Iraqi Journal of Biotechnology, 2016, Vol. 15, No. 1 , 46-52

Cytotoxic Effect of Pyomelanin Pigment Produced from Local *Pseudomonas aeruginosa* Isolates on Different Cell Lines Using MTT Assay

Huda M. Mahmood[1], Alaa K. Mohammed[2], May T. Fleih[3]

[1]Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, University of Baghdad

[2]Biochemical Eng. Department , AL-Khawarizmi Engineering College , University of Baghdad

[3]Biology Dep., College of Science, University of Baghdad

Received: August 26, 2015 / **Accepted**: November 24, 2015

Abstract: This study was designed to determine the most significant (IC_{50}) values of purified pyomelanin pigment which was produced by local isolates of *Pseudomonas aeruginosa* on four cell lines using3-(dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay). 143 samples from burns, urine and sputum were collected from patients hospitalized into eleven hospitals which are located in Baghdad, Al-Anbar and Karbala provinces during the period of July 2013 to April 2014. All isolates were identified as *Pseudomonas aeruginosa* according to morphological, cultural, biochemical characteristics, VITEK-2 and 16S rRNA . Eight pyomelanogenic isolates (5.6%) were produced pyomelanin pigment on pyomelanin production medium. The results were showed that the isolate of sputum from cystic fibrosis (CF) was greatest in production of pyomelanin. The cytotoxicity of extracted and purified pyomelanin was assessed on Lung A 549, Skin A375, Macrophage RAW264.7 and VERO cell lines. The results showed that pyomelanin has cytotoxic effect on all tested cell lines but the most significant was on Skin A375 cell line with dose dependent effect.

Key words: MTT, pyomelanin pigment, *Pseudomonas aeruginosa*, cell line.

Corresponding author: should be addressed (Email: msc_hmm@yahoo.com)

Introduction

Pyomelanin production has been reported in *Pseudomonas aeruginosa* isolates mainly from urinary tract infections and chronically infected Cystic Fibrosis (CF) patients (1,2,3). Melanin is a group of negatively charged hydrophobic macromolecules formed by the enzymatic oxidation and subsequent polymerization of phenolic and/or indolic compounds (4, 5). In addition to *P. aeruginosa*, production of pyomelanin and other forms of melanin has been described to occur almost in every taxon of living organisms ranging from bacteria to human (6), which are classified into four categories based on the intermediates of melanogenesis: eumelanin, pheomelanin, allomelanin and pyomelanin.

Measurements of cell viability and proliferation forms are the basis for numerous *in vitro* assays of a cell population's response to external factors. The MTT is a tetrazolium salt

(3- (dimethyl thiazol-2-yl)-2, 5-biphenyl- tetrazolium bromide) which is an indicator that reduced to a colored formazan product by reducing enzymes present only in metabolically active host cell. MTT assay a rapid colorimetric semi- automated enzyme based assay. The reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD (P) H flux (7, 8). Therefore, the reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation (9).

This is the first study in Iraq on the assessment of pyomelanin cytotoxicity effects which was produced by local isolates of *P. aeruginosa* on different human cell lines. Therefore, this study was designed to determine the most significant half inhibition concentration (IC_{50}) values of purified pyomelanin pigment on four cell lines by using MTT assay.

Materials and Methods

Bacterial Isolation

One hundred forty three samples were obtained from different source (burns, sputum and urine), which collected from patients from different hospitals in Baghdad, Al-Anbar and Karbala provinces. The collected samples were inoculated on MacConkey agar at 37˚C for 48 h. to select pale colony, then sub-cultured on Cetrimide agar to obtain pure colonies. These isolates diagnosed by morphological and biochemical tests, and re-confirmed by VITECK-2 and 16Sr RNA (10, 11).

Pyomelanin Detection

All *P. aeruginosa* isolates were cultured on tyrosine production medium to obtain pyomelanogenic isolates and incubated at 37°C for 48h. then incubated at room temperature for one week. Mutants producing a dark-brown pigment after incubation refers to positive result of pyomelanin production were selected (11, 12, and 13).

Extraction and Purification of Pyomelanin Pigment

Bacterial strains capable of producing high amounts of pyomelanin on pyomelanin production medium supplemented with L-tyrosine were isolated. Then the plates freezed and thawed to extract the pyomelanin pigment. The disrupted broth was acidified with 1 N HCl to pH 2 and allowed to stand for one week at room temperature. Then this suspension was boiled for one hour to prevent the formation of melanoidins and then centrifuged at 8,000 ×g for 10 min (14). The formed black pigment pellet was washed three times with 15 ml of 0.1 N HCl, and then washed with water. To this pellet, 10 ml of ethanol was added and the mixture was incubated in a boiling water bath for 10 min and kept at room temperature for one day. The pellet was washed with ethanol two times and then dried in air. The extracted pigment was pooled for use in subsequent analysis. The chemical analysis of melanin pigment was carried out by Fava modified method (14). The standard synthetic pyomelanin pigment which used for comparison with extracted pigment was obtained from

Sigma Aldrich Company (St. Louis, USA).

MTT Cell Assay Protocol

A- Cancer Cell Lines

This experiment was carried out at CENAR center (Center for National Product Research and Drug Discovery) / University of Malaya / Malaysia. The cell lines used in this study were Lung A549, Skin A375, Macrophage RAW264.7 and VERO cell line.

B-General Procedure

This assay was accomplished according to Aghdassi (15). Controls and test compounds were assayed in triplicate for each concentration and replicated three times for each cell line. The data of optical density taken from plate reader were then subjected to multiple regression analysis using the general linear models procedure of SAS (V 9.1) to develop least-squares polynomial equations (16). These equations were then used to calculate the concentration of compounds required to cause 50% reduction (IC_{50}) in growth (cell number) for each cell line, According to the following formula IC_{50} = (Mean of test / Mean of control) x 100.

Results and Discussion

All isolates were identified as *Pseudomonas aeruginosa* according to morphological, cultural, biochemical characteristics, VITEK-2 and 16S rRNA.

Figure (1) showed one band of 198 bp (product size) in all the eight pyomelanogenic isolates and the control as a result on agarose gel compare with DNA marker (100- 10000 bp).

Specific 16S ribosomal RNA (*16S rRNA*) was used which is the signature sequence of *P. aeruginosa*. It is stable part of the genetic code. PCR was performed for diagnosis of pyomelanogenic and wild type local isolates of *P. aeruginosa* using *16S rRNA*.

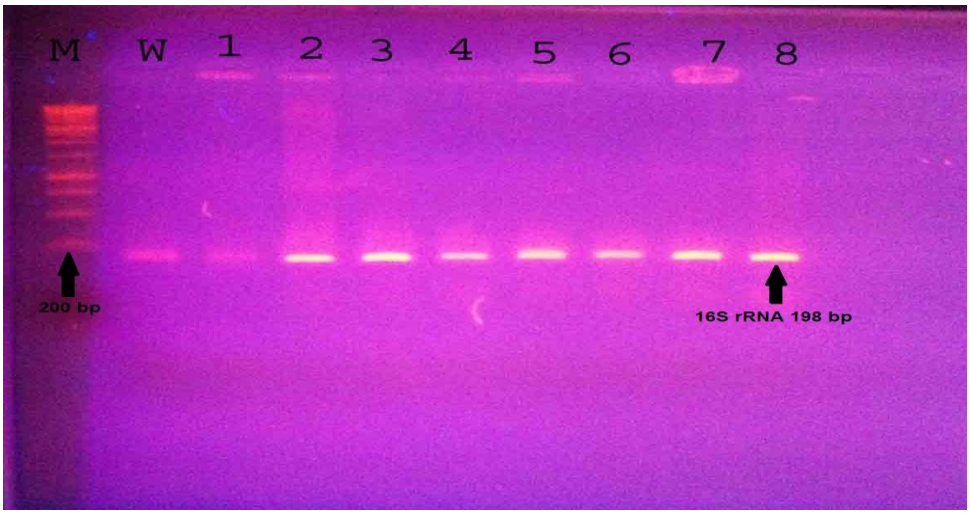

Figure (1): Agarose gel electrophoresis for *16S rRNA* gene(198 bp), M= Markers100-10.000bp, W=wild type , lanes (1-8) pcr product for pyomelanogenic isolates (mutated)

MTT assay was achieved to determine the most significant (IC_{50}) values of purified pyomelanin pigment on four cell lines. Data analysis carried out in g/L and log values of g/L is being plotted in graph pad prism using log (Inhibitor) versus normalized response curve. Effects of pyomelanin on cell viability were investigated according to the condition reported by Dellai *et al*. (17) using the MTT which is the end point assay that detect cell viability at certain-time point. Assays were performed against Lung cancer cell line (A549), skin cell line (A375), Macrophage (RAW264.7), and Vero cell line. The cytotoxicity was expressed as IC_{50} which is the concentration that reduces the absorbance of treated cells by 50 % with reference to the control (untreated cells). After 24 h. of incubation, cytotoxic effects of pyomelanin pigment were measured by MTT assay. Results have shown a dose-dependent inhibition of all tested cell viability at dilutions ranging between (1:2, 1:4, 1:8, 1:16, 1:32, 1:64) of pyomelanin pigment. As shown in Figure (2-A) the half-maximal inhibition concentration (IC_{50}) values of standard synthetic pyomelanin pigment-treated A 549 cells after 24 h. of incubation at 37º C was 363.1 µg /ml, while the IC_{50} value of extracted pyomelanin pigment - treated A 549 cells after 24 h. of incubation at 37 ºC was 410.16 µg /ml (Figure2-B).

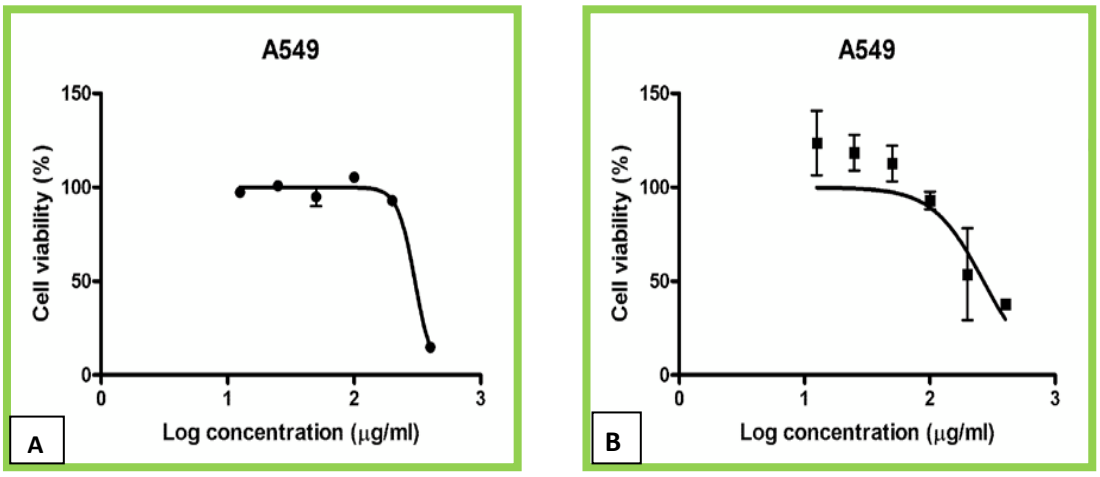

Figure (2) :The half-maximal inhibition concentration (IC_{50}) values of pyomelanin pigment-treated A 549 cells after 24 hours of incubation at 37ºC (A) Standard pyomelanin, (B) Extracted pyomelanin pigment

Results from Figure (3-A) showed that the IC_{50} of standard pyomelanin pigment- treated A375 cells after 24 h. was 105.3 µg/ml while the IC_{50} of extracted pyomelanin pigment-treated A375cells after 24 hours was 234.4 µg /ml Figure (3-B).

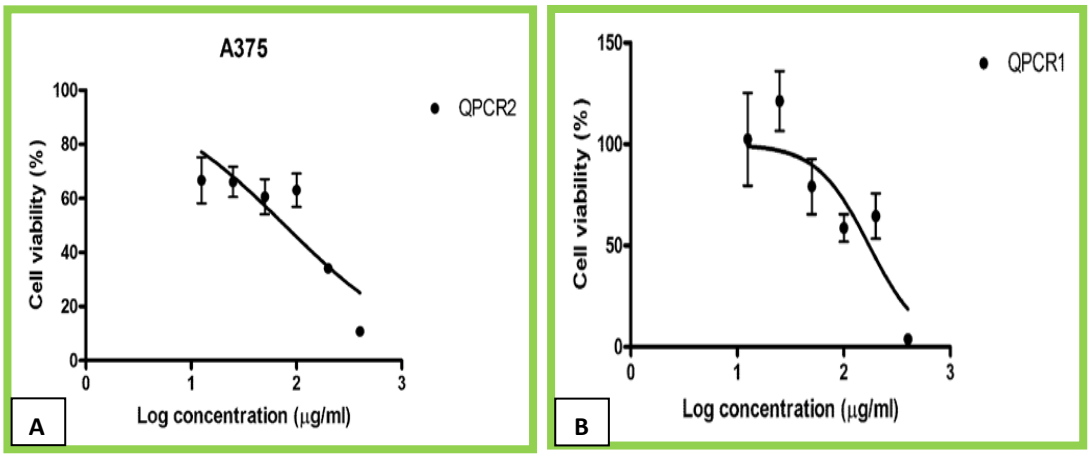

Figure (3): IC_{50} of pyomelanin pigment- treated A375 cells after 24 hours of incubation at 37ºC (A) Standard pyomelanin, (B)Extracted pyomelanin pigment

Figure (4-A), showed that the IC_{50} of standard pyomelanin pigment –treated RAW 264.7 (macrophage) cells after 24 hours was 295 µg/ml while the IC_{50} of extracted pyomelanin-treated RAW 264.7 (macrophage) cells was 235.4 µg /ml after 24 hr. as shown in Figure (4-B).

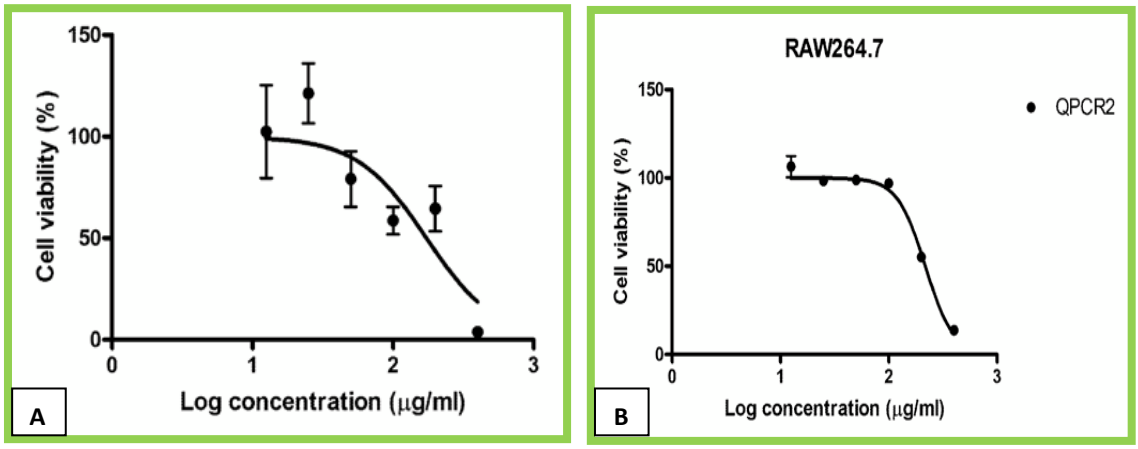

Figure (4): The IC_{50} of pyomelanin pigment–treated RAW264.7 cells after 24 hours of incubation at 37ºC (A) Standard pyomelanin, (B)Extracted pyomelanin pigment

The IC_{50} of pyomelanin pigment - treated VERO cells after 24 hours was 100 µg /ml while the IC_{50} of Standard pyomelanin pigment-treated VERO cells was 100 µg/ml after 24 hours of incubation at 37ºC Figure (5).

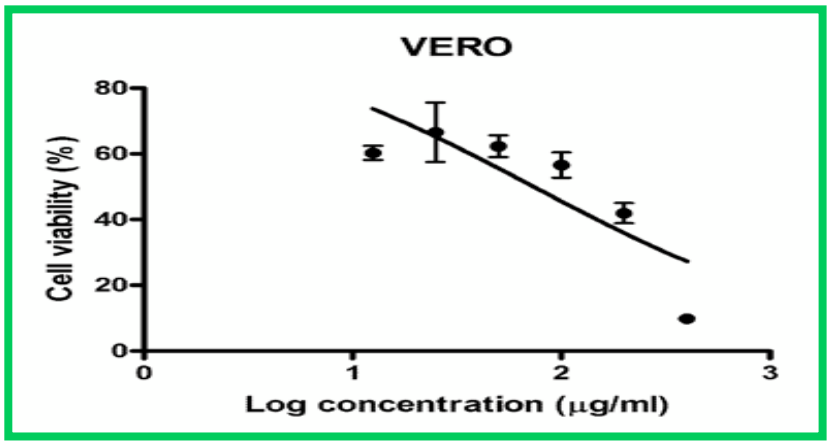

Figure (5): The IC_{50} of Standard pyomelanin pigment-treated VERO cells after 24 h. of incubation at 37°C.

As a conclusion, it can be stated that the MTT assay is more reliable and most suitable assay for measuring a decrease in cell viability caused by cytotoxic chemicals when metabolic events lead to apoptosis or necrosis, the reduction in cell viability. The cytotoxicity of soluble and insoluble melanin from *Streptomyces lusitanus* DMZ-3 was studied using brine shrimps, the study showed that both pigments exhibit higher cytotoxic activity as LC_{50} value less than 500µg/ ml (18).

References

1. Bodey, G. P.; Bolivar, R.; Fainstein, V. and Jadeja, L. (1983). Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis.*, 5: 279–313.
2. Doring, G.; Holder, I.A and Botzenhardt, K. (1987). Basic Research and Clinical Aspects *of Pseudomonas aeruginosa* .International Symposium, Tubingen, Germany, Basel Karger.
3. Gilligan, P.H.(1991). Microbiology of airway disease in patients with cystic fibrosis. *Clin Microbiol Rev*., 4: 35-51.
4. Butler, M., and Day, A. W. (1998). Fungal melanins: a review. *Can. J. Microbiol.* 44:1115–1136.
5. Wheeler, M. and Bell, A. A.(1988). Melanins and their importance in pathogenic fungi. *Curr. Top. Med. Mycol.* 2:338–387.
6. Wang,H.; Qiao,Y.; Chai,B.; Qiu, C. and Chen, X. (2015). Identification and Molecular Characterization of the Homogentisate Pathway Responsible for Pyomelanin Production, the Major Melanin Constituents in *Aeromonas media* WS. *PLoS One 10*: 1-27.
7. Vistica,D,T.; Skehan,P.; Scudiero, D.; Monks, A.; Pittman, A., and Boyd,R.,M.(1991) . Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production . *Cancer Research, 51*:2515-2520.
8. Berridge, M.,V.;Herst P.,M. and Tan AS.(2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol Annu Rev.*, 11: 127-152.
9. Berridge, M. V. and Tan A.S.(1993). Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Arch Biochem Biophys*. *303*:474-482.
10. Washington, W. J.; Allen, S. ;Janda, W. ;Koneman, E.; Procop, G. ;Schreckenberger, P. and Woods, G. (2006). Koneman's color

atlas and textbook of diagnostic microbiology, 6th Ed. Lippincott Williams and Wilkins, United States of America.pp 359-360.

11. Collee, J. G.; Miles, R. S. and Watt B. (1996) . Test for the identification of bacteria. 131-146. In: Collee. J. G.; Fraser, A. G.; Marmion, B. P. and Simmons, A. (Eds). *Practical Medical Microbiology*.14th Ed. Churchill Livingstone, New York.
12. Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Staley, J.T. and Williams, S.T. (1994). Bergey's Mannual of determinative bacteriology. ($9^{th\ ed.}$). A Waverly company. Baltimore, USA.
13. Forbes ,A.; Sahm , F.and Weissfeld ,S. (2007). Bailey and Scott's , Diagnostic Microbiolog. International Twelfth Edition Mosby Elsevier page 172- 176.
14. Fava, F.; Gioia D. D., and Merchetti, L.(1993). Characterization of a pigment produced by Pseudomonas fluorescens during 3-chlorobenzoate co-metabolism. *Chemosphere*. *27*: 825-835.
15. Aghdassi, A. (2007). Measuring cell viability / cytotoxicity using MTT cell proleferation assay. *Cancer Res.*, *67*: 616-623.
16. SAS Version 9.2**.** (2002). SAS Institute, Cary, North Carolina, USA.
17. Dellai,,A.; Deghrigue , M.and Bouraoui ,A.R. (2012). Evaluation of antiproliferative and anti-inflammatory activities of methanol extract and its fractions from the Mediterranean sponge. *Cancer Cell International*, 12:18-25.
18. Madhusudhan, D. N.; Mazhari, B. B.Z.; Dastager, S.G. and Agsar, D. (2014). Production and cytotoxicity of extracellular insoluble and soluble melanin by *Streptomyces lusitanus* DMZ-3. *BioMed Research Internatioy nal., PP* 1-11.