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IN-VITRO PHOTOPROTECTIVE EFFECT OF AN ACTINOMYCETE DERIVED MELANIN AGAINST MELANOMA

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ABSTRACT: Actinomycetes are biotechnologically important microorganism as they produce many secondary metabolites and natural pigments, including a dark-brown pigment melanin. Melanin is produced in melanocytes that provides pigmentation to skin. Although many roles have been attributed to melanic pigments, the main role in nature seems to be photoprotection damage. Melanin possesses anti- UV radiation property by absorbing the electromagnetic spectrum and preventing optical damage in the living organisms. Melanoma is a malignant of melanocytes that controls the pigment melanin in the skin. The aim of the present study is to isolate the melanin producing actinomycetes from the agricultural soil and to assay the cytotoxicity using murine B16 melanoma cell line and normal mouse L929 fibroblast cell line. The results showed that the melanin obtained from the actinomycetes showed good anticancer activity against murine B16 melanoma cell line and nontoxic against normal mouse L929 fibroblast cell line. Hence it can be used as a photoprotective agent.

INTRODUCTION: Melanin originates from a Greek word Melanos means black. Melanin is a dark- brown pigment which is negatively charged composed of polymers and phenolic compounds. The pigment melanin is ubiquitous, found in human beings, animals and microorganisms. Microorganisms in the culture media by fermentative oxidation, produces dark- brown pigment referred to as melanin or melanoid[1]. Melanin protects the skin from UV radiation and from external physical changes through its physiological activity and maintain intracellular homeostasis [2, 3].

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Melanin also serves as: (a) an antioxidant and a free radical scavenger [4, 5], (b) a photoprotector that efficiently absorbs and dissipates heat from the solar radiation [6], (c) an absorbent of organic compounds and chelates metals [7, 8] and (d) an organic semiconductor [9]. Melanins are used in a wide variety of applications in day-to-day life including cosmetics, optical lenses, pharmaceuticals, and batteries to name some [10-15].

UV radiation primarily ultraviolet B (UVB) and ultraviolet A (UVA), has a range of harmful effects in biological systems especially at the DNA level. The undesirable effects connected to UV exposure in humans includes skin cancer, photoaging and immunosuppression [16]. The screening for photoprotective compounds that filter UVB and UVA, is must to the minimize skin cancer development. Generally, a compound's absorbance depends on its structural characteristics, larger molecules will absorb longer wavelengths.

The aromatic compounds are considered better UV screeners as their maximum absorption is 2 usually within the UV range, and their efficacy increases with the number of substituents they possess [17]. The melanin is a chemically diverse and most widespread pigments present [18] having complex polymeric structures with aromatic rings making UVB and UVA screeners [17] and the stable free radical scavenging character makes them to act as a sink for other free radicals [19].

The aim of the study is to isolate and identify the melanin producing actinomycete from rhizosphere soil and to test its anticancer activity against melanoma.

MATERIALS AND METHODS:

Screening and Isolation of Melanin Producing Actinomycetes: Actinomycetes were isolated from the rhizosphere soil samples collected from the agricultural fields in Vellore, India. The samples were serially diluted and plated on starch casein agar with nystatin and actidione. For screening of the melanin producing actinomycetes, the isolates were inoculated inpeptone iron medium. The inoculated plates were incubated for 5 days at 30°C. After the incubation period the plates were observed for colonies showing diffusible pigment.

Melanin Production:

Inoculum Preparation: An isolated black pigmented colony, from the culture plate was transferred in to 50 ml Erlenmeyer flask containing peptone iron broth. The flask was incubated at 30°C for 48 hours at static condition. The freshly grown culture with 1.0 OD at 600 nm is used as inoculum to inoculate the production medium.

Production Medium: The pigment production by submerged fermentation in 100 ml of the Peptone-iron medium inoculated with 5% inoculum of selected strain was carried out in 250 ml Erlenmeyer flask and then incubated at30 ºC for 5-6 days. After the black pigment formation culture suspension was centrifuged at 5000 rpm for 20 min. The cell free supernatant was used for the extraction of the pigment.

Assay for Melanin Production: The pigment was characterized by taking 2 ml of the supernatant and 1 ml of 0.4% substrate solution (L-dopa). The reaction mixture was incubated at 37°C for 5 min and read spectrophotometrically at 200-800 nm (UV-1601, Shimadzu) [20, 21].

FT-IR of Melanin Extract: The extracted melanin was characterized by FT-IR spectroscopy (Fourier transform infrared spectroscopy, Perkin Elmer Spectra GX). The FT-IR analysis was carried out in the mid IR, region of 400–4,000 cm^{-1}. The extract was mixed with spectroscopically pure KBr in the ratio of 5:95 to form uniformpellets, which was then fixed in sample holder, and the analysis was carried out.

Characterization of the Isolate: The isolate was characterized by studying its morphological (colony characters and Grams nature) and biochemical characteristics using standardized protocol. The observations were noted and compared as described in Bergey's Manual of Systemic Bacteriology.

***In-vitro* Cytotoxicity and Anticancer Assay:** Both safety or toxicity and the anticancer activities of the purified melanin pigment of the actinomycetes were measured *in vitro* on both cancerous (murine melanoma cell line B16) and non-cancerous cells (mouse fibroblast L929) procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA).

B16 cell line was used to determine the inhibitory effects of melanin pigment on cell growth and L929 cell line was used to determine the cytotoxic effects of melanin using standard 3-(4, 5 dimethythiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MTT assay is a colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells [22]. The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of the cells was evaluated by direct observation of cells under Inverted phase contrast microscope. Any detectable changes in the

morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cells Seeding in 96 well Plate: Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of Compound Stock: 1mg of melanin was weighed and dissolved in 1ml 0.1% DMSO using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

After 24 hours the growth medium was removed, freshly prepared each compound in DMEM were five times serially diluted by twofold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained.

MTT Assay: Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the contents in wells were removed and 30µl of MTT solution was added to the test and control wells, the plate was shaken gently, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently to solubilize the formazan crystals. The absorbance was measured by using microplate reader at a wavelength of 540 nm [23].

The percentage of growth inhibition was calculated using the formula:

% of viability = Mean OD Samples / Mean OD of control group × 100

RESULTS AND DISCUSSION: The three quarters of the surface of the planet is covered with soilis the home to more than 80 % of life and yet remains largely unexplored. Soil microorganisms with great potential are expected to produce bioactive compounds that represent a valuable source for the development of novel therapeutic agents. An attempt has been made to isolate and screen the melanin pigment producing bacteria from the agricultural soil. Overall, ten different isolates were isolated by serial dilution technique and spread plate method on the starch casein agar (SCA) medium. Plates exhibiting pigmented colonies were selected. Among all the isolates, a black pigment producing colonies were selected and propagated on the same medium until pure cultures were obtained. The colony characteristics of the isolate have been studied and photographed **Table 1** & **Fig. 1.**

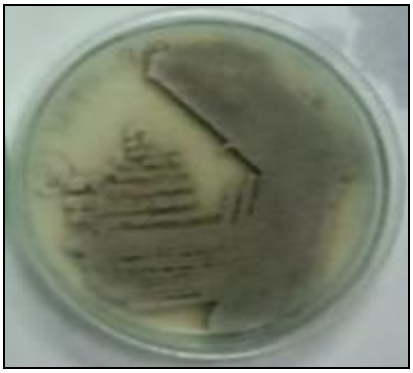

FIG. 1: MORPHOLOGICAL CHARACTERISTICS ON STARCH CASEIN AGAR

FIG. 2: PIGMENT PRODUCTION IN PEPTONE IRON BROTH

UV–Visible Spectrophotometric Analysis of the Extracted Pigment: Among the different solvents used for pigment extraction methanol: acetone mixture 5:1 (v/v) was found to be superior in comparison to other solvents. The supernatant was collected and the process was repeated until the

pellet turned white **Fig. 2.** The UV-Visible absorbance spectrum (200–800 nm) of the purified melanin is shown in **Fig. 3.** Maximum absorption peak was observed in the UV region at 235 nm which then decreased towards the visible region, which is the characteristic property of melanin. The peak at 235 nm was similar to the peak for melanin pigment extracted from *Phyllosticta capitalensis* [24]. The *Chroogomphus rutilus* melanin which had maximum absorption peak at 212 nm [25] and *Actinoalloteichus* sps. MA-32 melanin had produced the peak at 300 nm [26]. Whereas, absorption peak of melanin synthesized by *Streptomyces bikiniensis* M8 exhibited an absorbance in the UV region with highest absorption peak was found at 230 nm, the peak decreased towards the visible region due to the presence of the very complex conjugated structure, which is the characteristic property of melanin [27].

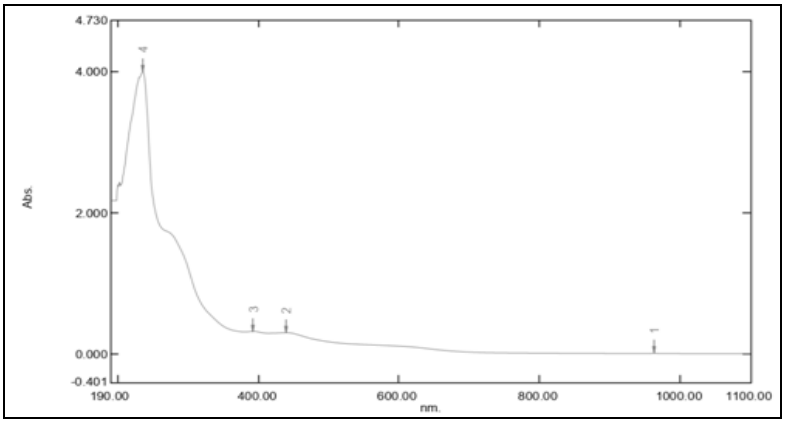

FIG. 3: UV- VIS CHARACTERIZATION OF THE PIGMENT

FTIR Analysis of Melanin: The FTIR spectrum of the extracted melanin **Fig. 4** showed peaks around 3546.45 cm^{-1} and 3264.13 cm^{-1}, correspond to the OH group, small band at 2923.21 cm^{-1} can be assigned to stretching vibration of aliphatic C-H group. The signals in the 2412.90 cm^{-1} area are attributed to the stretching vibrations (O-H) of the carboxylic acid. Peak observed around 1786.09 cm^{-1} and 1733.91 cm^{-1} are attributed to bending of C=O group. The characteristic strong band at 1676.74 cm^{-1} attributed to vibrations of aromatic ring C=O of esters. The N-H bending vibration peak at 1536.65 cm^{-1}, indicates that the pigment had typical indole structure of melanin. The peak centered at 1380.84 cm^{-1} N=O characteristic of nitro group. Phenolic COH stretching at 1230.75 cm^{-1} relates to phenolic compounds. The peak centered at1044.92 cm^{-1} is the indication of C-O characteristic of esters. The peak observed at 829.79 cm^{-1} due to aromatics C–H group ascribed to alkene C-H substitution in the melanin pigment. The functional groups of the pigment extracted from *Streptomyces* sps. correlated with those of melanin produced by various microorganisms as reported previously. On the basis of the presence of the functional groups, it was concluded that the pigment was melanin.

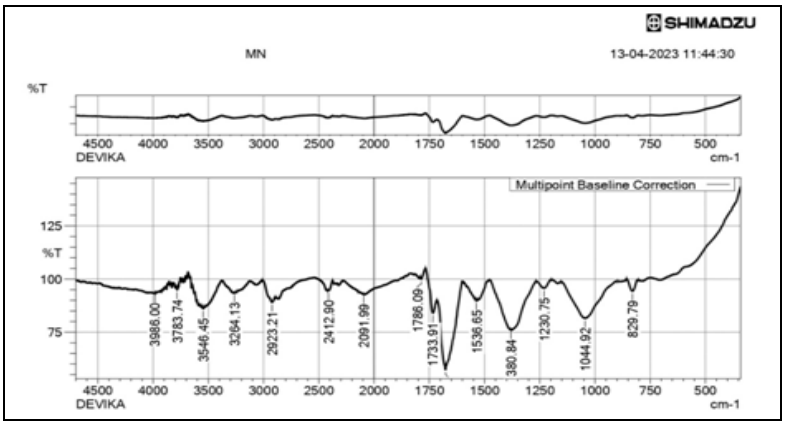

FIG. 4: FT-IR CHARACTERIZATION OF THE PIGMENT

TABLE 3: ABSORBANCE VALUE AT 540NM

Sample Concentration (µg/ml)	OD I	OD II	OD III	Average Absorbance @ 540nm	Percentage Viability
Control	0.5827	0.5823	0.5874	0.5841	100.00
			Sample		
6.25	0.5248	0.5217	0.5206	0.5224	89.13
12.5	0.5142	0.5008	0.5025	0.5058	86.03
25	0.5607	0.457	0.45	0.4892	77.04
50	0.3481	0.3402	0.3419	0.3434	58.53
100	0.3057	0.3052	0.2908	0.3006	49.79

LC_{50} Value:

LC_{50} Value- 90.0144µg/mL (Calculated using: ED_{50} plus V1.0 Software):

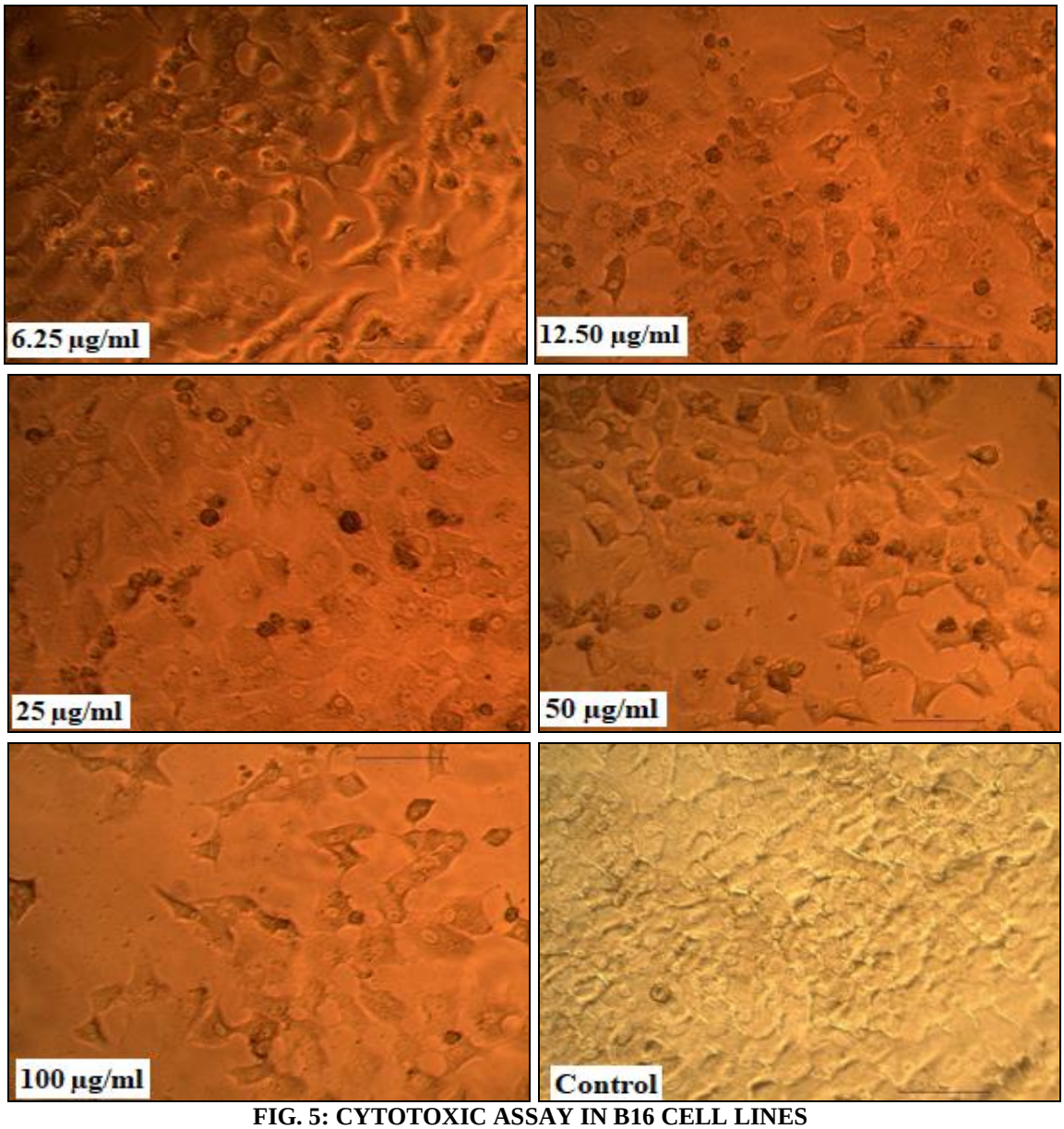

FIG. 5: CYTOTOXIC ASSAY IN B16 CELL LINES

TABLE4 ABSORBANCE VALUE AT 540NM

Sample Concentration (µg/ml)	OD I	OD II	OD III	Average Absorbance @ 540nm	Percentage Viability
Control	0.951	0.9523	0.9562	0.9532	100.00
			Sample		

6.25	0.9406	0.9402	0.9417	0.9408	98.79
12.5	0.9337	0.9306	0.9325	0.9323	97.83
25	0.8427	0.8428	0.8447	0.8434	88.62
50	0.7991	0.7981	0.7842	0.7938	82.27
100	0.7523	0.7547	0.7321	0.7464	76.80

LC_{50} Value (Hypothetical):
LC_{50} Value: 203.907µg/mL (Calculated using ED_{50} plus V1.0 Software):

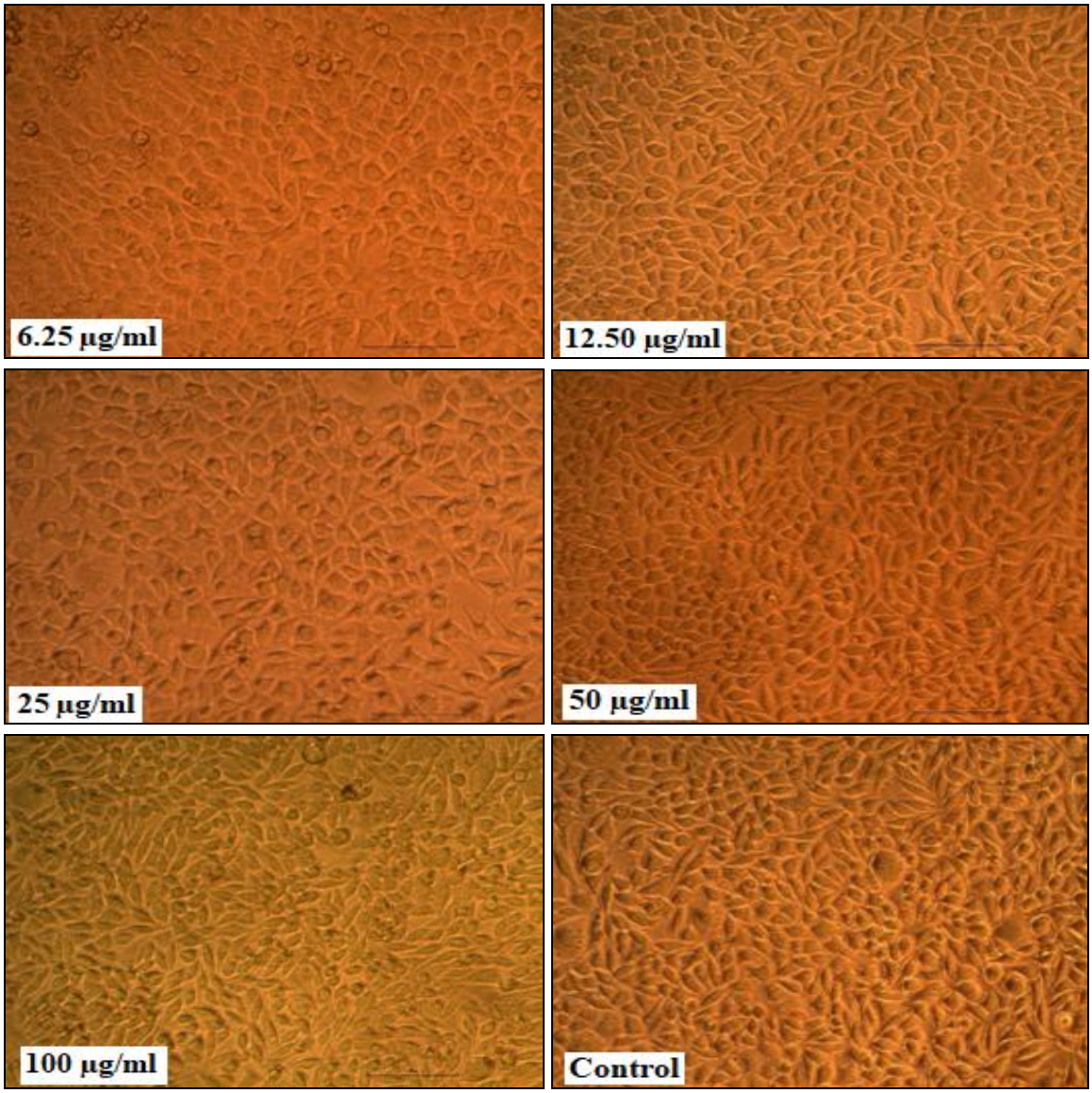

FIG. 6: CYTOTOXIC ASSAY IN L929 CELL LINES

The melanin pigment extracted from the Actinomycetes showed that it has the ability to destroy the B16 Melanoma cells and non toxic against the normal L929 fibroblast cells **Fig. 5** & **6.** Hence it can be used as an photoprotective agent.

CONCLUSION: Microbial metabolites and their derivatives have proved to be an excellent choice for therapy. As the actinomycetes have the ability to produce many biological active compounds that possess antibacterial, antifungal, antioxidant and anticancer activity. In this study it was found that the melanin pigment produced by the actinomycete isolated from the rhizosphere soil has a very good growth inhibition effect on B16 melanoma cell lines and nontoxic against L929, normal fibroblast cell lines. Hence it can be used as a safe and potential photoprotective agent against melanoma.

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