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Teratogenicity of Pyocyanin Pigment Isolated from Local Pseudomonas aeruginosa Isolates on MiceNeural Tube Defects (NTDs) and other Abnormities

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ABSTRACT

The primary objectives were to examine the association between pyocyanin pigment and neural tube defects and other different malformations in mice embryos. In mice, NTD may result from genetic mutations or from exposure to teratogenic agents, several of which are identified as risk factors in humans. Pyocyanin is a blue-greenish pigment was extracted from local Pseudomonasaeruginosa which is an extracellular virulence factor that is widely accepted to interpret the pathogenicity of Pseudomonas aeruginosa.

To achieve this, Swiss white mice exposed to pyocyanin pigment, mice were 30 females and males, average age (8-14) weeks, and average weighted (27 ±2) g for females and (30 ±2) gm. for males. Pyocyanin was given intraperitoneally in the concentrations of 75 µg/kg of live body weight for group I,125 µg/kg of live body weight group II and 200 µg/kg of live body weight for group III, whereas the control received only distilled water all those doses were given on day 8th of pregnancy.

The results have failed to demonstrate any association between pyocyanin and neural tube defects

(X2= 5.32, d.f= 3, P≤ 0.14). Whereas, It is evident that the pyocyanin has a real effect on mice embryos which it caused hypoxia (X2= 12.45, d.f. = 3, P≤0.006). Also, the results showed that the relationship between pyocyanin and resorption was highly significant (X2= 10.45, d.f=3, P≤ 0.01). The influence of pigment was highly significant (P≤0.01) on body weight, body length, and tail length.

These significant findings of a low percentage of NTDs and other malformations, with a high percentage of hypoxia and resorption, are excellent evidence that pyocyanin pigment has a strong property of toxicity.

Keywords: Neural tube defects, Pyocyanin, Hypoxia, Resorption.

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INTRODUCTION

Most neural tube defects (NTDs) in human beings are multifactorial, resultant from an additive contribution of numerous risk factors, which are genetic and environmental in addition to the interaction between these factors (Mahendra. 2017). There are two types of NTDs open which are common and closed (Zohn, 2012). Numerous teratogens are known that may induce neural tube defects and other malformations in experimental organisms. In some cases, a teratogen may act synergistically with genetic factors to increase the abnormalities or on other hand, the teratogen may cause a decrease in the incidence of neural tube defects and other malformations.

Secondarymetabolites are a broad class of compounds produced at stationary stages of microbial growth cultures;*Pseudomonas aeruginosa* is one of the microorganisms that have the ability of secondary metabolites production. *Pseudomonas aeruginosa* is aGram-negative,motile, aerobic, rods and not surrounded by sheaths (Garrity, 2004) and the major opportunistic human pathogen.

The genus *Pseudomonas* arenotable by production of colorful secondary metabolites called phenazine, about 90 to 95% of *P. aeruginosa*strains produce pyocyaninwhich was the main phenazine pigment-related with certain organism (Smirnov and Kiprianova, 1990; Zodpe, 2019), which had influential antimicrobial activity (Chakraborty, 1996).Pyocyanin (PCN) is a blue-greenish pigment of nitrogen-containing heterocyclic compounds with low molecular weight belongs to phenazine group with redoxactive properties (Parson *et al.*, 2007).

Reports on the effect of PCN onneural tube defect (NTDs) are limited which is associated with infection.The effect ofpyocyanin pigment on the central nervous systemis associated with high motility of *Pseudomonasaeruginosa*,which is rare,there is restricted supported evidence.The Infection of the central nervous system(CNS) by *P. aeruginosa*, though uncommon, is related to high death. The toxicity of pyocyanin is related to the development of acidic vesicular organelles (AVOs),which is encouraged oxidative stress, apoptosis, and cellular senescence in astrocytoma cells, these seemed to get up secondary to AVOs formation.Furthermore, PCN may affect with the intrinsicautophagicroutes of these cellsMcFarland et al (2012).Therefore, due to a very limited study on the effect of PCN on the neural tube defects and other malformations, the study was designed to discover furtherthe association between PCN and neural tube defects and different abnormalities in mice embryos. Hoping thereby, to understand more of the causal pathogenic mechanism.

MATERIAL AND METHODS

Subjects (Mice Model)

The current study was conducted on the Swiss white mouse, with 30females and males, average age (8-14) weeks, and average weighted (27 ±2) g for females and (30 ±2) gm. for males. During the study period, the

environmental conditions were under controlof the temperature of 23±2 C°. Mating day was considered day zero of pregnancy and the next day is the first day of pregnancy.Females who have not been fertilized were used again and they were re-checked for the presence of vaginal plug every 12 hours.

Sample Collection (*Pseudomonas aeruginosa)*

A total of 55clinical samples of *Pseudomonas aeruginosa* cultures were collected from 5 hospitals of 2 provinces Baghdad and Al-Anbar, from July 2018 to December 2018. The samples were burns (n=38), respiratory tract infection (n=1), urinary tract infections (n=14) and cystic fibrosis (n=2). All *Pseudomonas aeruginosa* isolates were grown on King A broth or King B medium and incubated for a 72 hr. at 37 C. Only the isolates producing greenish blue pigment (pyocyanin) were selected on king A medium (Atlas and snyder2006).

Extraction of pyocyanin pigment

The pigment extraction followed the procedure described by Ingledew and Gumpblle,(1969)and Al-Azawi (2006) with some modifications**;** a quantity of 200 ml of King A broth medium has been inoculated by culture from *Pseudomonas aeruginosa* incubated at optimum temperature at 37 in a rotary shaker at 110 rpm for 72 hr.Medium from*Pseudomonas aeruginosa* has been centrifuged for 20 minutes at 10000 rpm to take off the cells. The chloroform was added to KingA. Medium containing pyocyanin in the ratio 2:1(v/v). After the period, the chloroform color was changed to blue because pyocyanin was dissolved in it (Baron and Rowe 1981).Collection of the blue chloroform and extraction of pyocyanin by washing blue chloroform with acidified water (0.1)ml HCL. Such treatment converts the blue pigment to red acid, using vortex to extract whole pyocyanin found in chloroform.The acidified layer has been collected, then the pH was adjusted to 7.1+- 1 byTris base pH=11 to be returned pyocyanin pigment to their natural color.Re-extraction of pyocyanin from acidified layer of water containing pyocyanin +acidified water +Trisbase by adding chloroform and many times combining the two solutions to extract most pyocyanin from the mixture.The acidified water layer has been discarded and pyocyanin has been takenoff from chloroform by 0.05M (HCl). Repeated stepsseveral times to promote the purity of pyocyanin.The isolated water layer has been adjusted pH to 7.5 by adding a few drops of NaOH solution.

Purification of pyocyanin pigment by using gel filtration chromatography

The silica gel 60 was prepared by suspension of 35 mg of silica gel 60 powder in 500ml of distilled water for 1 hour in continuous shaking and washing.The gel was packed intothe column by using a small funnel with dimension of (2-35)cm and washing for 24 hours with (Methanol chloroform) in ratio 1:1(v/v). A quantity of 5ml of crude pyocyanin was added to the column,which was packaged by silica gel as (stationary phase) accurately and eluted with chloroform and methanol (1:1) v.The flow rate was 1 ml /3 min pyocyanin in the eluted fractions was spectrophotometer at a wavelength of 520 nm. After the completed elution, the column was re-washed by methanol: chloroform.

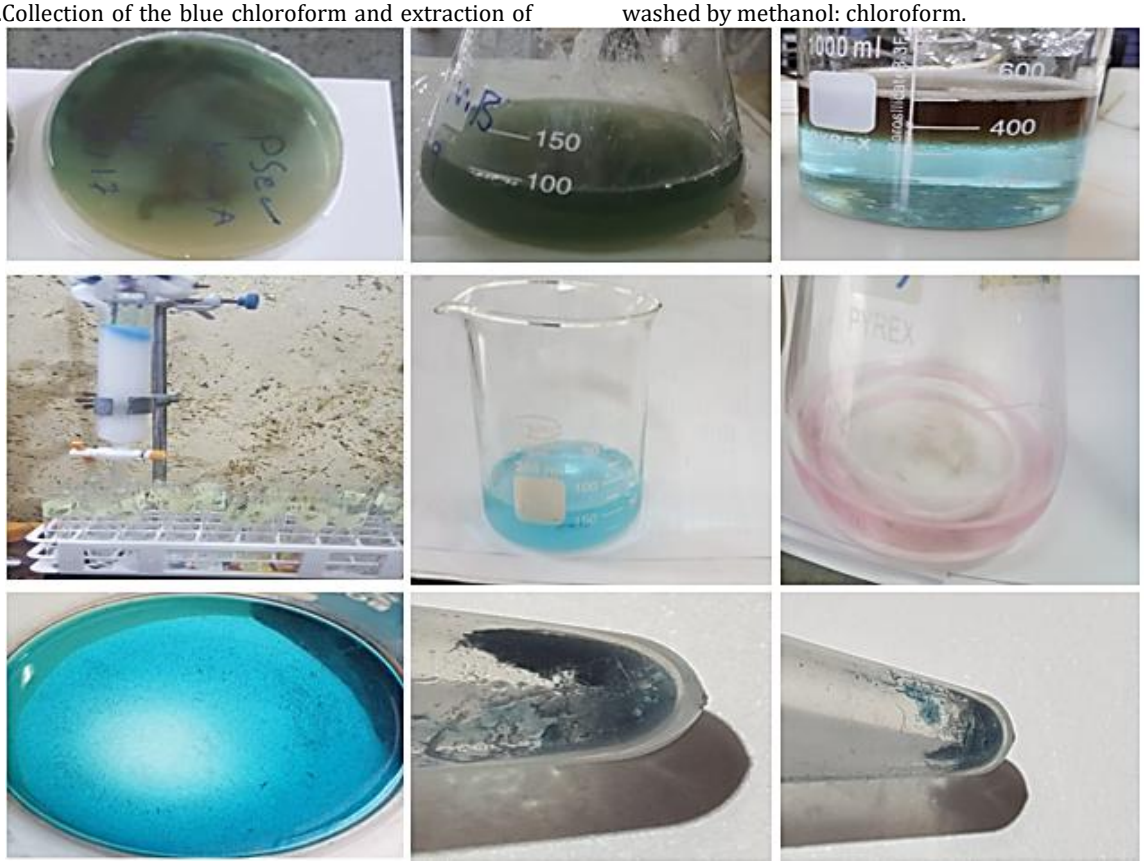


Figure 1. Purification steps of pyocyanin pigment extracted from local *Pseudomonas aeruginosa*isolates by using gel filtration technique.

Thin layer chromatography (TLC)

TLC was carried out according to Genevieve *et al*., (2006) with some modifications as follow:

The silica gel sheet (60f-254, 0.2mm layer thickness; aluminum support; 0size (20×20) cm spin has been used to analyze samples. The slotting line from the bottom edge of the plate was marked 1 cm.The liquid of standard

and extracted pyocyanin was dissolved in chloroform with a final volume of 20 ml,was detected in the first position and the test samples were displayed in a different position .Before development, the plate was left day at room temperature in the dark.Using solvent system chloroform, the TLC plates were developed chloroform: methanol 1:1 (v/v) in the TLC glass tank until mark distance from the spotting line was reached by the solvent front to check the purity and anR.f value from a partially purified pigment has been tested. The fraction from the eluted sample has been also treated with a few drops from (0.1 N) NaOH then kept for 2 -3 hours at 4C for crystallization (El-Shouny W.A. and *et. al*, 2011). The crystals have been separated by using membrane filtration, dried at room temperature. The crystals have been stored or suspended in water when it is used.

Characterization of pyocyaninpigment

Infra-Red (IR)

Infra-red spectrum of extractedpyocyanin and cultures compared to standardpyocyanin recorded by IR spectrophotometer,Shimadzu (Japan 4000-400 cm^{-1}) (Aunchaleebet. al .2009).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC mass was to diagnose the chemical compounds of pyocyanin pigment after the extraction. (Silverstein and Basseler, 1968).

Injection of Pyocyanin pigment

Stock solution of pyocyanin (after gel filtration) was prepared as (75,125, 200) µg/kg live body weight respectively. The concentration and dose were given intraperitonealaccordingto mouse body weight on day (8^{th}) of pregnancy in comparison to control mice which were injected with distilled water only. Female mice were divided into four groups.The control groups were injected intraperitoneal (IP) with distilled water.Three experimental groups were injected intraperitonealfor one time as below.The mice were killed on the day 18^{th} of gestation by cervical dislocation. The uterine horns of females were opened and examined for evidence of fetal death or reabsorption and then placed on the dissecting, the front and back limbs were fixed. The anatomy was performed from the beginning of the abdominal cavity, uterine horns were removed and opened to examine the embryos, after removing the fetal membrane and washing with distilled water. Embryo placed on a petri dish and examined by dissecting microscope to check for any external abnormalities, such as neural tube defects (NTDs) including (spina bifida, exencephaly, microcephaly), cleft palate, small body, eye and ear size, tail deformation (short or curly) which an indication of NTDs.

Statistical analysis was performed using the SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Pyocyanin is an extracellular virulence factor that is widely known to interpret the pathogenicity of *P.aeruginosa*. In comparison to the several studies identified thePCN as an oxidative substance which underlying the mechanism of PCN to induce toxicity. There is few information about the toxicity of this pigment on the CNS, but recently, the first *in vivo* evidence of PCN toxicity on the NTDswas reported (McFarland et al; 2012).

After 18 hours of incubation pyocyninwas produced, which was recognized byalteration in color to bluish green by adding of chloroform, which is soluble. The maximumabsorption of the extracted pyocyanin pigment at 270 nm was similar to that of standard pyocyanin .The result was agreed with (Sudhakar et al. 2013). The extracted pyocyanin was subjected to further characterization was by investigating their IR spectrum. The pyocyanin spectrum in Figure (2) showed the existence of phenazine as identified by side chains of the molecule. The peak at 3448.59 cm^{-1} shows the presence of the O–H bond. The peak at 2951.18 cm-1 relates to the C–H– aromatic bond. The peak shown at 1637.34 cm^{-1} represents the C=N bond and the peak at 130.7.02 cm^{-1} corresponds to C–O bond. This is similar to the information of standard FTIR spectra.

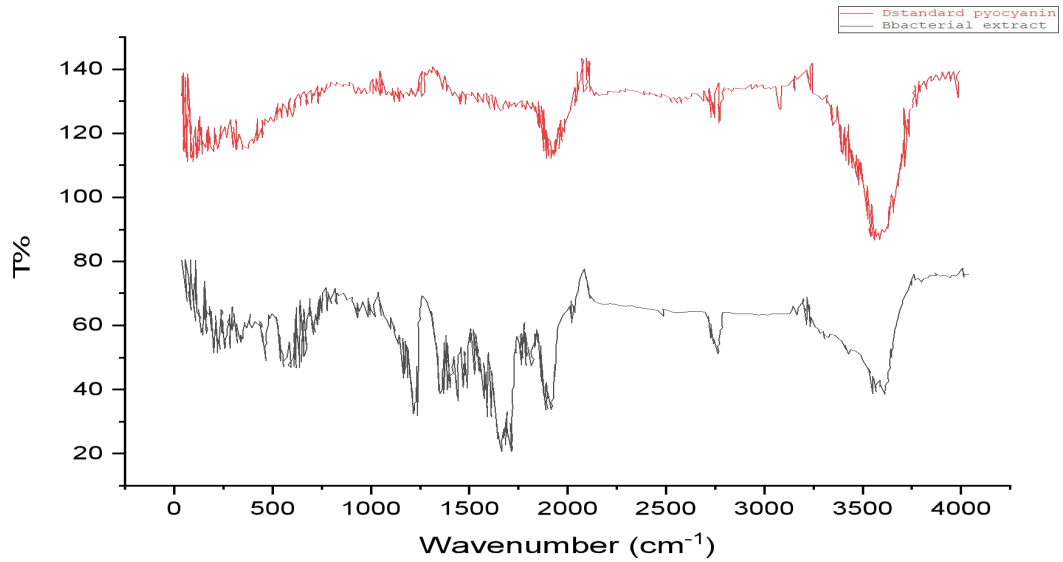


Figure2. FTIR spectra of standard (red) and purified (black)pyocyanin pigment

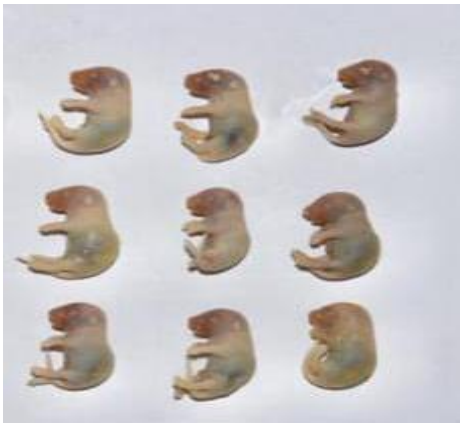
NTDs

The search for the effect of pyocyanin on the NTDs showed that this pigment might not contribute to NTDs and the results have failed to demonstrate any association between pyocyanin and NTDs (X2= 5.32,d.f= 3,P≤ 0.14). This indicates no significant relationship

between these two factors in inducing NTDs, although is no relationship the results showed a very low percent of microcephaly3.2% for dose 200 mg, whereas it showed 0% of any types of NTDs in all other doses and control.

Hypoxia

The pyocyanin has a real effect on mice embryos which caused hypoxia (X^2= 12.45, d.f. =3, P≤0.006) Figure (3-A). The relationship between hypoxia and pyocyaninwas highly significant at a level of P≤0.006. It can be observed that the highest percentage of hypoxia was produced at a concentration of 200 µg/kg ofpyocyanin which was 12.5%, whereas the percentage at doses 75 and 150 was1.8% and8.9% respectively. This result showed a gradual increase in hypoxia with increment of pyocyanin doses concentration.



(A)(B)

Figure3. A- Embryos with hypoxia B- Embryos with pyocyanin, it is very clear in the abdomen.

It could be argued that embryos are more sensitive to the cell-killing effect of pyocyanin but with different responses to the concentration of pigment administered to pregnant female mice.Since pyocyanin is a water-soluble blue-green pigment and its redox-active secondary metabolite and an extracellular pigment produced by (Lau et al., 2004). Hassan and Fridovich (1980) indicated that the reduced form of the pigment which can react with molecular oxygen and give rise to reactive oxygen imposing oxidative stress on host cells which cause cellular damage.

Hypoxia may be resulted from decreasing in EDU-positive neurons in the parietal cortex that maybe lead to a decrease in neuroblastdivision rates, which were not able to migrate into proper position in the II-III critical layers (Vasilev et al., 2016).

Also, it increases the intracellular oxidative stress and exhibits a redox cycle, which plays an important part in the toxicity of the substance. The redox-active properties play an important part in toxicity, pyocyanincan exist in an oxidized, blue form or in a reduced color which it can easily cross cell membranes (Hall et al., 2016). Therefore, it is easy to see the shadow of the pigment, blue green, in the abdomen of the embryo through the skin, Figure(3-B).

Resorption

As indicated by the analysis of chi-square, it showed that the relationship between pyocyanin and resorption was highly significant (x^2= 10.45, d.f=3, P≤ 0.01). There was a significant increase in the percentages of resorption, and the results showed this pigment had a toxic effect on mice embryo at all doses but with different percentages. The toxicity increased gradually with increasing the concentrations of the pigment. The percentages of resorption for the doses75, 150, or 200 µg /ml were 2.9%, 5.9%, or 8.8% respectively.The results of comparisons between pyocyanin – treated embryos and control groups showed that the percentage of resorbed fetuses were significantly greater in the pyocyanin treated group than in control at all doses.

Pyocyanin causes a wide range of cellular impairment such as the inhibition of cell respiration, epidermal growth, and dispersion of calcium homeostasis (Denning, 1998). It helps in oxidative damage to different types of cells that led to apoptosis (Lau, et al., 2004). Pyocyanin has been shown to have several pathogenic effects and causes a wide range of cellular damage such increasing IL-8, depressing host response, and inducing apoptosis in neutrophils throughout inhibition cell respiration and disruption of calcium inhibition cell respiration and disruption of calcium homeostasis which helps in oxidative damage to different cell types.

The variability in response to pyocyanin for different doses may be due to variations in the biotransformation of the administered pigment. The hepatic microsomal monooxygenase system plays a role in over 70% of metabolized pyocyanin. The rate of breakdown or the type of by-product produced by this system may explain some of the effectsof dosesconcentrations (Alani, 1991).

Bodyweight, Body length, and Tail length

The impact of pigment was highly significant (P≤0.01) for body weight, body length, and tail length. It can be seen that pyocyanin has a strong effect on the b body weight, body length, and tail length and caused a decrease in the measurements of those traits. The percentage of body weight, body length, and tail length decreased by a percentage of 15%, 11%, and 2% in comparison tocontrol. The effect of pigment on the embryonic growth may be due to secondary cessation of DNA synthesis by placental enzymes. Also, the well-known effects of the pigment on permeabilityof membrane mayobstruct with developmentof placenta, whichcause growthretardationin embryo and edema (Al-Ani, 1991).

Other malformations

In conclusion, these significant findings of a low percentage of NTDs and other malformations, with a high percentage of hypoxia and resorption are excellent evidence thatpyocyanin pigment has a strong property of toxicity. The results of this study, also participate in building an idea of the relationship between bacterial pigment and different malformations.In addition to

that,this research removessome ambiguity about some essential knowledge of humanson the toxic effects of natural pigment on embryos developments.

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