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EXTRACTION, PURIFICATION AND CHARACTERIZATION OF PYOCYANIN PIGMENT FROM *PSEUDOMONAS AERUGINOSA* AND TESTING ITS BIOLOGICAL EFFICACY

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ABSTRACT : ***Pseudomonas aeruginosa*** **was isolated and diagnosed from wound and burn injuries. It included 20 isolates from burn injuries and 18 wound injuries, which were diagnosed based on phenotypic characteristics and their ability to produce Pyocyanin pigment. The ability of** ***Pseudomonas aeruginosa*** **isolates to Pyocyanin pigment production after its growth on King A agar media was tested. The results showed the ability of 29 isolates out of a total of 38 isolatesto producepigment, in ratio 76.31%. Pyocyaninwas purified using Thin Layer Chromatography technique after phenotypic and quantitative detection of Pyocyanin pigment produced from** ***Pseudomonas aeruginosa.*** **The components of Pyocyanin pigment were confirmed and functional groups were identified using the FTIR assay. The efficacy of Pyocyanin pigment in hematolysis was assay by test tubes, as was the efficacy of the pigment as an antioxidant. The efficacy of Pyocyanin pigment inhibitory against isolates of pathogenic bacteria isolated and diagnosed from various clinical sources including burns and wounds was tested. Where showed results the Pyocyanin pigment a high inhibitory effect against isolates pathogenic bacterial.**

Key words : *Pseudomonas aeruginosa*, pyocyanin pigment, antioxidant, antibacterial, hemolytic.

INTRODUCTION

Pseudomonas aeruginosa bacilli, an negative gram's stain, possesses the ability to produce a very distinctive Pyocyanin pigment in addition to other pigments and most studies indicate that approximately 90 to 95% of the isolates of *Pseudomonas aeruginosa* bacteria are able to produce that pigment (Dahah, 2017). The Pyocyanin pigment, also called 5-N-methyl-1-hydroxyphenazine or 1-hydroxy-N-methylphenazine, is a product of secondary metabolism, belonging to the phenase family because it contains the nucleus of phenazine and is also described as a virulence factor for the bacteria produced from it and it keeps cells alive The bacterial produced to it and supports the formation of the biofilm, as well as its anti-bacterial and anti-fungal activity (El Feghali and Nawas, 2018). It is also considered to be an electronic transfer factor and an extracellular respiratory pigment, as having several properties made it a promising substance that could be employed in the future in some scientific, medical, nutritional, environmental and energy production fields (Tang *et al*, 2017; Alwohaili and Alaawad, 2019). In addition, Pyocyanin is a blue-colored phenase, an electronic receptor that stimulates the Redox cycling in germ cells and epithelial tissues of the human body (Esmat *et al*, 2020). This pigment has two positive and negative charges and thus it is Zwitter ionic and therefore it is able to dissolve in chloroform, hydrochloric acid, water, Toluene, n-heptane, petroleum ether, benzene, hexane, methylene chloride, and diethyl. ether, and other solvents (Ranio *et al*, 2020). Pyocyanin pigment .is blue in base and neutral solvents, red in acidic solvent, its toxicity is lost when the media becomes acidic and is reduced to a colorless compound called Leukopyocyanin, either by reducing agents, or under anaerobic conditions, and this compound resulting from reduction can oxidize. Easily when exposed to air (Laura *et al*, 2019).

This pigment is characterized by its toxicity of cells and its role in inhibiting other bacterial cells, because of its double charge and its low molecular weight (210.23) Dalton, it can penetrate the biological membranes easily to bacterial cells and inhibit the growth of most bacterial isolates, which are negative and positive gram's stain pigment and this action may also confirm through Depletion of the oxygen supply of the cell, production of H_2O_2 and modulation in the mechanism of the regular flow of the transporting electrons within the respiratory chain (Karakonstantis *et al*, 2020).

Provides the importance of pyocyanin pigment as an antibacterial agent, as well as its pharmacological applications, this study aimed to extract and purify the pyocyanin pigment from *Pseudomonas aeruginosa* and test its biological efficacy.

MATERIALS AND METHODS

Collection and diagnosis of isolates *P.aeruginosa*

Isolates were collected from burns and wounds for patients in and out of hospitals in the following governorates (Baghdad, Mosul, Diyala) and bacterial isolates were diagnosed based on their implant and microscopic characteristics in addition to a molecular diagnosis.

Phenotypic detection of pyocyanin produced from *P. aeruginosa*

The phenotypic green pigment, which is considered a secondary metabolism of *Pseudomonas aeruginosa* was detected as it was inoculated in the media of Nutrient broth using a micropipette, after it was growth and diagnosed on the king - A agar media and incubated at 37°C for 24 hours (Rani *et al*, 2018).

Quantitative detection of the pyocyanin pigment

Pseudomonas aeruginosa inoculated on nutrient media at 37°C for 24 hours, in test tubes, after which the tubes were placed in the centrifuge at a speed of 1500/ rpm, for 10 minutes, then the clearer was taken and chloroform was added, at a ratio of 1.6: 1%. It was mixed continuously for 10 seconds, as the lower layer turned blue, then a volume of the blue layer was added to one volume of the solution of the pyocyanin pigment reagent. Pink-reddish. Then the absorbance was measured at a wavelength of 520 nm with the spectrophotometer to find out the pigment concentration based on the following equation. The concentration of the concentration of pyocyanin = O.D × 17.072 (Khadim and AL Marjani, 2019).

Extraction of pyocyanin

Followed the method of Nawas and ELfeghali (2018) with some modifications, as he prepared 500 ml of Nutrient broth media in sterile flask and then vaccinated *Pseudomonas aeruginosa* bacteria, at a rate of 5% of the volume of the liquid media and was placed in the incubator shaker and incubator at a temperature of 37°C. For 72 hours, with a speed of 150 jolts/minute after the preparation period has ended, place the media containing the bacterial cells in test tubes and place these tubes in the central centrifuge at a speed of 1500 rpm/ 10 minutes, then take the clarifier and add chloroform to it, at a ratio of 1.6: 1%, then mix continuously for 10 seconds, until the lower layer turns blue, then add a volume of the blue layer with one volume of HCl pigment reagent solution at a concentration of 0.2 standard with mixing for 10 seconds as the blue turns pink-red.

Add the Borat – NaoH pyocyanin pigment reagent at a concentration of 0.4 molar, with a pH 10, to the above solution unless the red color changes to blue, the chloroform addition step and subsequent steps are returned to it, then the extract is left so that the chloroform evaporates at room temperature to obtain the required substance in powder form for pigment.

Analyses the pigment, read the Spectrophotometer - UV light device along the 278 nm wavelength and record the absorbance. The dye was then measured with an FTIR system to confirm its components.

Purification of pyocyanin by thin layer chromatography

Thin Layer Chromatography technique was used, according to the method mentioned by Farouq *et al* (2018), with some modifications, as a drop of pyocyanin pigment was placed on the bottom line depicted on the thin layer as a fixed phase using the capillary tube, then left the drop to dry and placed. Another drop of pigment, repeated 5 times. Then, the thin layer was placed in an anaerobic jar sealed glass container vertically, after the glass container was prepared by placing 75 ml of chloroform and 75 ml methanol as a moving phase, then continuing to move the pigment spot towards the upper end with a gradual color change, and after 3 hours removed the layer from the glass container and left it at the lab temperature for 24 hours to dry, as Resolution Front, RF was measured.

And compared with previous studies, then the effect of the purified pigment was scraped as a powder from the separation layer using sterile scraper, and the pigment was collected in a test tube with the addition of 3 mL of distilled water to it. And placed in the central centrifuge at a speed of 2000 rpm/15 minutes, after which the formed flask was determined and measured by the optical density spectrum at ranging wavelengths from 200 to 800 nm and compared the results with previous studies.

Characterization of Pyocyanin pigment using a FTIR analysis

After confirming the absorption of the Pyocyanin pigment by the optical density spectrum and to further characterize this pigment and know the chemical composition and types of chemical bonds in the pigment, the FTIR assay was done as reported in Savitha *et al* (2020). After taking 0.05 g, of Pyocyanin pigment in powder form, after the process of extracting that pigment , it was placed in an FTIR-4600 device, as the chemical

compounds that make up the Pyocyanin pigment were identified.

As well as types of chemical bonds, their number and functional groups, after extracting the values using the Jasco Spectra Manager II program.

Assay of Hemolysis Pyocyanin by test tubes

Preparing the sticking

Hemolytic activity : The test was according to ALjaafreha (2019) with the modifications performed, stuck red blood cells were present, from blood samples collected from healthy, non-smoking graduate students and putting blood in test tubes for blood collection and non-coagulation. Then, the tubes were placed in the centrifuge at a speed of 1500 rpm/5 minutes. In order to separate the plasma from the other blood components, the sediment was neglected and the erythrocyte precipitate was taken, the precipitate was washed with normal saline, 3 times using the centrifuge at a speed of 1500 rpm/5 minutes for one time, taking into account the accurate balance of test tube volumes to ensure that red blood cells do not break down and dissolve, then red blood cells are suspended with normal saline, so the final concentration of the sample becomes 0.5%. By adding 50 µL of erythrocyte precipitate to 9.95 mL of normal saline.

Assay of the efficacy pyocyanin pigment of hemolysis

Preparation 6 dilution of pyocyanin pigment and add to the stained red blood cells by 1.5 ml from each dilution to 1.5 ml of normal saline solution to reach a total volume of 3 ml. then add 1.5 ml of normal saline to 1.5 ml of the red blood cells stuck, as a negative control solution. Triton-x 100, 1.5 milliliters add to 1.5 milliliters red blood cells stuck of a substance, as a positive control solution. after incubator at 37°C for 30 minutes. The suspension was centrifuge at 1500 rpm/10 minutes, the supernatant was taken and the precipitate was discarded. The absorbance of the supernatant absorption of the optical density spectrophotometer was measured at a wavelength of 520 nm, and the results were calculated using the formula for hemolysis values.

Determination of the antioxidant activity of pyocyanin

This test was performed as reported by Rani (2018) with modification, to determine the efficacy of pyocyanin pigment as an antioxidant.

Free radical scavenging activity or antioxidant activity of pyocyanin was estimated by DPPH radical scavenging assay DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) free radical assay method is based on the transfer of electron that produces a violet solution in methanol. Then The efficacy of pyocyanin pigment as an antioxidant was calculated from the formula of free radical activity.

$$\text{Free radical} = \frac{\text{A(control)} - \text{A(sample)}}{\text{A(control)}} \times 100$$

Antimicrobial activity of pyocyanin by well agar diffusion method

The sensitivity of 16 isolated pathogenic bacteria diagnosed with burn and wound injuries was tested for 3 different concentrations of Pyocyanin pigment. The method of Diffusion in Agar was followed by drilling according to Bezalwar *et al* (2019), Transfer 0.1 ml of the bacterium that (compared to the MacFarland solution) was transported and homogeneously spread over the Muller Hinton agar media. Then, make 4 holes of equal dimensions in the middle of the test with a diameter of 5 mm with a cork hole, 0.2 ml of Pyocyanin pigment solution were added according to the three concentrations identified in the study and a hole in which distilled water was placed as a comparison point. Then the plates were incubated at 37°C for 24 hours, after which the inhibition zone around the holes were measured in millimeters. The inhibition regions were compared for each concentration of each pathogenic bacterial isolate.

RESULTS AND DISCUSSION

Collection and diagnosis of isolates *Pseudomonas aeruginosa*

The culture diagnosis was performed using the phenotypic properties of bacterial isolates when growth on the MacConkey agar media, which is a differential media. The bacteria growth on it are distinguished by the production of small, yellowish-soft, round colonies because they do not ferment lactose. The isolates showed their ability to grow on the media of the solid Cetrimide agar, under aerobic conditions, due to their resistance to the material, the bacterial colonies appeared in the green-yellow color and in a mucous form. The isolates after DNA extraction were subjected to the diagnosis by PCR method and by using primers to represent 16S-rRNA regions.

Phenotypic detection of pyocyanin pigment

Pyocyanin, a secondary metabolite of *Pseudomonas aeruginosa* was detected. After its growth on Nutrient agar media, King - A agar and Citrmaide agar media, incubation at 37°C for 24 hours. It was observed that the pyocyanan pigment was produced at ratio 76.31% of the isolates of *Pseudomonas aeruginosa* under study and the highest incidence of pigment was found on the King-

A agar, while the clarity of the pigment was lower on the media Citrmaide and nutrients agar. King –A agar media is selective for the appearance of pyocyanin pigment and preventing the appearance of other pigments belonging to *Pseudomonas aeruginosa* because it consists of peptone, nitrogen, vitamins and essential amino acids. This helps in the production of this pigment as well as magnesium sulfate, which increases the activity of cells to produce pyocyanin pigment (King, 2017).

Quantitative detection of pyocyanin pigment

The production of pyocyanin was detected by quantitative assay of 20 isolates of *P. aeruginosa* isolated from burn infections. As the optical intensity absorption values were shown using the spectrometer 1.819 and after applying the pigment quantitative detection equation, the amount of Pyocyanin pigment reached, 31.071 mg/ml of burns isolates, while the pyocyanin pigment of wound inflammation isolates ranged from 9 isolates from 7.301 to 16.417 mg/ml.

The addition of two volum of pyocyanin pigment detector, HCl at a concentration of 0.2 NM, to one volum of the blue pigment mixed with chloroform, and the color of the pigment turned to pink - reddish, which reveals the transformation of the media into acidic. The quantitative pyocyanin pigment detection assay depends on the amount of pigment extraction and the amount of light intensity absorption at a wavelength of 520 nm in an acid solution (El-Fouly *et al*, 2015).

Extraction of pyocyanain

Pyocyanin was extracted using the organic solvent chloroform, which produced a blue solution after adding it to the Nutrient broth green in a ratio of 1: 1.6., which was confirmed by the addition of a pyocianain HCL pigment of hydrochloric acid at a concentration of 0.2 standard. The blue color changed to red, through this reagent confirmed the pyocyanin pigment by color detection.

This extract matched with Queeneth *et al* (2018) that pyocyanin pigment is a bioactive compound that is water-soluble. With a blue color when extracted with chloroform, after making sure of it in color detection.

Purification of pyocyanin pigment by Thin layer chromatography

According to the method mentioned by Farouq *et al* (2018) the pyocyanin pigment which was extracted with chloroform was purified. using thin layer chromatography (Fig. 1). To verify the pigment extracted after measuring the optical density of the dry pigment on the thin layer was 2.776 at a wavelength of 280 nm and the value of R F = 0.75 as the results are shown in Fig. 2.

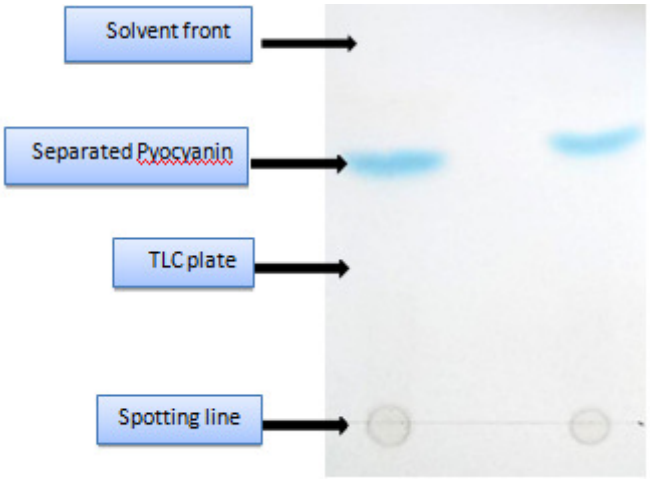

Fig. 1 : Separation of the TLC thin layer and purification of the pyocyanin pigment.

These results were consistent with that Chinmayi *et al* (2018) mentioned about purification of pyocyanin pigment, as the highest value of light intensity was obtained at 278 nm.

Abdul-Hussein and Atia (2016) mentioned that the pyocyanin pigment extracted from *Pseudomonas aeruginosa* isolates. It was validated by using the thin layer chromatography technique and obtaining a value of R F = 0.71.

Pyocyanin pigment analysis by FTIR assay

A FTIR assay was conducted to confirm the chemical components of pyocyanin pigment as well as know the number and types of chemical bonds and active groups in that pigment. These results are approach to the findings of the researcher, AL-Shamary (2018), as analysis of pyocyanin pigment from the isolates of *Pseudomonas aeruginosa* FTIR that is performed the presence of the aliphatic chain, the C = C aromatic compound in addition to the methyl group CH_3, the carboxyl group C-O and the C-H group. The results of the FTIR assay for pyocyanin pigment also showed that they are composed of carboxylic acids 1461.12 cm^{-1} and aromatic compounds 738.54 cm^{-1}.

The results of the FTIR test for pyocyanin pigment also showed that they are composed of carboxylic acids 1461.12 cm^{-1} and aromatic compounds 738.54 cm^{-1}, cm^{-1} aromatic aromatic ring, 1717.92 cm^{-1} carboxylic acid, and 738.54 cm^{-1} phenazine.

Assay of Hemolysis Pyocyanin pigment by test tubes

This assay was conducted to verify the toxicity of pyocyanin pigment on red blood cells for use as a treatment for wound injuries. As the results of the effectiveness of pyocyanin pigmentin hemolysis showed

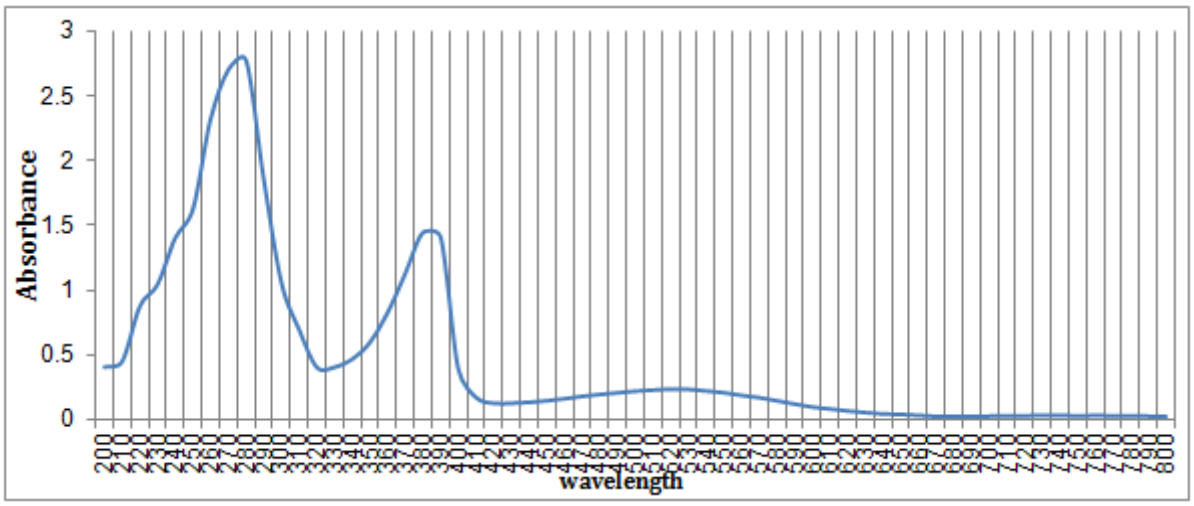

Fig. 2 : The optical intensity absorption values of the purified pyocyanain pigment using TLC technique.

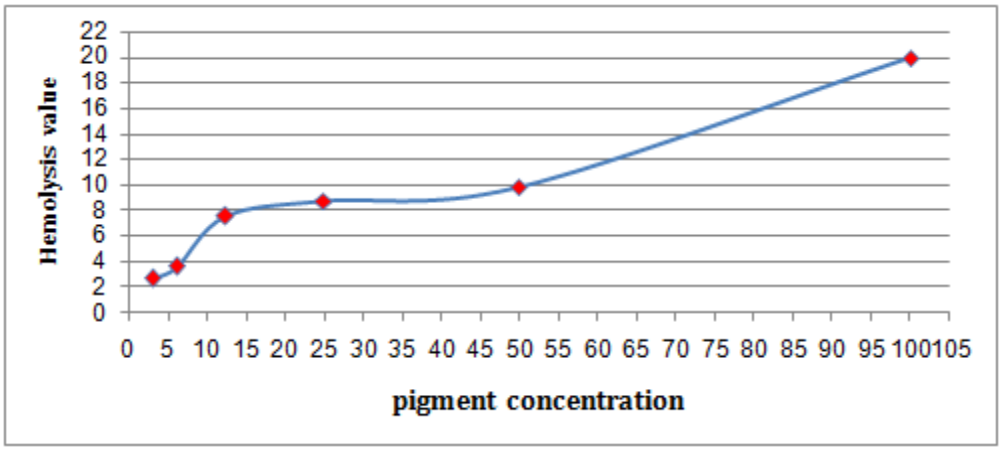

Fig. 3 : The results of Hemolysispyocyanin pigment.

that the values of hemolysis were graded according to the concentration of pyocyanin pigment, after measuring the optical density of each concentration. A blood analyzer. It results showed that blood analysis value appeared at a concentration of 100% (1.125 mg/ml) 19.97% and 9.87% with a concentration of 50 , Whereas absorbance appeared to decrease in conjunction with decreased dye concentration as shown in Fig. 3.

Haemolysis is the process of decomposing red blood cells, affecting host cells for their toxicity on eukaryotic cells. It is considered one of the virulence factors of *Pseudomonas aeruginosa*, Chinmayi *et al* (2018) indicated that *Pseudomonas aeruginosa* possessed two types of decomposition enzymes, the analyze and toxic type of eukaryotic cells phospholipase and lecithinase. The second type is thermally stable enzymes that consist of L-rhamnose and 1-b-hydroxydecenoic acid that do not affect human cells.

Determination of the antioxidant activity of Pyocyanin

This assay was done to confirm that Pyocyanin was an antioxidant and is intended as an antioxidant it is a molecule that is able to slow or prevent oxidation of other molecules *in vivo* and oxidation is a chemical reaction that converts a particular substance into an oxidizing agent that damages cells. Antioxidants end this chain of reactions by completely removing the primary medium, and preventing other oxidation reactions from oxidizing themselves. As a result, antioxidants usually remove the phenolic or polyphenol oxygen factor.

The results showed that Pyocyanin pigment is highly active as an antioxidant, by converting the dye color with DPPH To the dark yellow color according to the hue concentrations of the pigment under study, and after measuring the optical density of each concentration along a wavelength of 520 nm and by applying the free radical equation. It gave the highest result at a concentration of 100% 1.125 mg/ml. As it reached 83.707% and that value decreased by decreasing the concentration of Pyocyanin pigment as shown in Fig. 4.

Those results converged with Laxmi and Bhat (2016) with the highest pyocyanin pigment value as an antioxidant at pigment concentration of 0.002 g was 65.542%.

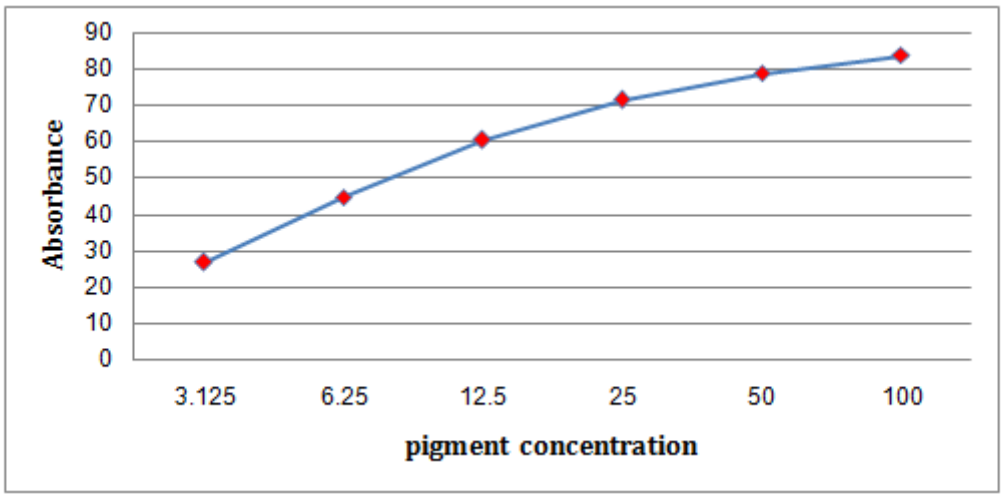

Fig. 4 : The activity of Pyocyanin pigment as an antioxidant.

Matteo *et al* (2018) also used DPPH (2-2diphenyl-1-picrylhydrazyl) as an antioxidant It contains stable free radicals, depending on the inhibition of the ion that has an unmarried electronic pair, so it is unstable, DPPH is a violet solution when dissolved in methanol.

Antimicrobial activity of Pyocyanin by well agar diffusion method

Selected 16 isolates of pathogenic bacteria forming the biofilm that are resistant to most antibiotics have been identified, to test the efficacy of Pyocyanin pigment in inhibiting the growth of pathogens isolates. For three concentrations of that pigment, using the Well agar Diffusion Method, as results were obtained Inhibition of measuring the diameters of inhibition zone of growth isolates on the test media and the results shown in Table 1.

The efficacy of the inhibitory pigment included both positive and negative bacteria of gram stain, as the results showed that *S.aureus.* The most isolated was sensitivity the pigment efficacy, as the diameter of the inhibition zone reached 37 mm for concentration 100%, 1.125 mg / ml, 30 mm for concentration 50%, and 22 mm for concentration 25%. While the results of inhibition of isolates bacteria *Klebsiella pneumoniae*, 27, 16, 11mm, respectively, for the three concentrations of the pigment and isolates of *Enterococcus faecalis* 16, 10 and 7 mm, whereas *Burkholderia cepacia* isolated 15, 9 mm and *Escherichia coli* isolated 14, 10, 5 mm, respectively. While the isolates of *Pseudomonas aeruginosa* bacteria were resistant to the effect of Pyocyanin inhibitance in its high concentration. This bacteria has an enzyme system to protect its cellular components from the influence of

Table 1 : The pyocyanin inhibition zone against pathogenic bacteria.

Isolates bacterial	Diameter of inhibition zone (mm)		
	The concentration of Pyocyanin (100% mg/ml 1.125)	The concentration of Pyocyanin (50% mg/ml 0.562)	The concentration of Pyocyanin 25% (0.281 mg/ml)
S.aureus	37	30	23
S.aureus	34	28	20
E. faecalis	15	10	8
E. faecalis	17	13	10
E. faecalis	16	10	7
E.coli	15	11	7
E.coli	14	10	5
E.coli	12	9	5
K. pneumoniae	25	15	9
K. pneumoniae	26	17	12
K. pneumoniae	27	16	11
K. pneumoniae	15	11	7
P. aeruginosa	-	-	-
P. aeruginosa	-	-	-
P. aeruginosa	7	5	-
B.cepacia	15	9	-

the pigment produced by SOD and CAT (Wontae and SunYoon, 2019).

When the bacterial cells are exposed inconditions of oxidative stress due to the influence of Pyocyanin pigment, Superoxide disututase (SOD) levels increase, Catalais (CAT) and glutathione peroxidiase (GSH-px). SOD works in *P. aruginosa* cells to convert O_2 to H_2O_2 and thus acts as a protective mechanism and a first line of defense. Against the oxidative stress caused by the effect of Pyocyanin pigment, then the high concentrations of H_2O_2 are removed by the CAT enzyme, after the degradation and inhibition of the efficacy of Pyocyanin pigment (Susan *et al*, 2016).

It was shown results this studying the inhibitory activity of Pyocyanin pigment towards the growth of pathogenic bacteria and their formation of the biological membrane. The interaction of the pigment with the respiratory chain of the cell membrane sensitive to the effect of this pigment, which affects the performance of cellular metabolism naturally (Limin *et al*, 2019). therefore, the pigment had an inhibitory effect on most bacterial isolates, negative and positive to the gram's stain and this action may be confirmed by depleting the oxygen supply of the cell , and production of H_2O_2 and modulation in the mechanism of the regular flow of transporting electrons within the respiratory chain as mentioned by Jagmann *et al* (2016).

CONCLUSION

Pyocyanin pigment is produced as a secondary metabolite to protect of the bacteria *Pseudomonas aeruginosa* from other microorganisms. Pyocyanin pigment demonstrated high inhibitory action against pathogenic bacterial isolates. It also possesses an effective antioxidant effect. Therefore, it can be used in the pharmaceutical industry and biotechnology applications.

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