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Original Article

Characterization, metal chelation and anti-cancerous activity of extracelluar melanin pigments produced by *Pseudomonas mosselii* STSGRDS1

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Abstract

Melanins are ubiquitous pigments which are naturally produced and distributed throughout the biological kingdom. *Pseudomonas mosselii* strain STSGRDS1 producing melanin was used in the study. STSGRDS1 melanin reported to have metal chelating potential of increasing effect with increase in the concentration of melanin. The anti-proliferative activity of the purified melanin by MTT assay had IC_{50} value 87.15µg/ml towards A431skin cancer cell line for standard 5-flurouracil and also showed substantial apoptotic activity. The minimal inhibitory concentration (MIC) against bacteria and fungi were also evaluated. Hence, the purified melanin having anti-proliferative properties can be exploited in therapeutic applications.

Keywords: melanin, *Pseudomonas mosselii*, anti-cancer, metal chelation, MIC

1. Introduction

Melanins are macromolecules formed by oxidative polymerization of phenolic or indolic compounds which are are hydrophobic and negatively charged. Color of melanin is usually observed to be black or brown but many other colors have also been observed (Langfelder, Streibel, Jahn, Haase, & Brakhage, 2003). Melanins are habitual pigment found in nature and distributed throughout the animalia (Turick, Knox, Becnel, Ekechukwu, & Milliken, 2010). Melanin biosynthesis is initiated by enzyme tyrosinase i.e. from L-tyrosine through series of enzymatic and non-enzymatic reactions (Ikeda *et al.*, 1998). Melanins are basically of three types, the black eumelanins produced via O-dihydroxy phenylalanine (DOPA), red pheomelanins produced cysteinylation of DOPA quinone and allomelanins comprising a category of melanins which are hetergenous including DHN-melanin, catechols, pyomelanins, ɣ- glutaminyl-4-hydroxybenze & 4-hydroxy-phenylacetic acid (Plonka & Grabacka, 2006). Melanin producing bacteria are commonly allomelanin producers, although they will produce other sorts of melanins. There's *Vibrio cholerae* (Ivins & Homes, 1981) a pheomelanin producer, *Klebsiella* sp. a eumelanin producer (Sajjan *et al.*, 2010).

There are numerous obscured factors for accurate characterization of melanin. Generally, melanins have low purity having various compositions and the determination of the ratios in melanin of miscellaneous units is not available yet. This led to the heterogeneity of their structural features, where the organization and its molecular structure of melanin are still complicated and not known completely (Subianto, 2006; Tan *et al.*, 2019). Though the number of investigations on melanin has been researched, but it is still not well understood. The meticulous definition of melanin biopolymer does not exist, as its structure is not well documented so far. (Arun, Angeetha, Eyini, & Gunasekaran, 2014; Ganesh Kumar, Sahu, Narender, Prasad, Nagesh, & Kamal, 2013; Meng & Kaxiras, 2008).

The biological functions of melanin remains mostly perplexed. It is factual that melanin acts as a strong antioxidant through heavy metal sequestration (Korytowski *et*

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al., 1995). In human skin, melanin plays an important role in light absorption; that the pigment could able to disperse over 99.9% of absorbed UV radiation. Owing to this property, melanin has an important role in protecting skin cells against the UV radiation, which consequently reduce the cancer risk (Subianto, 2006; Tan *et al.*, 2019). Melanins modulate the expression of cytokines like interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) (El-Obeid, Al-Harbi, Al-Jomah, & Hassib, 2006). IL-6 could inhibit the growth and development of colon cancer. (Xiao, Jing, Zhang, Zhou, & Zhang, 2000). Toxicity of melanin from *Aspergillus niger* for lung cancer cell line showed concentration dependency. (Arun, Jegatheesan, Eyini, & Gunasekaran, 2013). Thus melanin can be utilized in pharmaceutical applications. The present study therefore delineates the metal chelation, anti-proliferative and Minimal Inhibitory Concentration of purified melanin produced by *Pseudomonas mosselii* STSGRDS1.

2. Materials and Methods

2.1. Chemicals, cell lines and bacterial isolates

Fungal melanin (Mykotech, Goa), L-tyrosine (Hi-media Laboratories Pvt Ltd, Mumbai, Maharastra, India) and all other chemicals used were of analytical reagent grade. A431 cell line were maintained in DMEM- High Glucose (Himedia) supplemented with FBS (Fetal Bovine serum (Himedia) and grown to confluence at 37 ℃ incubator with humidified atmosphere of 5% CO_2 (Healforce, China). Melanin producing *Pseudomonas mosselii* STSGRDS1 was isolated from garden soil, from Tiruppur, Tamilnadu, India. Producers of melanin was initially screened by plate-based assay followed by assay in tyrosine broth (Yabuuchi and Ohyama, 1972). The bacterial isolate were identified by biochemical and 16S rDNA sequencing and analysis (Reddy, Prakash, Matsumoto, Stackebrandt & Shivaji, 2002; Altschul, Gish, Miller, Myers and Lipman, 1990).

2.2. Production, extraction and purification of melanin

Tyrosine basal broth (Yabuuchi and Ohyama, 1972) containing 0.2% tyrosine was used for production of melanin. 5 ml of culture suspension were taken as primary inoculum for 50 ml of production medium and kept in environmental shaker at 140 rpm at 37±2 ℃ for 180 hrs. After 180 hrs of incubation, the cell free supernatant was further acidified to pH 2 using 1 N HCL. Visualization of black precipitation of melanin was observed at the bottom of the flask at lower pH. Further treatment with acid, water and ethanol helped to render purified melanin (Sajjan *et al.*, 2013).

2.3. Characterization of melanin

Spectroscopic techniques such as UV-visible (Fava, Di Gioia and Marchetti, 1993), FTIR (Yuan, Burleigh and Dawson, 2007), XRD (Casadevall, 2012) were used to evaluate the biophysical properties of the pigment. The surface morphology of black melanin was examined using scanning electron microscopy followed by EDAX (or EDS), an X-ray spectroscopic method for determining elemental compositions like qualitative and quantitative analysis (Sajjan *et al.*, 2013).

2.4. Metal chelating activity

The chelation of ferrous irons by the melanin pigment was estimated by the method of Huang *et al.* (2011). Different concentrations of melanin such as 25 µl, 50 µl, and 100µl were mixed with the solution of 2 mM $FeCl_2$ (0.05 ml). Further, reaction were initiated by the addition of 5 mM ferrozine (0.2 ml) and therefore the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. All the tests and analyses were done in duplicate and averaged. The inhibition of the melanin pigment, metal chelating activity in percentage (%) was calculated by the following equation:

$$\text{Metal chelating effect (\%)} = [(A_0 - A_1) / A_o] \times 100$$

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of the sample of the melanin pigment or standards. The control used contains $FeCl_2$ and ferrozine.

2.5. MTT assay

Different concentration (31.25, 62.5, 125, 250 and 500ug/ml) of melanin were added to A431 cells and incubated for 48 hours at 37 °C in a 5**%** CO2 atmosphere**.** Percentage difference in viability were determined by standard 3-**(**4,5 dimethythiazol-2-yl**)**-2, 5-diphenyl tetrazolium bromide **(**MTT**)** assay after 24 hours of incubation**.** The cells were washed with phosphate buffer saline **(**PBS**)**, and 0**.**5mg/ml final concentration of MTT reagent were added and incubated for three hours**.** MTT was removed by washing with PBS and 200 µl of solubilization solution **(**DMSO**)** were added**.** Occasionally pipetting in and out were required to dissolve the formazan crystals**.** Absorbance were read on a spectrophotometer or an ELISA reader at 570nm and 630nm used as reference wavelength (Mosmann (1983), Wicaksono, Handoko, Kusuma, Yulia and Sandra (2009).

2.6. Apoptotic staining

A431 cells were cultured in a 6-well plate and incubated in a CO2 incubator overnight at 37 °C for 24 hours**.** Cells were then treated with melanin at a concentration 87.15µg/ml and controls for 24 hours**.** After incubation, the cells were washed with PBS and 200 µl of trypsin-EDTA solution were added and incubated at 37 °C for 3-4 minutes**.** 2 ml culture medium was added and cells were harvested directly into 12x75 mm polystyrene tubes**.** Tubes were centrifuged for five minutes at 300xg at 25 °C**.** Pellet was washed twice with PBS and 5 µl of FITC Annexin V were added and cells were vortexed and incubated for 15 min at RT (25 °C) in the dark. 5 µl of PI and 400 µl of 1X Binding Buffer were added to each tube and vortexed gently. Immediately after addition of PI, the staining was analyzed by flow cytometry (Andree, Reutelingsperger, Hauptmann, Hemker, Hermens, & Willems (1990); Casciola-Rosen, Rosen, Petri, & Schlissel (1996); Homburg, de Haas, Von

dem Borne, Reutelingsperger, & Roos (1995); Koopman, Reutelingsperger, Kuijten, Keehnen, Pals & Van Oers (1994); Martin, Reutelingsperger, McGahon, Rader, Van Schie, LaFace & Green (1995); O'Brien & Bolton (1995); Raynal & Pollard (1994); Schmid, Krall, Uittenbogaart, Braun, & Giorgi (1992); van Engeland, Ramaekers, Schutte, & Reutelingsperger (1996); Vermes, Haanen, Steffens-Nakken, & Reutellingsperger (1995).

2.7. Determination of MIC of the purified melanin against bacteria

A stock solution was prepared by adding 10mg of the purified melanin in 10ml of phosphate buffer having concentration 1000µg/ml. Each stock solution was diluted to 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.12 µg/ml, 1.56 µg/ml, 0.78 µg/ml, 0.39 µg/ml, 0.19 µg/ml, 0.095 µg/ml with phosphate buffer. *Kocuria kristinae*, *Staphylococcus haemolyticus*, *Burkholderia cenocepacia*, *Enterobacter cloacae*, *Escherichia coli*, *Enterobacter*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus* was used as reference strains. An aliquot of 80 µl of each dilution of purified melanin was released into a well of 96-well microtitre plate, 100 µl of LB broth, 20 µl of bacterial inoculum and 5 µl of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC). The plate was incubated at 37 °C for 18 hrs and color change was observed. Minimum inhibitory concentration (MIC) of the purified melanin pigment were determined against *Aspergillus flavus*, *Fusarium* sp, *Scedosporium*, *Cylindrocarpon* isolates from mycotic keratitis patients based on broth micro dilution method as recommended by the Clinical and Laboratory Standard Institutes (CLSI) (Laxm, Kurian, Smitha, & Bhat, 2016).

3. Results and Discussion

3.1. Characterization of melanin

The isolated bacterial strain STSGRDS1 had shown typical melanin UV-visible spectrum in the 280-800 nm range. The isolate is compared to that of the standard melanin STSGRDM1. The isolate showed considerable similarity with the standard melanin as shown in Figure 1. El-Naggar *et al.* (2017) reported that the higher absorption of the purified pigment was observed in UV region at 250nm. Santhanalakshmi *et al.* (2017) reported that *Pseudomonas* sp. showed UV visible spectrum of the pigment showing absorbance in the UV region 350 nm.

FTIR spectrum of the melanin STSGRDS1 was compared with the standard melanin STSGRDM1 as shown in Figure 2. Both melanin showed considerable similarities as those in earlier reports (Guo *et al*., 2014; Sajjan *et al*., 2010; Turick *et al*., 2002). The spectrum showed a broad absorption spectrum around 3,400 cm^{-1}, which is corresponding to the phenolic -OH and -NH stretching vibrations. The peaks observed between 1,600-1,400 cm^{-1} was attributed to the aromatic ring C=C stretching. This confirmed the polyphenolic and aromatic nature of melanin. Shoumita *et al.* (2018) stated that the functional groups were at 1,636, 3,199 that indicates C=O and C-H stretching, respectively. These observations of FTIR indicate that the extracted melanin pigment is melanin. These characteristics were found to be

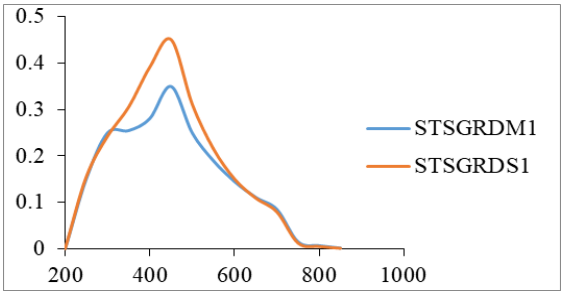

Figure 1. UV visible spectrum of STSGRDM1 and STSGRDS1

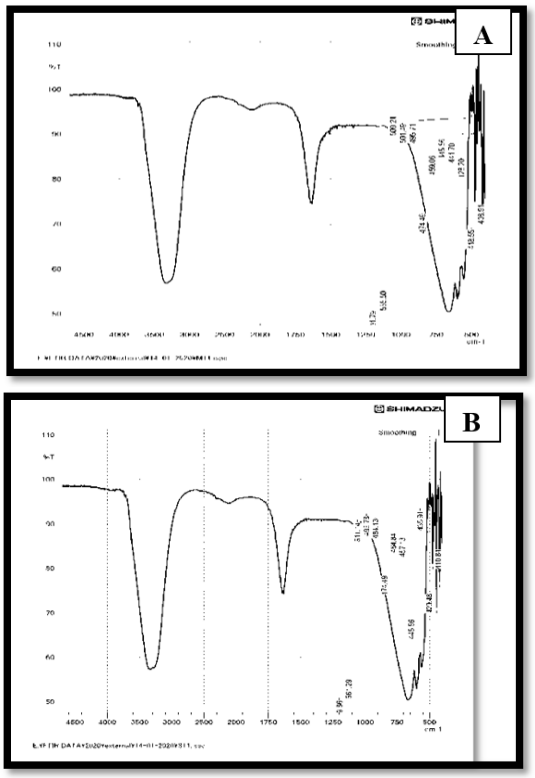

Figure 2. FTIR analysis of (A) STGRDM1 and (B) STSGRDS1

much similar to that of bacterial melanin- pheomelanin. El-Naggar *et al* (2017) reported that the spectroscopic properties of the pigment extracted from *Streptomyces glaucescens* NEAE-H correlated with those of melanin produced by various microorganisms as reported previously and was concluded that the pigment was eumelanin.

X-ray diffraction studies of sample STSGRDS1 was compared with the standard STSGRDM1 as shown in Figure 3. Casadevall *et al.*(2012) reported that the scattering of X-rays by highly ordered structures like crystals produces sharp peaks in the spectrum. In contrast, the amorphous materials like melanin produce broad features within the diffraction spectrum which is understood as non-Bragg features resulting from the absence of coherent scattering from regular and repeating structures (e.g. crystals).

SEM analysis was carried out to determine the morphological structures of the isolate as well as bacterial consortium. The ultra-structure of the bacterial strains was

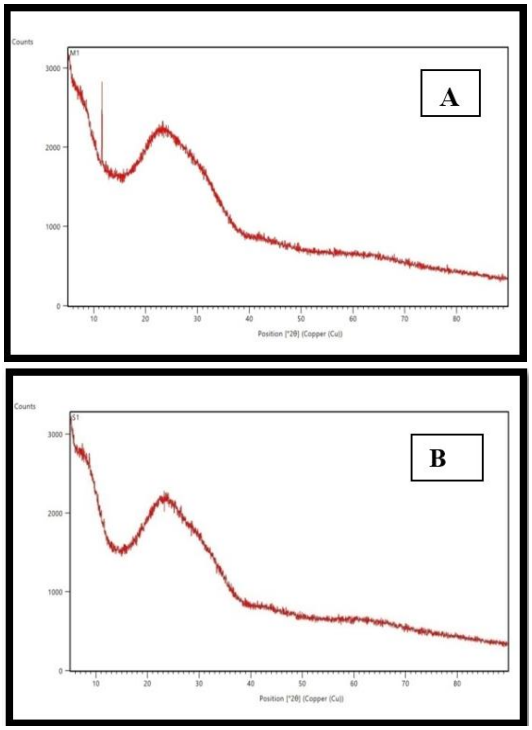

Figure 3. XRD analysis of (A) STGRDM1 and (B) STGRDS1

observed under magnification. The results showed that comparing the standard STSGRDM1 had proven to be similar to that of STSGRDS1 as shown in Figure 4. Other reports indicated that commercial sepia melanin appeared to be formed as spherical particles having different sizes (Mbonyiryivuze *et al.*, 2015).

The elemental compositional study was observed in both STSGRDM1 and STSGRDS1. STSGRDS1 contained similar elemental composition compared to that of the standard STSGRDM1 as shown in Figure 5. Earlier reports Ye *et al*. (2014) indicated that Lachnum YM404 produced pheomelanin which had high S content (14.83). Mbonyiryivuze *et al.* (2015) reported that the commercial sepia melanin consisted of C, O, Na, Mg, Cl, S, and Ca.

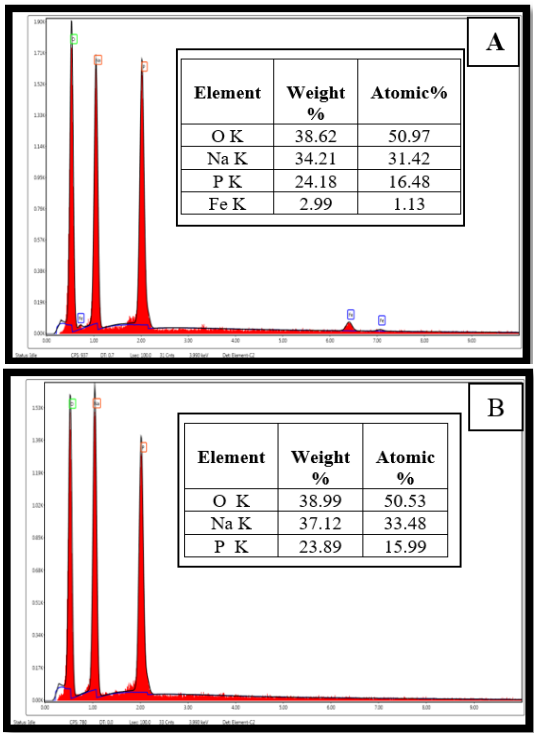

Figure 5. (A) Elemental composition of standard melanin STSGRDM1, (B) Elemental composition of purified melanin STSGRDS1

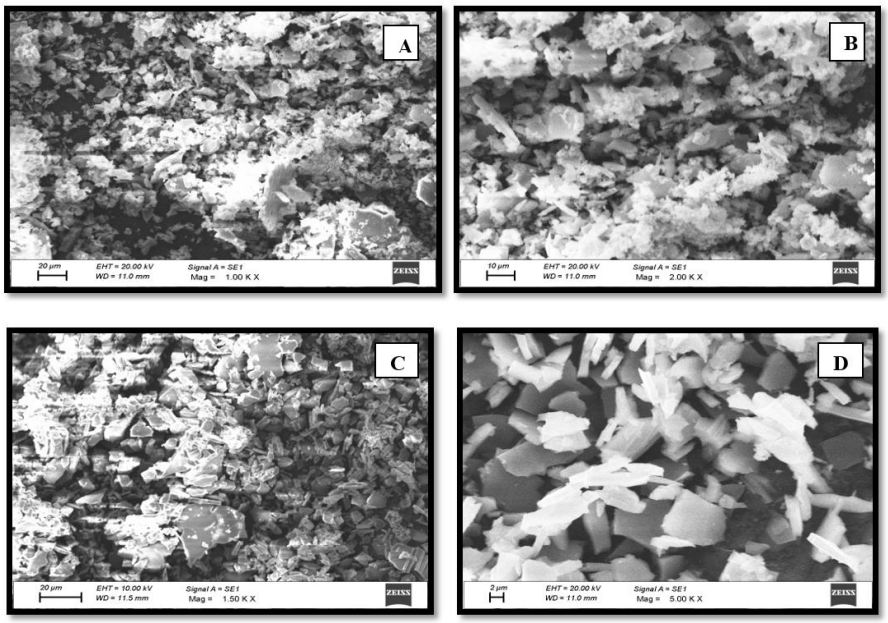

Figure 4. A, B showing the SEM analysis of standard STSGRDM1 and C, D showing SEM analysis of standard STSGRDS1

3.2. Metal chelating property

Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents the complex formation was disrupted resulting in decreasing red color of complex. Melanin STSGRDS1 also showed Fe^{2+} chelating activity when compared with the standard fungal melanin STSGRDM1 as shown in Figure 6. Tarangini and Mishra (2014) reported that a microbial isolate showed Fe^{2+} chelating activity of 64% at a specific concentration 0.2 mg/ml. Riley (1997) stated that melanin structure was 2-carboxyl indole that link with metal ion that made melanin to have Fe^{2+} chelating power. Karadag *et al*. (2009) reported melanin also binds to metal ions and minimizes generation of ROS. Therefore metal chelating potential can be consequently related to radical scavenging ability.

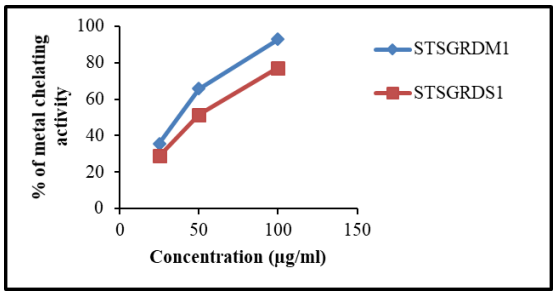

Figure 6. Metal chelating activity of standard melanin STSGRDM1 and purified melanin STSGRDS1

3.3. Anti-proliferative activity of melanin

Pseudomonas mosselii STSGRDS1 melanin was evaluated using skin cancer cell line A431 for its anti-proliferative activities. The sample melanin STSGRDS1 was compared with standard melanin STSGRDM1. Both samples exhibited cytotoxicity showing IC_{50} value of standard STSGRDM1 131.934 µg/ml and IC_{50} value of sample STSGRDS1 87.1534 µg/ml as shown in Figure 7. Since the IC_{50} value of sample STSGRDS1 was lower, which prove that it might have anti-cancerous activity; the sample was further analyzed for apoptotic staining as shown in Table 1 and Figure 8 the results revealed that STSGRDS1 may have possible therapeutic potential against Human epidermoid/skin

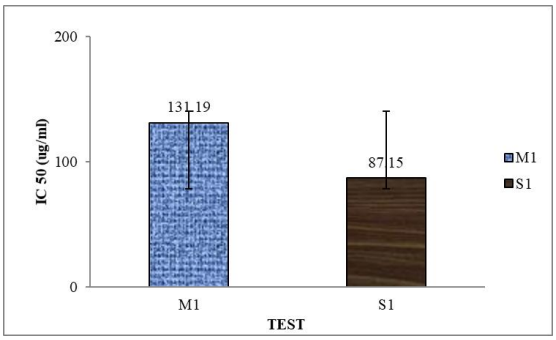

Figure 7. IC_{50} concentrations of standard melanin STSGRDM1 and purified melanin STSGRDS1

Table 1. Percentage of apoptotic cells

Quadrant	% of Necrotic cells	% Late apoptotic cells	% Viable cells	% of Early apoptotic cells
Label	UL	UR	LL	LR
Cell Control	0	0.03	99.83	0.14
Std Control	2.71	5.54	43.95	47.81
Test (S1)	12.27	27.61	31.96	28.17

Cancer derived diseases and further mechanism need to be proved with further studies. The results showed significance with earlier reports on melanin toxicity. El-Naggar *et al.* (2017) reported anticancer activity against skin cancer cell line using MTT assay and the IC_{50} value was 16.34 µg/ml and 8.834 µg/ml for standard 5-fluorouracil. Madhusudan *et al.* (2014) reported that the melanin produced by *Streptomyces lusitanus* showed a cytotoxicity of IC_{50} of 0.80 µg/mL tested in brine shrimps. Mahmood*et al.* (2016) reported the foremost significant IC_{50} value of melanin pigment produced by *Pseudomonas aeruginosa* on four cell lines Lung A549, Skin A375, Macrophage RAW264.7 and VERO cell lines. The results indicate the cytotoxicity was more significant on Skin A375 cell line. Gamal Shalaby, Ragab, Helal, and Esawy (2019) proved black melanin showed promising anticancer activity towards HEPG-2 and HCT-116 cell lines with IC_{50} values (6.5 µg/ml, 5.54 IC_{50} value) compared to Doxorubicin (4.05 µg/ml, 4.45 µg/ml) respectively.

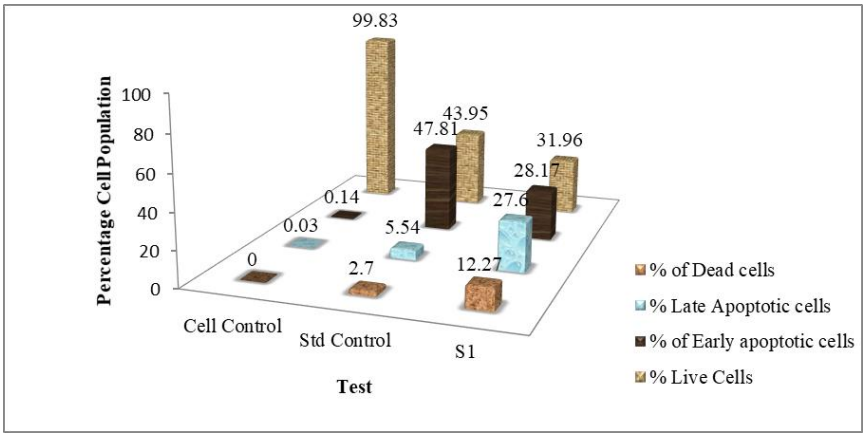

Figure 8. Bar graph representation the percentage of apoptotic cells gated in the different samples like untreated, standard (5-Fluorouracil), and test compound namely purified melanin STSGRDS1(S1) treated cells.

3.4. Determination of MIC of the purified melanin against bacteria and fungi

The MIC values of the purified melanin against the bacteria such as *Kocuria kristinae, Staphylococcus haemolyticus, Burkhoderia cenocepacia, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Klebsiella pneumonia, and Staphylococcus saprophyticus* can be assessed by ability to suppressed bacterial growth. The MIC values are tabulated in Table 2. The MIC values of purified melanin against *Aspergillus flavus*, *Fusarium* sp, *Scedosporium*, *Cylindrocarpon* isolates from human mycotic keratitis patients were determined and interpreted. The overall spectrum of minimum inhibitory concentrations (MICs) is provided as in Table 3. Laxmi, Kurian, Smitha, and Bhat (2016) reported that MIC of purified melanin from *Bacillus licheniformis* was 100µg/ml against nine food pathogens such as *Bacillus altitudinis*, *Bacillus pumilus*, *P.aeruginosa*, *Brevibacterium casei*, *Staphylococcus warneri*, *Micrococcus luteus*, *B. niacini*, *Bacillus* sp, *Geobacillus stearothermophilus*. Xu, Yang, and Ye (2017) reported that the MIC values of melanin for each bacterial strain, such as *E. coli*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Bacillus megaterium*, *Staphylococcus aureus* were 0.4, 2.4, 0.2, 0.6, 0.8, 0.3 mg/ml respectively.

Table 2. Standard melanin STSGRDM1 and STSGRDS1 showing minimum inhibitory concentration against bacteria.

Bacteria	MIC (mg/ml)	
	STSGRDM1	STSGRDS1
Escherichia coli	12.51	3.12
Listeria monocytogenes	0.09	6.25
Klebsiella pneumonia	0.09	0.19
Staphylococcus saprophyticus	0.39	0.39
Kocuria kristinae	1.56	0.39
Staphylococcus haemolyticus	25	0.19
Burkhoderiacenocepacia	0.09	0.09
Enterobacter cloacae	0.78	3.12

Table 3. Standard melanin STSGRDM1 and STSGRDS1 showing minimum inhibitory concentration against fungi.

Fungi	MIC (µg/ml)	
	STSGRDM1	STSGRDS1
Aspergillus flavus	0.78	1.56
Fusarium sp.	6.25	1.56
Scedosporium sp	3.12	3.12
Cylindrocarpon sp	0.78	0.78

4. Conclusions

Melanin producing strain *Pseudomonas mosselii* STSGRDS1 isolated from soil sample and purified were characterized and had profound bioactivities such as metal chelating, anti-proliferative and minimal inhibitory concentration. Thus, proving melanin has a potential to be a pharmaceutical agent.

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