Available online at http://www.journalijdr.com

IJDR

International Journal of
DEVELOPMENT RESEARCH

ISSN: 2230-9926

International Journal of Development Research
Vol. 07, Issue, 04, pp.12508-12511, April, 2017

Full Length Research Article

ANTIMICROBIAL EFFECT OF PYOMELANIN EXTRACTED FROM *PSEUDOMONAS AERUGINOSA*

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ARTICLE INFO

Article History:

Received 25th January, 2017
Received in revised form
09th February, 2017
Accepted 14th March, 2017
Published online 30th April, 2017

Key Words:

Pyomelanin,
Pseudomonas aeruginosa,
Identification,
Vitek analyss,
16SrRNA,
Antimicrobial Effects.

ABSTRACT

Pigmented bacterial isolates was isolated from various environmental samples. Vitek identification analysis and PCR analysis of 16SrRNA amplifying gene showed that this bacteria was related to *Pseudomonas aeruginosa.* Pigments was extracted with 1N HCl and ethanol.After extraction, pigment was subjected to various chemical analysis including thin layer chromatography, spectrophotometric analysis and gas chromatography –Mass spectrophotometric analysis. Chemical analysis showed that pigment was characterized as, pyomelanin which was evaluated biologically to examine its cytotoxicity against red blood cells, results showed that pyomelanin had no cytotoxic effect. Also pyomelanin was tested against pathogenic bacteria and fungi. Statistical analysisof present results showed thatpyomelanin gave an antibacterial and antifungal activity.

INTRODUCTION

Melanin is a well-known and universal pigment in living organisms, it has many benefits to human beings and plays a key role to protect internal tissues from the harmful effects of ultraviolet rays (Chedekel *et al*., 1995). Melanin have a high molecular wieght component. It has several biological function including skin color and also plays a role in protecting the skin against ultraviolet light (Huang and Chang, 2012). In the microbes, melanin act as a protective agent against environmental stresse; for example melanin makes the bacteria resistant to antibiotics (Lin *et al*., 2005). Melanin have a broad spectrum of biological roles, including, antioxidant (Hung *et al*., 2002). Antitumor activity (Hassib *et al*., 2006), liver protecting activity (Huang *et al*., 2003) and radio protective (Casadevall *et al*., 2007) etc. they are widely used in medicine, pharmacology, (cosmetics and other fields (Kiran *et al*., 2014). According to (Montefiori and Zhou, (1991) that synthetic soluble melanin's can inhibit replication of human immunodeficiency virus type 1 and 2 (HIV1 and HIV-2) in two human lymphoblastoid cell lines (MT-2 and Ha) and phytohemagglutinin – stimulated human T. cell s, effective concentration of 0.15-10 Mg /ml had no cell toxicity melanin is known for its absorbance capacity of radiation of all wave lengths will on optimum absorbance at UV rang (Ruan *et al*., 2004), which prevents photo induced damage. so it is used in the preparation of photo absorbing optical lenses and in bio plastic, and has biological activities such as radical scavenging, antioxidant, antitumor, anti- inflammatory (El-Obeid *et al*., 2006) and as immune stimulating agent (Sava *et al*., 2001).

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MATERIALS AND METHODS

Samples were collected from soil from different region of Basra city then samples were serially diluted with sterile distilled water. Processed samples were cultured on nutrient agar, blue green colonies grown on nutrient agar were picked out and sub cultured onto nutrient agar and Gram stained. Cultured plate were incubate for 24-48hrs at 25-37Cº.Bacteria was identified biochemically with catalase, oxidase and then subjected to Vitek 2 system analysis and PCR amplification of 16SrRNA gene using following primers. The melanin producing microorganisms was cultured onto nutrient agar enriched with L-Tyrosine (Yabuuchi and Ohyama, 1972). It was separated by observing a diffusible black pigment on nutrient agar plates (Aneja, 2003). Melanin producing bacteria was scraped by using sterilize razor blade and placed in screw capped bottles, melanin extracted from the cell free supernatant, following by acidification with 1N HCL with pH 2, and allowed to stand for one week at room temperature, 10

Primer	Sequence
Foreword	5′GGGGGATCTTCGGACCTCA-3′
Reverse	5′TCCTTAGAGTGCCCACCCG-3′

Steps	Temperature(^{0}C)	Time
Denaturation	94	4 min.
1-Denaturation	94	30 sec.
2-Annealing	60	30 sec.
3-Extension	72	2 min.
Elongation	72	7 min.

ml of ethanol was added and the mixture was incubated in a boiling water bath for 10 min and kept at room temperature for a day the plate was washed with ethanol twice and then dried (Sajjan *et al.,* 2013). Pigment was passed through TLC plates using Butanol: acetic acid: water as an eluent. For spectrophotometric analysis, pigment was extracted with methanol followed by centrifugation at 4000-70.000 rpm for 10-15 minutes and the cell debris was removed by a second centrifugation step and supernatant was transferred to a cuvette for the measurement of absorbance between 180-300 wave lengthGas chromatography(GC-Mass) analysis of pyocyanin were performed on a shimadzu Qp2010 quadrupole gas chromatography Mass spectrophto-meter (Gc-Ms) instrument equipped with a carbowax (30m x 0.25 mmID; o.25 im film thickness) capillary column (intercut DB5Ms. Japan). one microliter of sample was injected into the capillary column. Helium was used as the carrier gas. injector and detector temperatures were set at 280℃.Injection was performed in split mode (1:30), the column temperature was programed initially at 40℃ for 1 minuts and then increase at arate of 5℃ per min at final temperature of 280℃. pigments were separated at constant pressure (96.1 Kpa) 6 split ratio 30.0, column flow 1.71 ml/ min and peaks were identified by comparing the mass spectra with mass spectral database.

Antimicrobial activity: - Effect of pigment against pathogenic bacteria from wound (*Pseudomonasaeruginoas, Staphylococcus, E. coli*) and urinary tract infection (*Pseudomonas aeruginoas, Staphylococcus, E. coli and Bacillus*) and pathogenic fungi *Cryptococcus neoformans*, *Candidaalbicans* fungi *Aspergillus niger and, Aspergillus fumigatus*was performed by using agar diffusion methods. Data were summarized as mean ± SD. All data were subjected to statistical analysis using one way analysis of variance (ANOVA) by using mini tab program. The differences were considered significant if $P < 0.05$.

RESULTS

Dark brown pigment producing colonies were picked up from cultures grown on nutrient agar and subcultured on nutrient agar and gave name (B), purified colonies were Gramm stained, and catalase and oxidase tests examined, in addition to some biochemical tests. *Pseudomonas* suspected was further diagnosed by subjecting to Vitek system analysis. Results of vitek2 system analysis and PCR amplification of 16SrRNA revealed that bacterial isolates were identified as *Pseudomonas aeruginosa.* The chromatogram of colored spot on TLC plate showed an RF value of 0.73 for pyomelanin. While spectrophotometric analysis showed that pyomelanin exhibited maximum spectral peak at U.V region and decreased towards visible region. Because of the complex structure of pyomelanin extracted from *Ps. aeruginosa* 2, it need pyrolysis in which the heat break the phenolic compounds of this pigments revealed that it was heterogeneous complex of multiple ring structure, such as, n-node cyle-N-phnylanin, Benzenamin, 4-octyle Nonylalanin, Benzocar boxylic acid, tribenzyle, Benzenamin, Butoxy benzylidnn, pentylalanin, Glycyl – L – proline, Benzoat, Fornamid, pyrol [1,2] pyrazin, octylanin, N- dodecyl N-phenylalanin, Glysin. Figure (3).

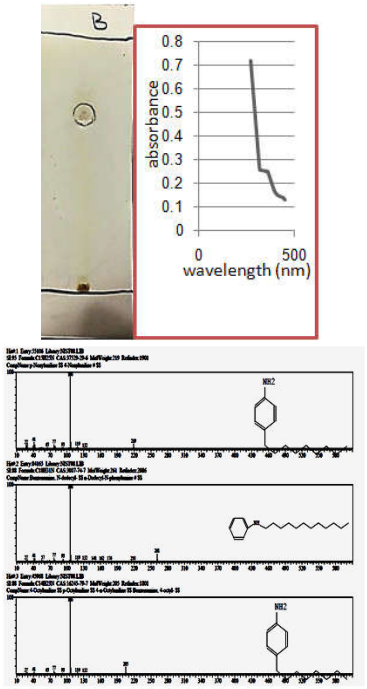

Figure 2. Left Thin layer chromatographs pyomelanin, right Spectrophotometric analysis pyomelanin,and lower GC-mass spectral analysis showed the presence of phenolic compounds which related to pyomelanin

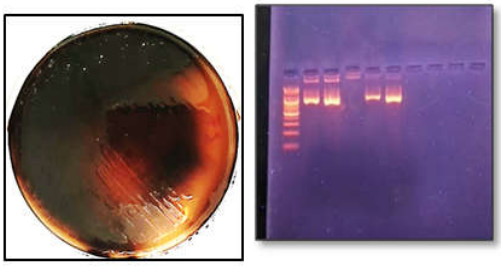

Figure 1. Brown pigment produced by *Pseudomonas aeruginosa (left)* PCR products of *Pseudomonas aeruginosa* Lane 1: ladder (100-2000bp) Lane 2,3,5,6 isolates of *Pseudomonas*, Lane 4 negative control

The effect of Pyomelanin pigment on UTI bacteria was more obvious against *E. coli*, *Staphylococcus* and *Bacillus* respectively while, wound bacteria were *E. coli* and *Staphylococcus* followed by multi drug resistant *Pseudomonas* the concentrations of pigment were between (10.000….100) μg/ml $P< 0.001$.pyomelanin pigment exhibited stunningly results against all tested fungi with different concentration. the highest effect was on *Aspergillus fumegatus* showed 36 mm Zone of clearance followed by *A. niger* gave 30mm Zone of inhibition, whereas *Cryptococcus neoformans* 23 Zone of inhibition, pyomelanin showed the same level of effects on *Candida Krusi* and *cadida tropicalis* 21mm Zone of clearance and lowest effect of different conceration of brown – black pigment was on *candida albicans* 14mm of clearance zone. P:< 0.001

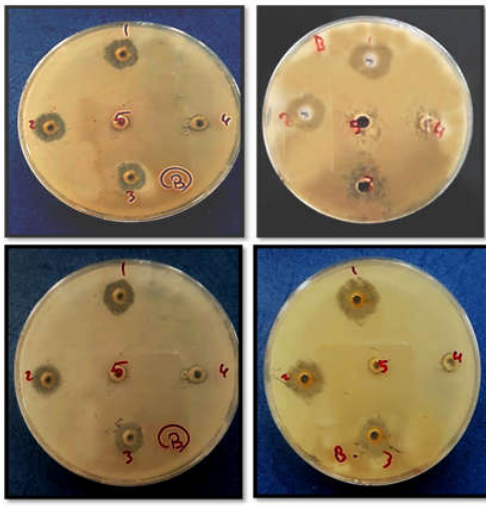

Figure 3. Effect of different concentration (1= 10000,2= 5000, 3= 1000, 4= 500 , 5= 100) of pyomelanin on pathogenic bacteria (from left to right) *Pseudomonas, E. coli* ,and down*Staphylococcus, and Bacillus*

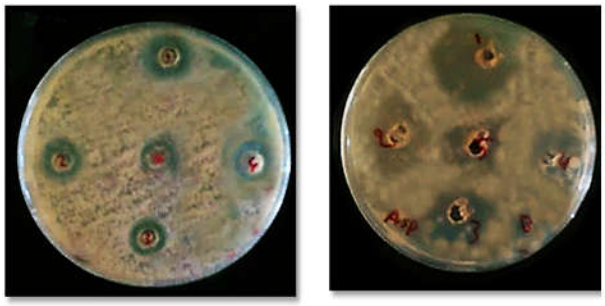

Figure 4. Effect of different concentration (1= 10000, 2= 5000, 3= 1000, 4= 500 , 5= 100) of pyomelanin against *Candida albicans* (left), and *Aspergillus niger* (right)

DISCUSSION

Results of current study showed that isolated pigmented bacteria with brown color exhibited phenomenal probability 99% and confidence excellent identification as *pseudomonas aeroginosa* using Vitek analysis with confirmation results from genetic analysis using 16SrRNA gene specific for *Pseudomonas aeruginosa* to confirm our study (Spilker *et al,* 2004). Pyomelanin production has been reported *P. aerogenosa* isolates mainly from urinary tract infection and chronically infeeted cystic fibrosis patients (Yabuuchi and Ohyama, 1972), Hunter and New man., 2010). Several bacteria are reported to produce melanin such as *Shewanella, Colwelliana, Vibrio cholera, Huphomonas* SP, *Azotobacter chroococcum, Alcaligenes eutrophus , Aeromonas Salmonicida, Burkholderia Cepacia, E. coli, Bordetella pertussis, Campylobacter jejuni, Yersinia pestis* (Tarangini and Mishra., 2013) and also certain marine bacteria like *Alteromonas nigrifaciens, Pseudomonas* SP, *Cellulophaga tyrosinoydans* and *Marinomonas mediterranea* (Soo-Jin *et al*., 2009; Solanol and Sachez – Amat., 1999;lvanova *et al*., 1996 ; Kotob *et al*. ,1995). Among *Pseudomonas* there are a few reports on melanin production *P. stutzeri* (Kumar *et al*., 2013), *P.maltophila* (Wang *et al*., 2002).

Pyomelanin produced by different bacterial species such as *Rizobium* SP. (Cubo *et al*., 2005), *Proteus mirabilis* (Agodi *et al*., 1996), *Klebsiella* SP.(Sajjan *et al*. ,2010), *Bacillus thuringiensis* (Aghajanyan *et al*., 2005) and *Modestobacter vericolor*(Reddy *et al*., 2007). Dark brown to black pigment which considered as pyomelanin depending on it chemical characteristics. for TLC, pigment exhibited RF value at 0.73 which have some result when compared with (Engstrom *et al*., 1993). Whereas the result of spectrophotometer revealed that pyomelanin extracted in this study was higher absorption in the UV region. (200-300 nm) then it decreased towards the visible region. this results was similar to results of previous study (Schaeffer, 1953, Aghajanyan*et al*., 2011 and Rosas *et al*., 2000). GC Ms analysis of pyomelanin extract in the present study showed the appearance of phenolic, indolic and aromatic compounds such as Nonylanin , octylalanin, Benzenamine, N-dodecyl N-phenyl alanine, Glysine, Butoybenzyliden, phenyl, pentylalanin, Glycyl – L- proline, tribenzyl, Benzocarboxylic acid, Fornamid, and Benzdicarboxylic acid. Appearance of Phenolic and indolic compounds GC –MS analysis is related to pyomelanin (Casadevall and Nosanchok, 2000, Hunter & Newman, 2010) pyomelanin is heterogeneous polymer containing compound with ring structures and it needed heat to break the complex phenolic and indolic compound into smaller fragments. in this study of pyomelanin extracted from *Penicillium chrysogenum*. Vasantha Kumar and his collages showed that pyomelanin hetrogenous aromatic polymer, that it made difficult to obtain uniform result when he compare his results with the information of NIST chemistry web book (Vasantha Kumar *et al*., 2013). In the present study pyomelanin pigment exhibited general effect on all pathogenic bacteria (wound and urinary tract infection) Gram positive and Gram negative, but gave higher effect against Gram positive *Staphylococcus* spp. followed by Gram negative *Pseudomonas*$_{11}$ (MDR) that may be due to the difference of nature of Gm+ve and Gm-ve cell wall (Tong *et al*., 2014). these results relative with the study of (Vasanthabharathi *et al*., 2011) they showed melanin extract was tested for antimicrobial activity against *S. aureus, E. Coli* and *C. albicans*. Our result exhibited the purified pyomelanin showed 17 mm Zone of inhibition against *E. coli* this result incompatible with (Mohagheghpour *et al*., 2000) who reported that effect of pyomelanin pigment showed 20mm zone of inhibition against *E. coli* and in our study the zone of clearance against *Staphylococcus* was 20 mm this result is in agreement with (Vasanthabharthi *et al*., 2011) who recorded that pyomelanin effects on *St*. was 18 mm zone of inhibition.

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