MOLECULAR DETECTION OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM MINCED MEAT AND STUDIES THE PYOCYANIN EFFECTIVENESS ON PATHOGENIC BACTERIA

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ABSTRACT

This study was aimed to collected Minced meat from the local markets in Baghdad governorate during 2018, and examined for the presence of *Pseudomonas aeruginosa*, in order to extract and purify pyocyanin and examined it as an antimicrobial activity against pathogenic bacteria in foods. Fifteen isolates were isolated from 50 samples and identified as *P. aeruginosa* using the API20E system and finally confirmed with PCR using *16SrRNA* gene. Four tested media were used for the production of pigment after incubation within 72 h, One strain which given a vigorous pigmentation was chosen and extracted with chloroform and HCl then analyzed with Gas chromatography (GC-Mass) which showed a sharp peak at the time of acquisition of 27.13 minutes at the chromatographic analysis recognized with mass spectrometry as Hemipyocanin (alpha-hydroxy phenazine) which produced molecular ion with intensive peak at 205 m/z. Agar well diffusion technique was applied for estimating the antimicrobial activity of purified (pyocyanin) with variable concentrations (25, 50, 75 and 100 mg/ml) which monitored toward Gram-negative and Gram-positive bacteria that isolated of minced meat. *Escherichia coli* and *staphylococcus aureus* was the most affected with pyocyanin were followed by *Serratia marcescens* and *Klebsiella sp*. at the same level. While *Enterobacter sp, Bacillus cereus, Proteus mirabilis*, and *Proteus vulgaris* showed intermediate sensitivity, the *Pseudomonas fluorescens* was shown low sensitivity to pyocyanin.

Keywords: pyocyanin purification, gas chromatography, inhibition zones, *16SrRNA* Gene.

مجلة العلوم الزراعية العراقية -2019 :50(4):1199-1207 قاسم

الكشف الجزيئي لبكتريا *Pseudomonas aeruginosa* المعزولة من اللحم المثروم ودراسة الفعالية البيولوجية لمادة البايوسيانين ضد البكتريا المرضية

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المستخلص

هدفت الدراسة الى جمع اللحوم المفرومة من الأسواق المحلية في محافظة بغداد خلال عام 2018 ، وبحثت عن وجود الزائفة الزنجارية، من أجل استخراج وتنقية البيوسيانين وفحصها كمضاد للميكروبات ضد البكتيريا المسببة للأمراض في الاغذية. تم عزل 15 عزلة من 50 عينة وتم تحديدها على أنها *P. aeruginosa* باستخدام نظام API20E وأخيراً تم تأكيدها باستخدام PCR باستخدام جين *16SrRNA*. تم استخدام أربعة اوساط زرعيةلانتاج الصبغة بعد الحضانة خلال 72 ساعة، اختيرت سلالة واحدة تعطى تصبغًا قويًا واستخلصت بأستعمال الكلوروفورم و HCl ثم تحليلها باستخدام تحليل كروماتوجراف الغاز (GC–Mass) الذي أظهر ذروة حادة في وقت الاستحواذ 27.13 دقيقة و التحليل الكروماتوغرافي للغاز الذي تم تحديده بواسطة تحليل الطيف الكتلي باعتباره alpha-Hydroxy phenazine (Hemipyocanin) والذي أعطى ذروة جزيئية مكثفة عند 205 z / m. استعملت طريقة الزرع إلانتشار في الحفر لتقييم النشاط المضاد للميكروبات للنقى (البيوسيانين) بتركيزات مختلفة (25 ، 50 ، 75 و 100 ملغ / مل) وفعاليتها ضد البكتيريا الموجبة لصبغة جرام والغرام سالبة المعزولة من اللحم المفروم. كانت البكتيريا الأكثر تحسس للبيوسيانين *Escherichia coli* و *staphylococcus aureus* تليها *Serratia marcescens* و *Klebsiella sp* في نفس المستوى. بينما أظهرت البكتريا المعوية *Enterobacter sp*، *Bacillus cereus* و *Proteus mirabilis* و *Proteus vulgaris* حساسية متوسطة، أظهر ال*Pseudomonas - fluorescens* حساسية ضعيفة للبيوسيانين.

الكلمات المفتاحية: تنقية البيوسيانين, كروماتوجراف الغاز , مناطق تثبيط, جين *16SrRNA*

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INTRODUCTION

The shelf life of foods is identified being the period when the food quality remains satisfying within severe conditions of storage, distribution, and display. Spoilage is the method in which food has degenerated and turns into unacceptable for humans being or its quality is diminished turning food improper for selling or consumption (13). Several bacterial isolates which are particular as spoilage organisms (SSO) of meat, fish and poultry that can be identified through the ability for analyzing the nitrogenous components and generating the volatile compounds such as (ketones, esters, and aldehydes) that responsible for the flavor that will be formed at the point of spoilage. Some organisms primarily cause a change in sugars by oxidation and producing alkali and other organisms produce a fluorescent pigment (3). From the total of microflora, *Pseudomonas spp*. may represent the minority at the beginning of shelf life of the food then become dominant at the end. Phenazines are comprised the most significant extracellular pigments that produce from genus *Pseudomonas*, *P. aeruginosa* which is rod shape, aerobic and a Gram-negative opportunistic pathogen. *Pseudomonas aeruginosa* has a distinctive feature through synthesized of the blue-green, chloroform-soluble compound called pyocyanin (1-hydroxy-s-methelphenazine) (9). A number of virulence factors are secreted by *P. aeruginosa* which is considered the physiological and pathological effects of these bacteria. Of these virulence factors, Pyocyanin is phenazine oxidation pigment with low-molecular-weight that produced by *P. aeruginosa* (14). The Pyocyanin production is regulated by sensing the quorum, which involves a cell-dependent synthesis of signaling molecules that modify the expression of virulence genes (19). In spite of the fact, that *pseudomonad* has repeatedly been described for its pathogenicity; the capability of these microorganisms to produce antimicrobial pigment has opened the opportunity to an application of this agent as a biological regulator (19). Pyocyanin has antimicrobial activity toward wide different microorganisms, which may assist *P. aeruginosa* through eliminating competing microorganisms; pyocyanin serve as an antimicrobial agent, selectively inhibitors for gram-positive and gram-negative bacteria rather than *Pseudomonas spp*. The redox-active phenazine compound (Pyocyanin) which kills bacterial cells by the production of reactive oxygen intermediates. *P. aeruginosa* resists pyocyanin because of the limited redox cycling of this compound and that under conditions favoring pyocyanin production; catalase and superoxide dismutase activities are increased.Researchers created numeral and substantial modern antimicrobial agents within the latest thirty years; simultaneously the resistance of bacteria to the antimicrobial agents has more progressed. The aim of this study is to isolate various isolates of *P. aeruginosa* from minced meat with purifying and discriminate the pyocyanin pigment by conventional methods and study the pyocyanin properties as antimicrobial activity toward some pathogenic bacteria.

MATERIALS AND METHODS

Sampling

This survey was carried out during 2018, 50 fresh minced meat samples were randomly collected from Baghdad supermarkets, Iraq. The samples were stored in the ice box while transport to the University of Baghdad/ laboratory of market research and consumer protection center for examination.

Isolation with identification of *P. aeruginosa* and target bacteria from clinical samples

P. aeruginosa is isolated out of minced meat specimens: blood agar, nutrient agar and *Pseudomonas* Cetrimide Agar (OxoidTM) and selective media for each microorganism. In beginning; Twenty-five gram of freshly minced meat specimens have been homogenized within peptone water (225 ml), samples were later cultivated on selective agar media through streaking and pour plate technique and incubated at 35 °C within 48 h. (16). Observed the distinguishing pigmentation and compare the physiological and microscopic aspects with biochemical characters of the isolates through the official description presented in "Bergey's Manual of Determinative Bacteriology", that were recognized as *P. aeruginos* then the positive isolates has been confirmed with (API 20E).

Brain heart infusion agar was used to preserve the pure strains as slants form (16).

DNA extraction

A genomic DNA of *P. aeruginosa* was extracted for PCR amplification depending on company instruction Kit of DNA (G-spinTm, INtRON, Korea). Bacterial culture was transported to the microcentrifuge tube and Centrifugation at 13.000 rpm for one min, a buffer of Lysozyme was insert into the (centrifuge tube) the lysozyme was completely dissolved by using a vortex, the lytic when finished, the centrifuge was repeated twice and washed with buffer, the extracted DNA was saved at 4˚C until use. 1.0 % agarose gel was used to Electrophoresis the purified DNA. Five microns of DNA was combined beside three µl loading dye of bromophenol blue then photos were taken through using U. V. light 350 nm (Sambrook and Russell, 2001).

Detection of *P. aeruginosa* using *16S Rrna*

The specific gene of *16S rRNA* was conducted as Partial amplification with applying the primer pairs as in table (1), producing an amplicon with 150 bp with 25 µl reactions that including 100 pmol of each primer, master mix was used which is includes (PCR buffer, Taq polymerase, MgCl2 and dNTPs) and 100 ng of template DNA, with the conditional of amplification: the first denaturation was 95 °C within 5 min following by 30 cycles of 95 °C within 30 s, 60 °C within 30 s, and 72 °C within 45 s, the last extension at 72 °C within 10 min. PCR amplicons have been analyzed through 2% agarose gel electrophoresis, then photos were taken through UV transillumination 350 nm (1). Nanodrop (1000) was used to determine the purity of all 15 *P. aeruginosa* extracted DNA and the concentration of DNA was measured with (260/280nm) (1).

Table 1. Primers sequences of *16S rRNA* gene

Target gene	Primer name	Sequence (5'–3')	Product size(bp)
***16S rRNA* gene**	***Pseudomonas*-F**	**5'- CTACGGGAGGCAGCAGTGG-3'**	**150**
	***Pseudomonas*-R**	**5'-TCGGTAACGTCAAAACAGCAAAGT-3'**	

Extraction, purification, and characterization of the pigment produced by *Pseudomonas* isolates

The isolates of *p.aeruginosa* those given a vigorous pigmentation were selected and grown with the broth of *Pseudomonas* at 37oC within 48 h for generation of pigment. The broth culture rich with Pigment was later centrifuged by (10,000 rpm within 15 min) then the supernatant was accumulated, later filtered within filter membrane pore sized (0.45µm) and applied as the crude extract (7). (Chloroform and HCl) was adopting for Extraction of pigment from the crude extract, Chloroform was combined within the broth culture at the proportion of (2:1). The extract was stirred well by utilizing a shaker for 2 min, then divided out into two discrete layers, one of them was the pigment (a blue solvent layer), and the other was a residual material of culture. The blue layer was accumulated, later solution of 0.1N HCl (20% for the blue layer's volume) was combined then vortexed, then generated an upper pink acidified layer. The pink layer was later neutralized by Tris-Base then the neutralized layer was treated with chloroform again. The entire technique was repeated for numerous times to turn into purified pigment (7).

GC-MS Chromatograph of *Pseudomonas aeruginosa* pyocyanin

Pyocyanin was analyzed by using gas chromatography (GC-Mass) spectrophotometer with autosampler system (PerkinElmer/USA) this device provided with a carbowax (30*0.25mm ID) and (0.25µm thickness of film) capillary column (intercut DB5Ms. Japan). One µl of extracted Pyocyanin was autosampler inside the capillary column. The carrier gas (Helium) was adopted. Temperatures of Injector and detector were arranged at 280°C. The temperature of the column was programmed firstly at 40°C to 1 min and later expands to a 5°C rate per min at a terminal temperature of 290°C. Pigments were separated with at (96.1 Kpa) constant pressure and the flow of column 1.71 ml/min. Peaks have been recognized by

comparing the mass spectra versus the mass spectral database (7).

Screening of *P. aeruginosa* pyocyanin as antimicrobial activity

Antimicrobial activity of pyocyanin toward each isolated bacteria was prepared by using well diffusion technique on Mueller-Hinton Agar following aerobic condition, 100µl of bacterial suspension was poured on the surface of MHA spread by L- shape glass rod and left for 10 minutes to settle down the bacteria and 120µl of different concentrations (25,50,75 and 100 ppm) of purified pyocyanin was added to the prepared wells in the same plate and incubated at 37˚C for 24h-48h, the diameter of the inhibition zone was measuring around the wells which represent the antimicrobial activity of pyocyanin (6).

Statistical analysis

The program of Statistical Analysis System-SAS (18) was employed to perform the different factors in investigation parameters. The LSD (least significant difference) test has been employed to significant compare within the means of this investigation

RESULTS AND DISCUSSION

Isolation and identification of *P. aeruginosa*

The Bacterial isolates that chosen from minced meat sample were cultivated on blood agar and MacConky agar medium, isolates which revealed positive hemolysis activity were elected and re-cultured on nutrient agar and selective agar, these bacterial isolates were identified morphologically and microscopically and the result shown that there are several isolates belong to several genera as in table (2). Out of 50 selected minced meat samples, only 15 strains have been predicted to be *P. aeruginosa*. While the other bacteria were (8 isolates for *Psedomonas fluorescens*, 24 for *Escherichia coli*, 13 for *Klebsiella sp*, 12 for *Staphylococcus aureus*, 10 for *Bacillus cereus*, 15 for *Proteus mirabilis*, 9 for *Proteus vulgaris*, 11 for *Serratia marcescens* and 12 isolates for *Enterobacter sp*) were chosen as target bacteria. *P. aeruginosa* is human's opportunistic pathogen, relating to the *Pseudomonadaceae* bacterial family which is popular within the environment; in the clean water, soil, and contaminated food. It has also been widely isolated from fish, meat products and canned food (4, 5).

Table 2. Total of bacterial strain isolated from minced meat specimens

Bacterial species isolated from 50 fresh minced meat	NO. of positive isolates	Percentage (%)of positive isolates
Pseudomonas aeruginosa	**15**	**30**
Psedomonas fluorescens	**8**	**16**
Escherichia coli	**24**	**48**
Klebsiella sp	**13**	**26**
Staphylococcus aureus	**12**	**24**
Bacillus cereus	**10**	**20**
Proteus mirabilis	**15**	**30**
Proteus vulgaris	**9**	**18**
Serratia marcescens	**11**	**22**
Enterobacter sp	**12**	**24**
Total isolates	**129**	

Morphological and biochemical features confirmed that *Pseudomonas aeruginosa* is a smooth, large, and irregular bacterium, surrounded by bluish-green coloration with grape-like odor. All the isolates were aerobic, catalase positive, nitrate reduction positive, showed oxidative metabolism on Hugh Leifson medium and the ability to stain with gram stain appear negative when examining microscopically with rods shape, motile. The results of the biochemical characterization, determined by means of the API 20 E for *P.aeruginosa* in figure (1), those results are consistent with results that observed via (20) who identified *Pseudomonas aeruginosa* that isolates from food

Figure 1. API 20E result of isolated *P.aeruginosa* Molecular characterization

In the microbiology laboratory, *P. aeruginosa* considered a very common isolate and its identification by conventional biochemical or commercial kits or by automated means may lead to a somewhat expensive process of identification. On the other hand, 24 hours or more may be needed to carry out for identification, so the identification of the *Pseudomonas aeruginosa* genus was confirmed by tests with the specific primer of *16S rRNA* by PCR. The ranges of purity of extracted DNA out of 1.7-2. 350 nm U.V was used for visualized the extracted DNA followed by electrophoresis with 1% agarose gel by 70 volts within 30 min. The genomic DNA of isolates have been detected with 2.0% of agarose gel electrophoresis which dyed via red safe stain and electrophoresed in 70 volts about 1:30 hr, the 15 lanes in figure (2) have been captured with ultraviolet 350 nm (UV) transilluminator with size of band of (150) bp plus (100) bp as DNA ladder and this result was reported previously by (12) study and confirmed by (11).

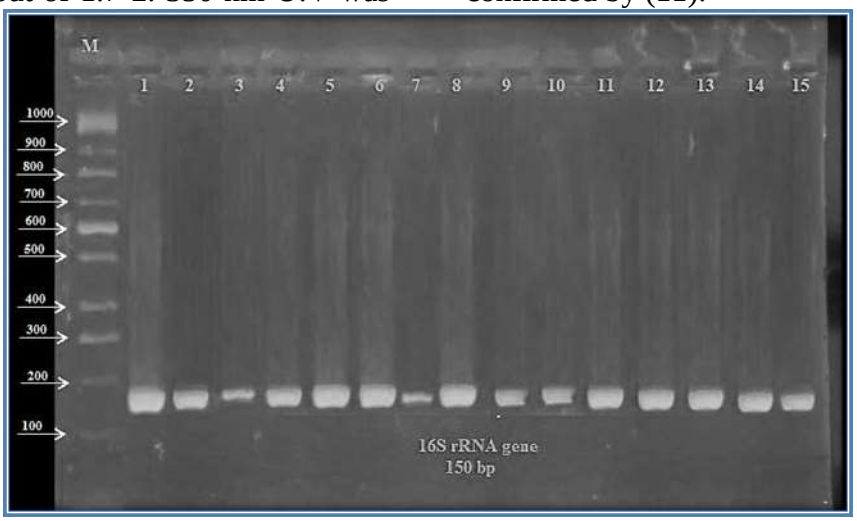

Figure 2. Amplification of *16S rRNA* gene with 150 bp on agarose gel of 2% with 70 volt and TBE buffer for 1:30 hours. M (100): DNA ladder, lanes (1-15), with 350 nm U.V light

The differentiation of the *16S rRNA* gene permits comparison at the genus level between organisms of bacteria, as well as to classifying isolates at multiple levels. The *16S rRNA* gene sequence was noticed by (15) who have analyzed of 5.0 isolates of *Pseudomonas* contain 99% nucleotide sequence comparable to *P. aeruginosa* despite its varied considerably in pyocyanin generation.

Production of pyocyanin

During growth of *P. aeruginosa* on the four tested media; blood agar, nutrient agar, Muller

Hinton agar, and Mac Conkey agar, it was concluded that there are various nutritional media can be utilized by *P. aeruginosa* for biosynthesis of pyocyanin. During this investigation, it was concluded that the pigment production produce throughout the first 24 hrs of growth and maximal pigment production was reached following 48 hrs. While, isolate No. 4 achieved the highest yield after 72 hrs. Among these examined strains, the characteristic of Pigment production was found in the 4 (26.6 %) out of 15 strain had this ability to produce pigment vigorously within 48 h of incubation as in figure (3).

Figure 3. Growth of *P. aeruginosa* on the tested media with produce pigment

Pyocyanin that generated by *P. aeruginosa* is employed in the various clinical microbiological laboratories as an adjunct test in the multiple testing procedures adopted for the identification of *P. aeruginosa*. In preceding researches, the pyocyanin production and catalase activity were enhanced when *P. aeruginosa* was grown in low- and high-phosphate succinate media under conditions of limited Phosphate (10).

Extraction and chemical analysis of pigment

In the current investigation, a chloroform solvent was the addition for departed of pyocanin from culture supernatants Chloroform extracted layer of pyocyanin showed converter in color from bluish to pinkish red during acidified by 0.1 (N) HCl, which indicated the presence of pyocyanin pigment. Chloroform extracted of *P.aeruginosa* revealed on gas Chromatographic analysis there is a sharp peak at acquisition period 27.13 minutes that recognized as (Hemipyocanin) alpha-Hydroxy phenazine through mass spectrum analysis which provided intense molecular ion peak at 205 m/z and its structure is presented in Figure(4)

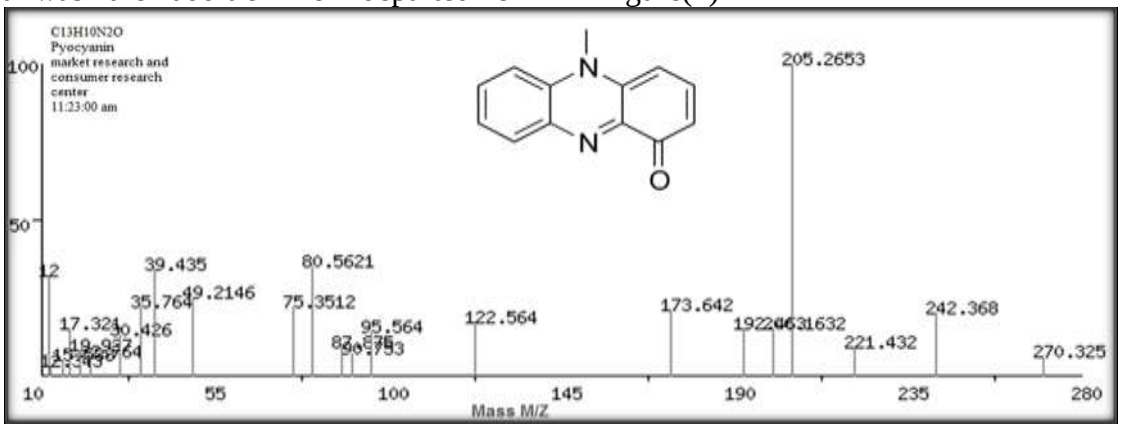

Figure 4. Illustrated the mass spectrum analysis of pyocyanin

GC-Ms of pyocyanin in the current investigation revealed the existence of phenazine and Hemipyocyanin compound. Prior analysis of GC-Ms by (14) confirmed these result that revealed the correlated hemi-pyocyanin pigment extracted of *P. aerogenosa* which recognized by mass spectrum following gas chromatography at ions peak (211 m/z) while the estimated one is 211.09 for C13H11N2O. And also consonant with the previous studies of (2) who demonstrated a molecular ion of the protonated purified compound of pyocyanin at m/z 196.

Antimicrobial activity against the target bacteria

The antimicrobial activity of purified pyocyanin at different concentrations (25, 50, 75 and 100 mg/ml) was observed towards Gram-negative and Gram-positive bacteria that isolated out of minced meat. One strain was chosen for the production of (pyocyanin) and estimated for its antibacterial activity by agar well diffusion technique. Out of various concentrations of pigments that used, 25 mg/ml revealed less activity with moderate inhibition zone on the agar plate. The remaining concentration of 50-75 mg/ml revealed the significant obligation with a higher zone of inhibitory activity, while the 100mg/ml which is considered the higher concentration and high purity recorded the higher inhibition zone compared with (25, 50 and 75 mg/ml) and the results are presented in table (3) and figure (5).

Table 3. Pyocyanin concentration of *P.aeruginosa* with Diameter of inhibition zone on target Bacteria

Bacterial isolates	Pyocyanin concentration of *P.aeruginosa* with Diameter of inhibition zone on target Bacteria (mm)				LSD value
	25%	50%	75%	100%	
Psedomonas fluorescens	**0**	**2**	**4**	**7**	**3.63 ***
Escherichia coli	**12**	**18**	**22**	**27**	**5.92 ***
Klebsiella sp	**8**	**11**	**17**	**26**	**5.77 ***
Staphylococcus aureus	**13**	**17**	**22**	**29**	**5.08 ***
Bacillus cereus	**4**	**7**	**11**	**14**	**4.42 ***
Proteus mirabilis	**3**	**6**	**8**	**13**	**4.68 ***
Proteus vulgaris	**4**	**6**	**9**	**11**	**4.09 ***
Serratia marcescens	**7**	**14**	**19**	**24**	**6.16 ***
Enterobacter sp	**5**	**8**	**13**	**17**	**4.51 ***
LSD value	**4.78 ***	**5.02 ***	**5.64 ***	**6.83 ***	**----**

*** (P<0.05).**

The common influenced bacteria to pyocyanin was *E. coli* and *staph.aureus* followed by *Serratia marcescens* and *Klebsiella sp*. at the same level. While *Enterobacter sp*, *Bacillus cereus*, *Proteus mirabilis*, and *Proteus vulgaris* showed intermediate sensitivity, the *Psedomonas fluorescens* was shown a weak low sensitivity to pyocyanin. These conclusions are in accordance with (10) they notify that phenazine compound has antimicrobial activity entirely toward *Bacillus subtilis* strains and *Escherichia Coli.* There was a considerable variation of the results regarding the bacterial resistance obtained from different strains and association with pyocyanin of isolated bacteria, This variation refers to the lipid of the cell wall content of Gram-negative and Gram-positive bacteria that may be accountable to the difference for the sensitivity of the pyocyanin antibiotic. Through expanding pyocyanin concentration from 50 mg/ml to 100 mg/ml, the antimicrobial activity is improved and enhanced; therefore the pyocyanin is concentration dependent as an antibiotic activity. Pyocyanin exhibits as a redox cycle and enhances intracellular oxidant stress and within the aerobic situation. This drives to reactive oxygen species (ROS) generation like hydrogen peroxide, and superoxide, these ROS compounds are able to inhibit the growth of microorganism (8).

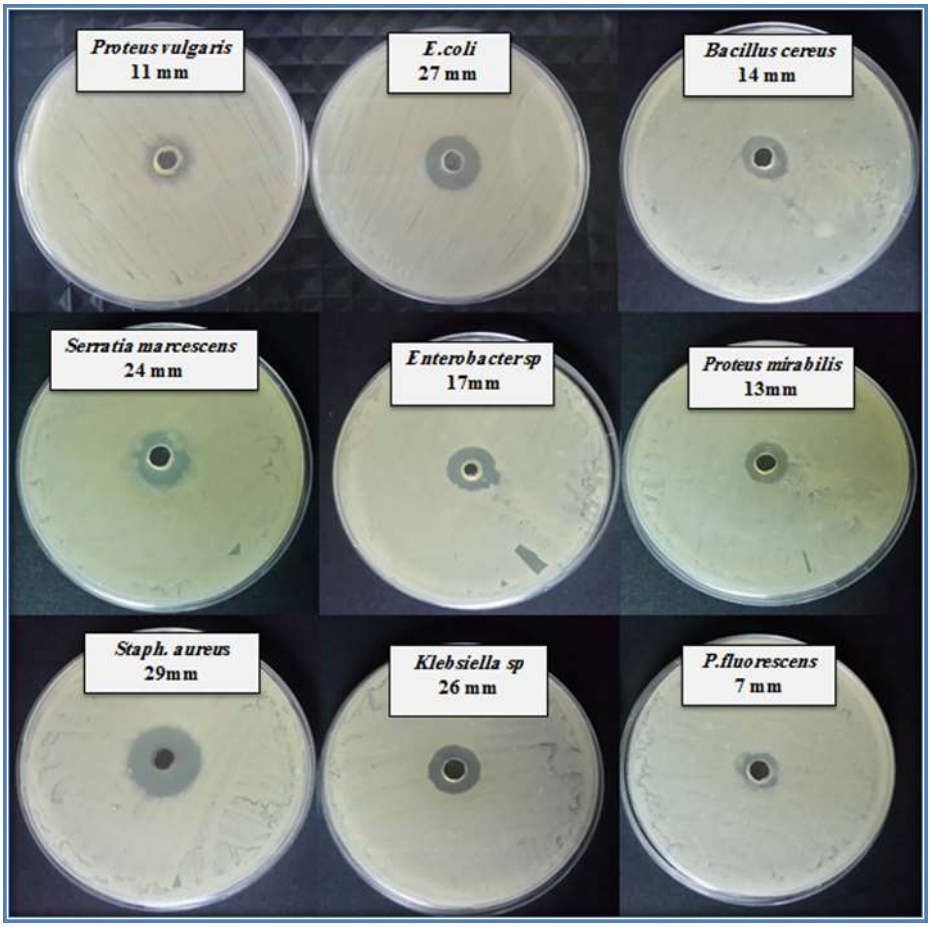

Figure 5. Inhibition zone diameter of purified pyocyanin with concentration (100mg/ml) against target bacteria

The current investigation concluded that the pyocyanin which extracted from *Pseudomonas aeruginosa* isolated out of minced meat was hemipyocyanin and has antimicrobial function as competitive agents infectious and pathogenic bacteria which contaminated food and these could assist as a signal to alert *P. aeruginosa* to the presence of another bacteria and the consequent progressed in pyocyanin production would help *P. aeruginosa* to compete with these microbes and save the food from contamination with pathogenic bacteria.

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