium of *Nocardiopsis* sp. isolated from marine sediment samples and their anticancer potential against breast cancer cell line

Running title: Melanin from *Nocardiopsis* sp and their anticancer activity

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ABSTRACT:

INTRODUCTION: Anti-Breast cancer medication is used to treat or prevent cancer. Breast Cancer-prevention drugs are known as anti-breast cancer drugs because they have an anti-cancer activity against cancer-causing stimulation and cancer cells. To test the anticancer activity of Melanin pigment producing marine actinobacterium of the *Nocardiopsis* species which was isolated from marine sediment samples using an assay known as MTT

MATERIALS AND METHODS: The sample was collected from the area Pichavaram mangrove forest area, Tamilnadu. Isolation of actinobacteria was carried out using Kuster agar medium. The colour of the mature sporulating aerial mycelium was recorded in naked eye. The melanoid pigments test was carried out on the media ISP-1 and ISP-7 as recommended by the International Nocardiopsis project. Reverse side pigment production of the isolate was determined on ISP-7 medium. Soluble pigment production of isolate was observed on ISP-7 medium. Spore morphological characters of the strain were studied by inoculating a loop of old cultures into solidified agar containing sterile glass slides. The chemotaxonomic characteristics were also analyzed by the method recommended by the ISP. The production of melanin from potential marine actinobacteria was estimated by the method of sivaperumal et al, 2018. The proliferation of MCF-7 cells was assessed by MTT assay safadi et al, 2003.

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RESULT: The melanin producing marine Nocardiopsis was isolated and identified. The production of melanin and other parameters were estimated. Further, the melanin showed more than 50% of inhibition after 72 hours by using the MTT assay in the MCF-7 cancer cell line.

CONCLUSION: From the current study .We conclude that the melanin from marine *Nocardiopsis* species showed potential anticancer properties and it might be useful for further research on marine drugs . As we have found that the *Nocardiopsis* species have over 65% of inhibition after 72 hours at 3µg/ml of extract by using the MCF-7.

INTRODUCTION:

Anti cancer is employed against or tending to arrest or prevent cancer. The drugs used for preventing cancer are called anti cancer drugs which have an anti cancer activity towards the cancer causing stimulation and cancer cells[1]. To check the ability of the anti-cancer activity of the extracted extracellular polymeric substances from a marine actinobacteria of *Nocardiopsis* species through an assay called MTT[1,2],[1–3]. The MTT/MTS in Vitro cell proliferation assay is one among the foremost widely used assays for evaluating preliminary anti cancer activity of both synthetic derivatives and natural products and natural product extracts[4],[5]

Melanin are termed as natural substances of high molecular weight released by microorganisms into their surroundings. Melanin establishes the functional and structural integrity of biofilms and is considered a fundamental component that determines the physicochemical properties of microbes [6]. Melanin are mostly composed of polysaccharides and proteins, but include other macromolecules such as DNA, lipids and humic substances [6,7] Exopolysaccharides are the sugar based parts of the EPS. Microorganisms synthesise a good spectrum of multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides[8] Previous research studies have shown that in addition to EPS sugars from Cyanobacteria to wastewaters remove heavy metals like copper, cadmium and lead. EPS sugars can physically stimulate these heavy metals and take them in through biosorption [9].

Melanins are termed as macromolecules made by oxidative polymerization consisting of phenolic or indolic compounds. Regularly the next shades are earthy colored or dark in shading however numerous different tones have additionally been noticed. Melanins are additionally hydrophobic and contrarily charged[10]. The biosynthesis of melanin is started from L-tyrosine by means of a progression of enzymatic and nonenzymatic responses by the protein tyrosinase. to start with, tyrosinase (monophenol monooxygenase EC 1.14.18.1) catalyzes oxidation of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA), which is additionally changed over into dopachrome. Dopachrome is modified over to melanin by a progression of non enzymatic oxidoreduction reactions. Tyrosinases from various natural sources are used

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for the amalgamation of L-DOPA and therefore the expulsion of phenolic compounds from waste waters [10,11]. There are three sorts of melanins for instance eumelanins, pheomelanin and allomelanins. Eumelanins are dark to brown shading colors created by oxidative polymerization of tyrosine (and additionally phenylalanine) to L-DOPA, which is additionally changed over into dopachrome and afterward to melanin. Eumelanin is the transcendent color incorporated in people and microorganisms[12]. Pheomelanin are red or yellow shading colors which are initially combined much like eumelanins, yet DOPA goes through cysteinylation and contains sulfur. The allomelanins framing the mediocre class are heterogeneous colors incorporating nitrogen free heterogeneous gathering of polymers shaped from an assortment of sources like dihydrofolate, homogentisic corrosive, catechols, then forth [12,13]

The majority of actinobacteria may be found in each aquatic and terrestrial habitat. The recent researchers screened intensively the marine plants, medicative plants, sediments and soil environments of actinobacteria, so as to elucidate their bioactive molecules. Although soils are screened by the pharmaceutical trade for fifty years, solely a minute fraction of the surface of the world has been sampled, and solely alittle fraction of actinobacteria taxa has been discovered [10] Actinobacteria are the foremost economically and biotechnologically valuable prokaryotes. regarding half the discovered bioactive secondary metabolites are created by them. Microorganisms are rumored to supply around twenty three,000 bioactive secondary metabolites and over 10,000 of those compounds are created by actinobacteria, representing forty fifth of all bioactive microbial metabolites discovered[14] Around 7600 compounds are created by actinobacteria species. or so 289 secondary metabolites from the marine-derived genus of actinobacteria are rumored within the Marin lit info, covering a good form of chemical structures, as well as peptides, macrolides, lactones, indoles, terpenes and quinones. These compounds show an in depth variety of industrially helpful activities, like cytotoxic, medicament, antifungal, antiprotozoal, anticancer, medicinal drug, anti-inflammatory drug, anthelmintic, herbicide [14]

The bioactive secondary metabolites from marine-derived actinomycete have attracted increasing interest throughout the last decades. Thanks to the wonderful record of accomplishment of actinomycetes during this regard, a big effort has been targeted on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programs within the past fifty years[14,15] Recently, there has been a decrease within the rate of discovery of latest compounds from terrestrial actinomycetes. On the opposite hand, there has been a rise within the rate of re-isolation of familiar compounds. so it's crucial for brand spanking new teams of actinomycetes from unknown or underexploited habitats to be pursued as sources of novel bioactive secondary metabolites [16]. Although there's a rare diversity of life within the terrestrial atmosphere, the best diversity is localized within the oceans. Marine actinobacteria are chemicals made from structurally various secondary metabolites [17]. Our team has extensive knowledge and research experience that has translate into high quality publications [18–22],[23],[24],[25],[26],[27],[28],([20,29,30],[31–35], [36],[37].[38]

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[39] [40] [41] [41,42] [43] [44] [45] [46] [47] [48] [49] [50] [51] [52] [53] [54] Aim of the study is to anticancer potential of EPS obtained from marine actinobacterium of *Nocardiopsis* species.

MATERIALS AND METHODS

Sample collection and preparation

The sediment sample was collected from the Pichavaram mangrove forest area, Tamilnadu. The collected sample was sun dried for 48 hrs and turned into fine powder by mortar and pestle.

Isolation of actinobacteria: Isolation and enumeration of actinobacteria were carried out in Kuster's agar medium (KUA) supplemented with 0.5% (W/v) NaCl. To minimize the fungal and bacterial contamination, KUA medium was supplemented with cycloheximide (10 µg/ml) and nalidixic acid (10 µg/ml) respectively (Kathiresan et al., 2005). Collected sediment samples were serially diluted and inoculated on KUA medium and incubated at 36°C for 7 days. The colonies were counted and the population density has been expressed as colony forming units per gram (CFU/g) of sediments. Morphologically distinct colonies were selected and pure cultures were obtained.

Identification of marine actinobacteria

Aerial mass colour: The colour of the mature sporulating aerial mycelium was recorded in naked eye. When the aerial mass colour fell between two colours series, both the colours were recorded. If the aerial mass colour of a strain to be studied showed intermediate tints, then also, both the colour series were noted. The media used were Yeast Extract-Malt Extract Agar and Inorganic-Salt Starch Agar.

Melanoid pigments: The grouping was made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown, pigment modified by other colours) on the medium. The strains were grouped as melanoid pigment produced (+) and not produced (-). In a few cases, the production of melanoid pigments was delayed or weak, and therefore, it was not distinguishable. This is indicated as variable (V). This test was carried out on the media ISP-1 and ISP-7 (Appendix I), as recommended by the International Nocardiopsis Project (Shirling and Gottlieb, 1966).

Reverse side pigments: Reverse side pigment production of the isolate was determined on ISP7 medium. The pigment production was noted as distinctive (+) and not distinctive or none (-). In case, a colour with low chroma such as pale yellow, olive or yellowish brown occured, it was included in the latter group (-).

Soluble pigments: Soluble pigment production of isolate was observed on ISP7 medium. The diffusible pigment production other than melanin was consider as positive (+) and not produced (-). The colour was recorded (red, orange, green, yellow, blue and violet).

Spore chain morphology: Spore morphological characters of the strains were studied by inoculating a loopful of one week old cultures into solidified agar medium contained sterile glass slide. The cultures were

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incubated at 28+20 C and examined periodically for the formation of aerial mycelium, sporophore structure and spore morphology.

Chemotaxonomical characteristics

Hydrolysis :Hydrolysis was done for releasing amino acids. Harvested cells of each strain weighing 20 mg (fresh) were placed in an ampo bottle and 1 ml of 6 N HCl was added and sealed with an alcohol blast burner. The samples were kept at 1210 C for 20 h in a sand bath. The bottles were cooled by keeping them at a room temperature of 28+20C. Hydrolysis was also done for releasing sugars. Harvested cells of each strain weighing 50 mg (fresh) were placed in an ampo bottle and 1 ml of 0.5N HCl was added and sealed with an alcohol blast burner. The samples were kept at 1100 C for 2 h. The bottles were then cooled by keeping them at a room temperature of 28+20 C.

Thin Layer Chromatography (TLC): Spotting of the whole cell hydrolysates was made carefully on TLC plate using a microliter pipette. Spots were 5-10 mm in diameter. This was done by multiple applications on the same spot of very small portions of the sample, which were dried by a hand dryer.

Amino acids: Each sample (3 µl) was applied on the baselines of the TLC plate (20 cm x 20 cm). Adjacent to this, 1µl of DL-diaminopimelic acid (an authentic material mixture of DAP isomers) and 1µl of amino acetic acid (glycine) were spotted as standards. The TLC plate was developed with the solvent system containing methanol: pyridine: glacial acetic acid: H2O (5: 0.5: 0.125: 2.5 v/v). It took approximately more than 4 h for development. The spots were visualized by spraying with 0.4% ninhydrin solution in water-saturated n-butanol, followed by heating at 1000 C for 5 min. Spots of amino acids ran faster than DAP. The sample spots were immediately compared with the spots of the standards since spots gradually disappeared in a few hours.

Whole-Cell sugars: On a cellulose TLC plate (20 cm x 20 cm), 5µl of samples was spotted along with 3 µl of sugar solutions as standards on the same plates. Galactose, arabinose, xylose and madurose were the sugars, which were used as standards. The TLC plate was developed with the solvent mixture containing ethyl acetate: pyridine: acetic acid: distilled water (8: 5: 1: 1.5 v/v). The development time was more than 4 h. Spots were visualized by spraying with aniline phthalate reagent (3.25 g of phthalic acid dissolved in 2 ml of aniline and made upto 100 ml with water saturated n-butanol). The sprayed plate was heated at 1000 C for 4 min. Hexoses appeared as yellowish brown spots and pentoses, as maroon coloured spots.

Assimilation of carbon source: The ability of the actinobacterial strain in utilizing various carbon compounds as a source of energy was studied, following the method recommended by the ISP (Shirling and Gottlieb, 1966). Chemically pure carbon source certified to be free of admixture with other carbohydrates and contaminating materials were used for this purpose. Carbon sources for this test were Arabinose, Xylose, Inositol, Mannitol, Fructose, Rhamnose, Sucrose and Raffinose. These carbon sources were sterilized by ether sterilization without heating. The media and plates were prepared and inoculated

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according to the convention of ISP project (Shirling and Gottlieb, 1966). For each of the carbon sources, utilization is expressed as positive (+), negative (-), or doubtful (±). In the 'doubtful ' strains, only a trace of growth slightly greater than that of the control was noticed.

Production and purification of melanin :The ISP-2 medium was prepared in seawater and was used for the development of inoculum. The 2ml of spore suspension from inoculums medium was inoculated in the fermentation medium containing 0.65 ml of glycerol, 0.63gm of Yeast extract, 0.55gm of glucose, .08gm of MgSO4.

The 2ml of spore suspension was inoculated into fermentation medium (ISP7) for 12days under the agitation for 200rpm at ambient temperature. Then cell free supernatant was collected by centrifugation at 10,000 rpm for 15min. The harvested cell free supernatant containing melanin was adjusted to pH 2 with con. HCl and kept at room temperature for 3hrs. After incubation the suspension was centrifuged at 10000rpm at 28°C for 20 mins to palletize the melanin pigment. The pellet was washed 3 times with distilled water and dissolved in a phosphate buffer (pH8).

Chemical analysis of the melanin: The chemical test was carried out with a little modification (Fava et al., 1993). The solubility test for the black pigment was tested by adding 100 µl of melanin pigment in 1ml of distilled deionized water, 1N HCl, 1M NaOH, absolute ethanol, acetone (warm), chloroform (warm), Phenol and Benzene. The reaction with the following oxidizing agents was also determined by adding 100µl of melanin to the 1ml of 30% Hydrogen peroxide (H2O2) solution. The precipitation test was carried out by adding 100 µl of purified melanin to 1ml of 1% Fecl3 solution and 1ml of Con. HCl.

MTT Assay: The proliferation of MCF-7 cells was assessed by MTT assay Safadi et al., (2003). MCF-7 cells were plated in 48 well plates at a concentration of 2x104 cells/well 24 hours after plating, cells were washed twice with 500µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with melanin in different concentrations for 24 hours. At the end of treatment, the medium from control and melanin treated cells were discarded and 200µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200µl of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan crystals formed were dissolved in dimethylsulfoxide (200µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nmof treated cells/A570 nm of control cells]×100.

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MORPHOLOGY STUDY: Based on MTT assay we selected the optimal doses (30 µg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 3×104cells were seeded in 6 well plates and treated with melanin (30 µg/ml for MCF-7 cells) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

STATISTICAL ANALYSIS: All data obtained were analyzed by Student's-t-test using MS-Excel, represented as mean ± SD for triplicates. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA) using one-way ANOVA. The level of statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Isolation of marine actinobacterium of *Nocardiopsis* species

The present study, isolation and identification of melanin producing marine Nocardiopsis species was done from sediment samples. The conventional identification features of spore chain morphology, sugar patterns and chemotaxonomic characteristics were done for the identification of the *Nocardiopsis* species (Table-1&2).

Table 1 Represents conventional finding of marine actinobacteria *Nocardiopsis* species

Color of aerial mycelium	Grey
Melanoid pigment	+
Reverse side pigment	-
Soluble pigment	+
Spore chain	Long chain
Assimilation of carbon source	
Arabinose	+
Xylose	+

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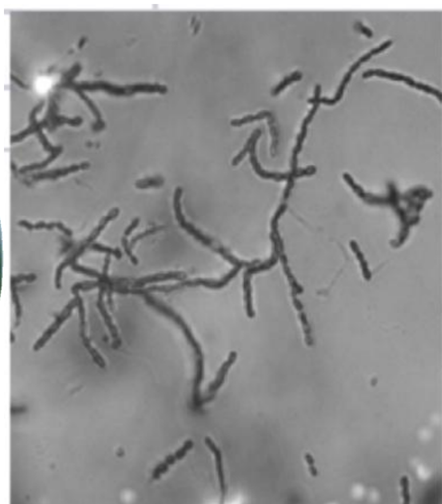
Inositol	-
Mannitol	+
Fructose	+
Rhamnose	-
Sucrose	-
Raffinose	+

Table 2 Represents chemotaxonomic findings of marine actinobacteria *Nocardiopsis* species

Cell Wall Amino Acids: Cell Wall Sugar: Cell Wall type Index:

LL-DAP	MesoDAP	Glycine	Arabinose	Galactose	III
-	+	-	-	-	*Nocardiopsis* sp.



(A) **(B)**

Fig.1A-B: Marine actinobacteria *Nocardiopsis* species strain and their spore chain morphology

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Anticancer potential:

Breast cancer is the second most frequent cause of death in women, according to a new study. Surgery, radiotherapy, immunotherapy, and chemotherapy are some of the clinical approaches for cancer patients. Although each of these treatments is helpful in its own right, when used together, they provide a more effective and comprehensive treatment for tumor conditions. Many antitumour compounds are derived from the EPS of marine actinobacteria, and these metabolites play a significant role in the identification of pharmaceutical compounds.[55]

The Actinobacteria are a phylum of Gram-positive bacteria. They can be terrestrial or aquatic. Marine actinobacteria are unique enhancing quite different biological properties including antimicrobial, anticancer, antiviral, insecticidal and enzyme inhibitory activities. Adaptation of marine actinobacteria to extreme climatic conditions such as high salinity, high pressure, and high temperature have modified their physiological conditions to survive and elaborate novel bioactive metabolites. They have attracted global in the last ten years for their ability to produce pharmaceutically active compounds[56]
Marine microorganisms, especially the actinobacteria, have produced the most genomic and metabolic diversity, and efforts should be made to explore them as a possible source for discovering novel bioactive products and developing newer drugs.[57,58]

Many persistent attempts to find an appropriate anticancer potential from natural resources have resulted in the creation of a new medicinal treatment in several instances. In this present original study, we have demonstrated and conducted a experiment based on MTT assay for anticancer potential for both the protein and organic extracts of marine actinobacteria nocardiopsis species were regulated and screened for anticancer potential against human breast cancer cell line and human invasive ductal carcinoma. It Is clearly shown that the effect of nocardiopsis species on the culture plate of Cancer cells have displayed a reasonable degree of anticancer potential, causing approximately over 65% inhibition after 72 hours. On using the MCF-7 cell line, the capacity of the tested compound has shown that almost over 60% of inhibition in 72 hours by using the MTT assay

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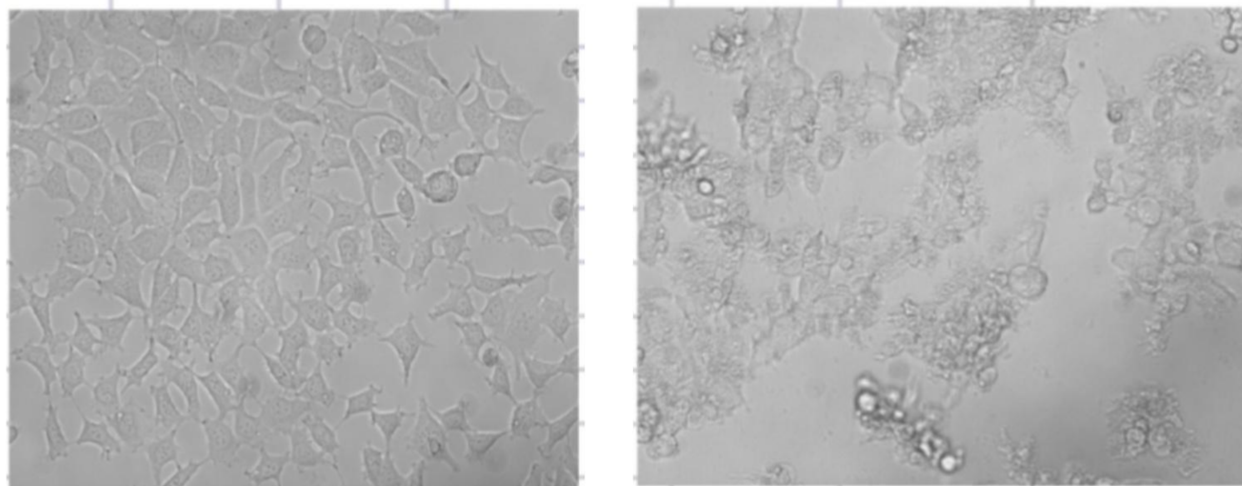

Control **Treated with melanin**

Fig 2 The anticancer potential of melanin obtained from *Nocardiopsis* species. against MCF-7 cell line

In a corresponding clinical study it has been reported that *Nocardiopsis* species, which are isolated and contaminated from terrestrial soils in the eastern ghats, have exhibited the anticancer potentials against the selected cancer cells. Secondary metabolites from actinobacteria, particularly the nocardiopsis is the most important marine source for acting as an anticancer reagent and has high anticancer potential (*Nocardiopsis* species: a potential source of bioactive compounds) [56,59][60][61].

CONCLUSION:

We conclude that the melanin from marine *Nocardiopsis* species showed potential anticancer properties and it might be useful for further research on marine drugs . As we have found that the *Nocardiopsis* species have over 65% of inhibition after 72 hours at 3µg/ml of extract by using the MCF-7.

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AUTHOR CONTRIBUTION:

Roshan A carried out the literature search, data collection, data analysis and manuscript writing. Dr Sivaperumal P and Dr Ezhilarasan have conceived the study, participated in its design and coordinated and provided guidance to draft the manuscript. All authors have equally contributed in developing the manuscript.

CONFLICT OF INTEREST:

The authors declare that there was no conflict of interest.

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