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Research Article

# Bioactivity of Phenolic Compounds Extracted from Strawberry against Formation of *Pseudomonas* aeruginosa Biofilm

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**ABSTRACT**

Background: Biofilm is a bacterial way of life prevalent in the world of microbes; in addition to that it is a source of alarm in the field of health concern. *Pseudomonas aeruginosa* is a pathogenic bacterium responsible for all opportunistic infections such as chronic and severe. Aim of this study: This paper aims to provide an overview of the promotion of isolates to produce a biofilm in vitro under special circumstances, to expose certain antibiotics to produce phenotypic evaluation of biofilm bacteria. Methods and Materials: Three diverse ways were used to inhibited biofilm formation of P.aeruginosa by effect of phenolic compounds extracts from strawberries. Isolates produced biofilm on agar MacConkey under certain circumstances. Results: The results showed that all isolates were resistant to antibiotics except sensitive to azithromycin (AZM, 15µg), and in this study was conducted on three ways to detect the biofilm produced, has been detected by the biofilm like Tissue culture plate (TCP), Tube method (TM), Congo Red Agar (CRA). These methods gave a clear result of these isolates under study. Active compounds were analyzed in both extracts by Gas Chromatography-mass Spectrometry which indicate High molecular weight compound with a long hydrocarbon chain. Conclusion: Phenolic compounds could behave as bioactive material and can be useful to be used in pharmaceutical synthesis. Phenolic contents which found in leaves and fruits extracts of strawberries shows antibacterial activity against all strains tested by the ability to reduce the production of biofilm formation rate.

**Keywords:** *Pseudomonas aeruginosa*, Biofilm, Strawberry, Phenolic Compounds.

## INTRODUCTION

*Pseudomonas aeruginosa* are the factors that cause opportunistic diseases that are usually originated in the environment, mostly in water and soil, also it can be found on animals, plants and human[1].

Gram-negative bacteria in the form of rod that is moving through the polar one whip, known to antibiotic resistance and can rise in a diversity of environments cruelty in general, usually via its capability to flexible biofilms creation. Bacteria frequently resulted from pyosyanin pigment green, blue, and phenazine are acknowledged in its effective oxidation, which are recognized as mammalian killing cells through the production of reactive oxygen intermediates. It is regularly false infection distinguishing sweet smell and consider as a major infection source HIV patients, It can be considered as major causes of infection in hospital-acquired like, Urinary Tract Infections (UTI)[2].

The *Pseudomonas* pathogenesis is complicate because both pseudo-invasive and toxic species go through three main stages:

Bacterial linkage to for colonies,
Restricted infection, and
Spread out in blood and in regular diseases

The significance of colonialism and stick is more pronounced once examined in the patients who suffer from infections in respiratory tracts especially those who suffer from cystic fibrosis and need the complexity of mechanical ventilation[3].

There are some factors that contribute the growth of *Pseudomonas aeruginosa* such as environment (host), type of infection, virulence, pigments, adhesions, toxin secretes, protease and proteins effectors. These factors disorder host organize and targeting the matrix of extracellular and the pathways of cellular.

*Pseudomonas aeruginosa* has the ability to develop biofilms on many surfaces that seem uninhabitable[4]. After attached to the surface, it moves crossways twitch move which lead to the micro colonies formation. The growth progress of biofilm affected with its matrix that composed from sugars. Matrix delays the deployment of some antibiotics in biofilm, making them effectively resistant to antibiotics. Phenolic compounds are secondary plant metabolites that are widely used in the plant kingdom. Strawberries are in the current phenolic compounds such as coumaric of ellagic acid and flavonoids such as quercetin, kaempferol and myricetin[5].

Bioactivity of microbes of phenols extracted from plant phenols were extensively studied, in addition to controlling the pathogens growth of the plants with its activities was investigated toward pathogens of human being as well for characterization and development of new food ingredients

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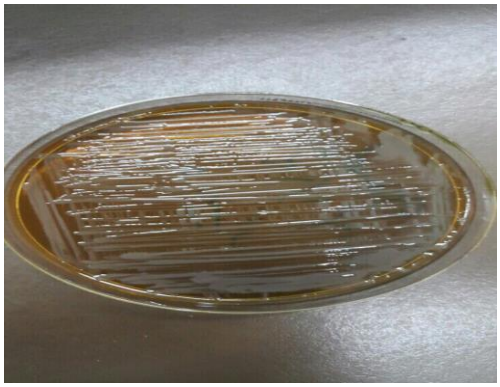

Figure: 1: The mucoid colony on macconckey agar after 96h.

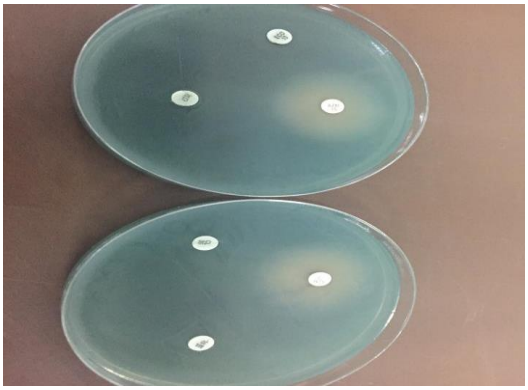

Figure: 2: Antibiotic susceptibility patterns of the *P. aeruginosa.*

Table 1: Antibiotic susceptibility measurements to isolated *P. aeruginosa.*

| Antibiotics | Ps1 | Ps2 |
|---|---|---|
| Azithromycin (AZM) | (S) | (S) |
| Cephalexin (CL) | (R) | (R) |
| Cefamandole (MA) | (R) | (R) |
| Nalidixic acid ( NA) | (R) | (R) |
| Ciprofloxacin (CIP) | (S) | (R) |
| Trimethoprim (TMP) | (R) | (R) |
| Nitrofurantion (F) | (R) | (R) |

Table 2: Readings (OD) to calculate biofilm formation.

| No. of Isolates | ODc | OD | Mean and SD. |
|---|---|---|---|
| Ps1 | 0.106 | 0.345 | 0.326±0.019 |
| Ps2 | 0.026 | 0.240 | 0.229±0.09 |

and health and medical complexes and strawberry extract inhibited the growth of Gram-negative bacteria[6].

The goal of this research is to estimate the bioactivity of phenolic strawberries against *Pseudomonas aeruginosa* microbes and to uncover the mechanism that is explain the activity of crude and pure phenolic extracts from strawberries.

## MATERIALS AND METHODS

*Test organism*

This study was conducted on two clinical isolates for *Pseudomonas aeruginosa* have been taken from patients whose have advanced infections in urinary tract, the samples were isolated by using morphological and biochemical tests in Kadhimiya hospital laboratories.

*Cultural media*

All the cultures were achieved by using modified quantitative method and semi-quantitative method (Maki *et al.*, 2006)[7]. Main separation was achieved using media like chocolate, blood and MacConkey agars. Primary detection was complete using gram staining which spread from the colonies and placed on media plates. This identification was tailed with biochemistry screening in suitable technique and sterilized environment[8].

Kirby Bauer Disc was used to complete the anti-microbial susceptibility test according to (Titball and Basak 2006). Diffusion method was used to accomplish it using (Himedia) anti-bioltic discs[8].

*Antimicrobial Susceptibility Testing*[9]

This investigation was taken place to find out the potency or sensitivity of anaerobic bacteria aerobic pathogens to a variety of anti-microbial materials to help the physicians to select patient medicament.

This investigation was done to determine 7 antibiotics e.g. Cefamandole, Cephalexin, Trimethoprim, Nitrofurantion, Azithromycin, Ciprofloxacin and Nalidixic acid. This test was achieved according to (Aaron *et al.*, 2002)[9].

*Test for production the polysaccharide from P. aeruginosa*[10]

Colonies of *P. aeruginosa* were streaked heavily onto plates of MacConkey agar modified by the addition of 3% (vol/vol) glycerol solution and incubate them for four days under 25°. (Leighand Alfred 1973)

*Extraction of phenolic components from strawberry leaves and fruits*[11]

Dissolve (5 g) of strawberry fruit were homogenized in 10 ml of acetone, then centrifuged (1500 rpm/min) for 10 minutes. The upper phenolic layer was separated and using 10 ml of acetone the phenolic compounds was re-extracted. Using nitrogen flow at 37°C, the acetone was detached from the extract and only the phenolic compound remain and top it iup to 10 ml with deionized water. The strawberry leaves were dried and well grind, and the same procedure was repeated to extract the phenolic compound[12].

Using Folin–Ciocalteu reagent (05 ml) was added to 0.1 ml of the extract and dissolved in 2 ml of deionized water and 2ml of gallic acid (0.029 mg/ml). Then, and after 1 minute, a 1.5 ml of sodium bicarbonate ($NaHCO_3$) (7.5 wight/volume) was added to the solution and to it up to 10 ml by water, the and then and for 2 hours and at room temperature, the solution was left in dark place. By means of UV-Vis spectrophotometer and at 760 nm, the absorptivity of the extract was measured and compared with control (blank solution)

*Biofilm Detection*[13]

The detection of early formed biofilms can assist to response before things developed and the biofilms turns to colonies and things get worse and be harmful to immune system, thus, three procedures to detect the biofilms were found, these are: Congo-Red Agar (CRA), Tissue-Culture(TCP) and Tube Method (TM).

*Congo-Red Agar Method (CRA)*

This method was to use a solution consist of the following components: (5 g/L) of sucrose, congo-red pigment (0.8 g/L) and agar No. 1 (10 g/L) all these components were mixed with brain heart infusion agar (37 g/L) and placed in aerobic autoclave at 55 °C for 15 minutes. And before incubation the plates for 24-48 hours at 37°C, all plates

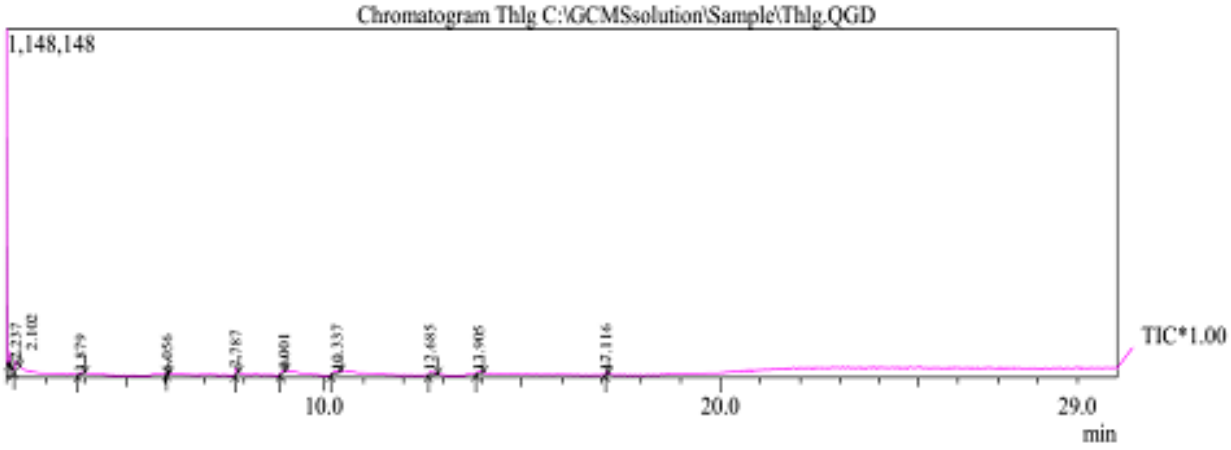


Figure 3: Gas Chgromotography for Strawberry fruit extract.

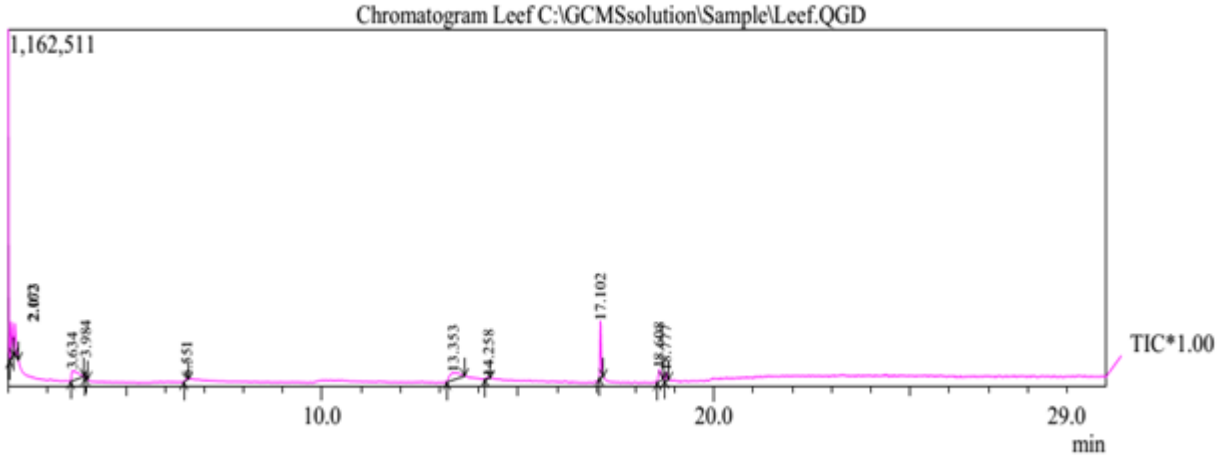


Figure 4: Gas Chgromotography for Strawberry leaf extract.

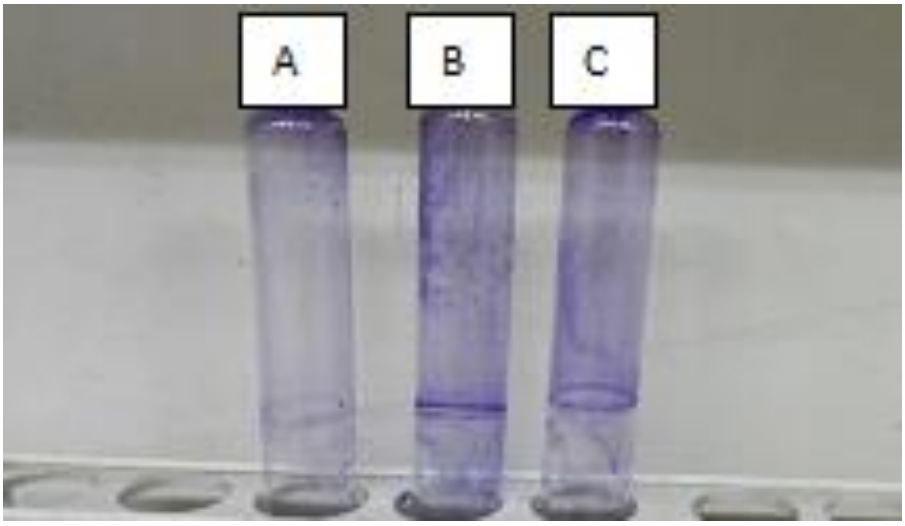


Figure 5: Tube method A. No visible film. B & C Visible film lining the wall and bottom of tube.

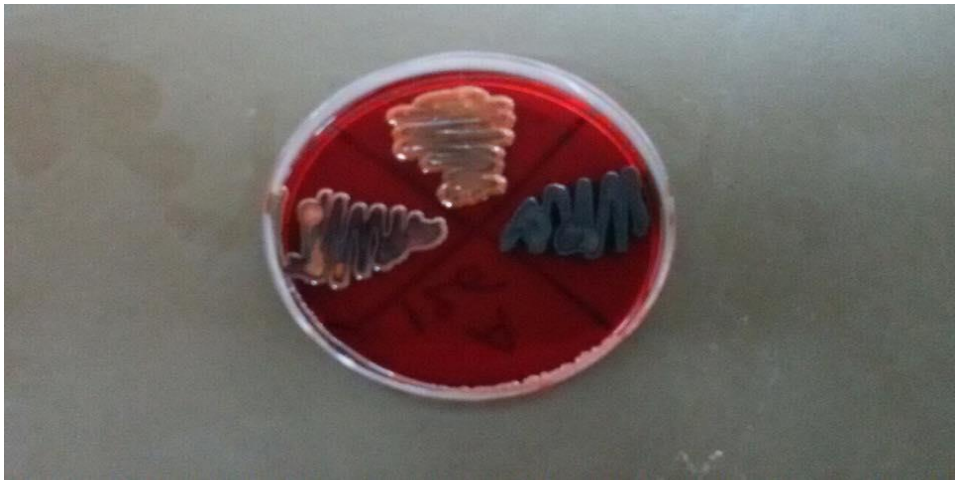

Figure 6: Congo red agar where A- weak (pink color), B- Dark indicate moderate and C- Black one represent the strong positive colonies.

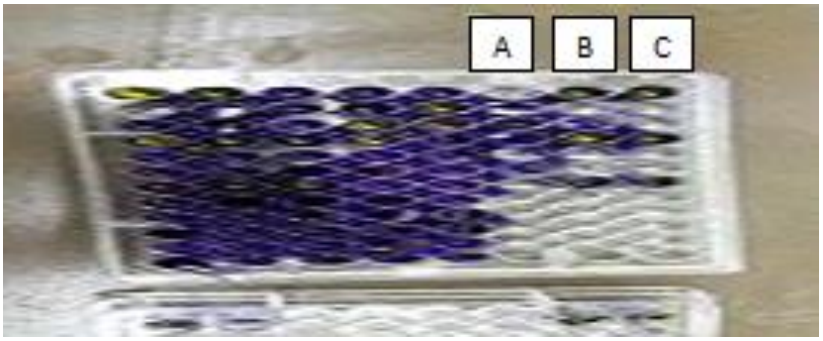


Figure 5: Tissue culture plate method A. No colour B & C. Violet colour indicating biofilm produce.

Table 3: Readings (OD) to calculate biofilm formation before and after treatment.

| No. of Isolates | OD Before treatment | OD after treatment (leaves) | OD after treatment (fruits) |
|---|---|---|---|
| Ps1 | 0.345 | 0.071 | 0.68 |
| Ps1 | 0.333 | 0.113 | 0.161 |
| Ps2 | 0.320 | 0.037 | 0.058 |
| Ps2 | 0.320 | 0.095 | 0.169 |

Table 4: mean and standard deviation of (OD) before and after treatment.

| No. of Isolates | Mean and SD Before treatment | Mean and SD after treatment (leaves extract) | Mean and SD after treatment (fruits extract) |
|---|---|---|---|
| Ps1 | 0.339±0.008 | 0.092±0.029 | 0.042±0.36 |
| Ps2 | 0.320±0 | 0.066±0.04 | 0.113±0.078 |

were inoculate by test organism. If the plates were in black colour indicate the formation of biofilms while pinky colour plates show weak production of it.

*Tube method*

In this method 10 ml of solution consist of trypticase soy concentrate with 1% of glucose in it (Solution One), this solution was inoculated with test organism from agar nutrient. The concentrate solutions were placed in incubator for 24 hours at 37 °C. The formation of biofilms in incaubated cultures were examined using certain tubes (violet colure tubes previously washed with phosphate buffer solution (PBS) (pH=7.3)), after pouring the cultures in this tubes, if a visible line appear in the tubes this indicate biofilms formation and this was scaled according to biofilms amounts, 3 is strong, 0 is no biofilms formed while 1 & 2 is weak and moderate respectively.

*Tissue Culture Plate method (TCP)*

Pour trypticase soy stock solution on the isolates that was collected from agars, and (TSB glu) media was added, and under 37 ºC for 18 hours was incubated and after that 100 ml of fresh media was used to dilute it.

The dilute (0.2 ml) of it was subjected in plates, stock solution plates were used as control and incubated at 37 ºC for 18-24 hours. Plates contents were stripped with gentle remove, plates wall were rinsed with (7.2 pH of PBS) to remove bacteria. Using (2%) of sodium acetate and 0.1% of crystal violet, biofilms were reinforced in the plates walls while the surplus stains in where washed with deionized water, then the plates were dried. Violet crystals on plate wall indicate the formation of biofilms and can be characterized using micro ELISA auto reader at 570 nm wavelength to show the Optical Density (OD) to the biofilms bacteria stains index that adherent.

*Biofilms Inhibition (formed from strawberry fruit and leaves extracts)[14]*

Micro plates test was used to measure the effect of extracts that was isolated from fruits and leaves on the inhibited

Table 5: Evaluation of compounds for strawberry fruit extracts.

| No. | Rt. | Area% | Name of compounds |
|---|---|---|---|
| 1 | 2.102 | 13.61 | Butanoic acid |
| 2 | 2.237 | 19.57 | Acetic acid |
| 3 | 3.879 | 6.42 | Furfural |
| 4 | 6.056 | 2.08 | 1-Pentene |
| 5 | 7.787 | 10.60 | Levoglucosenone |
| 6 | 9.001 | 5.90 | 1,4-Dioxane-2,6-dione |
| 7 | 10.337 | 11.54 | 4-Hepten-3-one |
| 8 | 12.685 | 16.87 | Propanedioic acid |
| 9 | 13.905 | 8.44 | 3-Nitropropanoic acid |
| 10 | 17.116 | 4.97 | 3,4-Dihydroxy-5-methyi-dihydrofuran-2-one |

biofilms. Using 96 wall plates the biofilms inhibition, a spectroscophotmtry test was achieved by adding 100µml of the extracts to the plates and incubate them for 72 hours at 37 ºC, after that the plate suspensions were detached and 100 µml of crystal violet aqueous solution was inserted. After half an hour the violet dye was detached and the wells were rinsed with ethanol (95%) and then the combination was subjected to spectrophotometer at 540 nm.

*Optical density (OD) calculations*[15]

The data of biofilms was collected by addition the standard deviation to the means of the readings (mean was determined by taking the average of the three readings), and the gathered data was classified the biofilms activity according to the following: Value more than 4 represent strong biofilms, while zero mean weak biofilms and from zero to four considered as moderate biofilms

*Gas Chromatography-Mass Spectrometry*[16]

This apparatus achieved two analyses in one instrument; the apparatus analyze gas chromatography and mass spectroscopy to the same sample

For gas chromatography high temperature column was used (Inert cap 1MS; 30 m × 0.25 mm id × 0.25µm film thickness) were the sample (5 µL) was injected in the detector that was placed at 280°C starting from 100 °C as a primary temperature. The column was operated uing (1/10) split mode, one minute after the temperature was set at 225°C, and adjusted at (12.5°C every minute with 4 minutes holding time), and temperature was then raised to 300°C (7.5°C every minute with 5 minutes holding time). The flow of helium gas (carrier) was adjusted at (17.5 mL/min), mass spectrum was processed using POSTRUN Software and GC-MS solution, compounds were identified and classified comparing with standard solutions.

## RESULTS AND DISCUSSION

*Morphological and Biochemical tests of the Pseudomonas aeruginosa*

The current study was based on routine cultural bacterial media such as: Blood agar to test the ability of bacterial isolates on blood heamolysis type β while macconky agar use to distinguish between lactose fermented and non-fermented bacteriaThe bacterial isolates growing on nutrient agar to detect the greenish-yellow colonies with production of green fluorescent pigment, finally the bacterial isolate was gram negative and it was positive for catalase and oxidase In the present study the phenotypic and biochemical analyses of the bacterial isolates screened from UTI Gram negative, motile, non-spore forming bacterial cells. It is unable to ferment lactose, produce fluorescent green pigments on nutreint agar and produce β-hemolysis on blood agar. In close consistence with these results[17].

Usually, and as mentioned in previous worker reports, the detection of *P. aeruginosa* was achieved using classical techniques depending on bacteria culture on a certain agars, tailed with chemical instrumental analysis to identify the concentration and the level of bacteria.

*Production of polysaccharide*

Slime production also resulted in the least reversion of the mucoid strains to the non-mucoid state. In all media tested, more slime was produced per cell at12Cº than at 25Cº and the least at 37ºC. Both variants produced most slime on plates with MacConkey agar containing sodium chloride or glycerol. However, excessive amounts of either sodium chloride or glycerol inhibited cell growth and slime synthesis. The addition of up to 5% glycerol increased slime production, but at 10% glycerol growth and slime synthesis was inhibited at 25 ºC or 37 ºC but not at 12 ºC. The addition of 0.25 M NaCl to MacConkey agar greatly stimulated slime production at all temperatures.

The addition of 0.5M NaCl was slightly inhibitory to growth and slime synthesis at 25 and 37 C but not at 12 ºC the mucoid strains obtained from chronic infections produced copious amounts of polysaccharide regard less of the media or conditions used, physiological conditions in the lesions must have favored the selection of slime-producing strains[18]. (Figurer 1).

*Antibiotic Sensitivity Test*

Figure 2 shows the patterns of the antibiotic susceptibility while all the collected data of this test were listed in Table 1.

Results in table 1 indicate the resistivity to most antibiotics exclude Ciprofloxacin and Azithromycin. The collected data in this research shows that using antibiotic for long time and the prolong stay in the hospital can affect the MDRPA frequency and increase it.

Zahra (Zahra *et al.*, 2015) pointed out that the MDR in *P. aeruginosa* can affect the activity mechanism as well as the synthesis of drug outflow system, protein in outer membrane default and also mutate the target[19]. The biofilms that formed due to infection in *P. aeruginosa* can be calculated due to its importance in pathogenic pathway of isolated biofilms.

Jabalameli (Jabalameli et al., 2012) studied the isolated biofilms in Iran, and it was found that it match the results in this work[20]. The correlation of antibiotic resistivity with biofilms development was studied and it was discovered that the Isolated MDR exhibit a major formation of biofilms (Bidi et al., 2013) comparing to isolated susceptible, this could be due to late in diffusion of the antimicrobials through the cell membrane of the bacteria[20].

*Determination of total phenolics*

Table 6: Evaluation of compounds for strawberry leaf extracts.

| No. | Rt. | Area% | Name of compounds |
|---|---|---|---|
| 1 | 2.073 | 16.60 | Ethanol,2-(2-chlorothoxy) |
| 2 | 2.162 | 13.40 | Acetic acid |
| 3 | 3.634 | 17.21 | 2-Pentene |
| 4 | 3.984 | 0.56 | 2-Butene |
| 5 | 6.551 | 1.14 | 5-Methyltetrazole |
| 6 | 13.353 | 19.16 | 1-Pyrrolid-2-one,N-carbamoyl |
| 7 | 14.258 | 1.22 | L-Pipecolinic acid |
| 8 | 17.102 | 18.51 | Hexadecanoic acid |
| 9 | 18.608 | 8.91 | 1,9-Nonanediol, dimethanesulfonate |
| 10 | 18.777 | 3.29 | 4-Methyloctanoic acid |

Usually the solution extracted from plants by using solvent extraction method is the most efficient way to produce the extract in addition to its easy to be applied. The yield of the extract depend upon the polarity of the solvent used in extraction in addition to the temperature, time and the physio-chemical characters of the solvent used in the extraction process, sometimes combination of solvents used for extraction in this case the ratio of the solvents also play a important rule too. The phenolics compounds specifications varies from plant to other, it may contain polymer substance, phenolic acid and anthocyanins, furthermore, the phenolic compounds can combined with plants components like proteins, carbohydrates, sugars, fats, organoacids compounds, thus the extraction process differ from plant to another.

Extraction trials of phenolic compounds from strawberries shows that the more polyphenols can be extracted by using solvent consist of 30% water and 70% acetone which was chosen in this research. The optimum quantity of phenolic compounds extracted from fruits was 50 mg GAE/g and 180 mg GAE/g from leaves which is less than what was reported previously 2009 by Andrade[22]. Figure 3 and 4 shows the GC-MS diagrams for struberry and leaves respectively.

*Detection of biofilm production[23]*

There above mentioned routes in materials and methodes were used to detect the biofilms, those are;

Tube method by Christensen G.

Congo red agar method by Freeman DJ.

Microtitration plate method by Christensen GD.

Figure 5 shows the presence of biofilms which appear as a violet dark line on the wall and the bottom of the test tube.

*2-Congo red agar Method (CRA)*

In this method, the weak-slim were pinky colored, while the moderate were in dark color and the positive were appeared as a block color as depicted in figure 6.

*Microtitration Plate Method (TCP)*

This method was measured the tissue culture plates where no color mean indicate the biofilms were not formed while the colored indicate the formation of bacteria as shown in figure 5, OD values collected from this method can be consider as index to adhering of bacteria.

The statistics of the detected biofilms in all three methods where listed in (Table 2)

The formation of colonies was due to antimicrobials drugs resistant strains to the bacteria which lead to the formation of biofilms[24]. these strains were responsible for the contamination to various medical devices used by patients which effect the treatment time and make it longer and more complicate. Increasing the genetic production can be achieved responding *P. aeruginosa* to anaerobic circumstances; this will discompose the antibiotics wall physically.

In this study the results that have been reached, the three methods in detecting the biofilm was good, effective and high specificity, and gave a clear result for the isolates under study and this is consistent[25].

*Inhibition of biofilm formation by Strawberry extracts (fruits and leaves)*

The response of animals and plants to the infection caused by bacteria that available in nature can be occur in various ways; like managing the genetic system or activate the mechanism of the defense system. Antibiotics that prepared from natural products increases the interest of chemists and seemed to be promising as an alternate to chemical synthesis of the drugs due to the side effect of the synthesized antibiotics which make threats on human health, natural antibiotic is also requested to protect food from contamination and infection. Phenols came from plants due to metabolism were considered due to its effect on the inhibition of the production of bacteria biofilm[26].

Because of the high content of phenols in strawberry plant, this study focused on the disclosure of its effectiveness against regardless bacterial pathogens and proves the biological effectiveness of high-phenols in the inhibition of the production of biofilm of the *Pseudomonas aeruginosa* (Table 3and 4).

After comparing the results that have been reached during this study demonstrates reduced biofilm formation rate from *Pseudomonas aeruginosa* after treatment of the crude extracts each of the fruits and leaves of the strawberry plant, and this shows the effectiveness of its high phenolic compounds in reducing the formation biofilm and in turn works to reduce the colonization of bacteria, which is the first step to start the infection ,these results are consistent with the Naïma (Naïma *et al.*, 2015)[27] which reported that in his study to the medical effectiveness of phenols against microbes, and[28] was prove that some of phenolic compounds does not show any activity against biofilms e.g., rosmarinic acid this compound which formed naturally by the root of *Ocimum basilicum* L. due to infections caused by *P. aeruginosa*, this phenolic compound cannot go through the biofilms and penetrate the cell membrane under both in vitro and in vivo conditions, while the phenolic compounds extracted from Ginger (*Zingiber offinale* Rosc) shows activity against microbes and was used by Arabs, Indians and Asian people as a treatment to several infections[29].

The inhibition of *P. aeruginosa* PA14 biofilms eventually will lead to reduce c-di-GMP which results to lower the formation of polysaccharides; however it is a big challenge to find compounds that can be used as anti-bioflims that can be used universally. The phenolic compounds affected with nutrition condition, thus, and as was recommended be

Blackledge (Blackledge *et al.,* 2013)[30]. This study recommend the theory that aimed two-component regulatory system (*GacS*/*GacA)* route is commendable to control the biosynthesis of exopolysaccharides (Psl) and Quality Scheme (QS) system.

The existing of a certain compounds could be harmful to all stages to the biofilms lifestyle circulation of *P. aeruginosa* and can associated to predictable antibiotic synthesis that has activity toward infection of bacteria (Biofilm-mediated Antibiotic)[30].

One results of the table is clear and there shows no significant differences in the production of the large rate biofilm

*Evaluations of Gas Chromatography-mass Spectrometry for strawberry extracts*

Acetone extract of Fruite and Leaf sample subjected to GC-MS analysis. The chromatogram showed that both sample extract were a mixture of at least 10 compounds (Figures 7 and 8) and their effects on (Table 5 and 6) respectively. Which accounted for 95.53% of the total mass, composition of the remaining 4.47% could not be ascertained due to their low abundance.

Results collected in this research assumed that long chain phenolic compounds posses as an active antibiotc and recommended to be used in pharmaceutical synthesis in drug industry. Seaweeds is recommended as a furtheroerk due to its natural cultvability which was also recommended by Kam (Kam *et al.,* 2013)[31] and Zhang (Zhang *et al.,* 2015)[32].

**REFERENCES**


1. Julia M. Plotnikova, Laurence G. Rahme, and Frederick M. (2000). Ausubel pathogenesis of the Human Opportunistic Pathogen *Pseudomonas aeruginosa* PA14 in Arabidopsis. Plant Physiology, Vol. 124, pp. 1766–1774.
2. Tsiry R., Quentin L., Pierre D. and Mondher E (2015) The Formation of Biofilms by Pseudomonas aeruginosa: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms. BioMed Research International Volume 2015 (2015), 3-Putty M, Pseudomonas aeruginosa. The Environmental Reporter, 5(3) (2007).
3. Qing W. and Luyan Z. Ma. (2013) Biofilm Matrix and Its Regulation in *Pseudomonas aeruginosa.* Int J Mol Sci. 2013 Oct; 14(10): 20983–2100.
4. Toder, K. (2002). University of Wisconsin-Madison – Department of Bacteriology, *Bacteriology at U.W*-Madison.
5. Tadesse, A. and Alem, M. (2006). Medical Bacteriology, Ethiopia Public Health Training Initiative. September, pp.249.
6. Thomsen, T.R.; Aasholm, M.S.; Rudkjøbing, V.B.; Saunders, A.M.; Bjarnsholt, T.; Givskov, M.; Kirketerp-Møller K. and Nielsen, P.H. (2010). The bacteriology of chronic venous leg ulcer examined by cultureindependent molecular methods. Wound repair and regeneration.18: 1; p38-49.
7. Maki DG, Kluger DM, Crnich CJ. 2006. The risk of bloodstream infection in adults with different intravascular devices: a systematic review of 200 published prospective studies. *Mayo Clinic* Proceedings Journal. 81(9): 1159-71.
8. Titball, R.W. and Basak, A.K. (2006). Membrane-damaging and cytotoxic phospholipases. In: Alouf JE, and Popoff MR. eds. The Comprehensive Sourcebook of Bacterial Protein Toxins. Academic Press; pp. 516–534.
9. Aaron, S. D.; W. Ferris, K.; Ramotar, K.; Vandemheen, F.; Chan, and R. Saginur. (2002). Single and combination antibiotic susceptibilities of planktonic, adherent, and biofilm-grown *Pseudomonas aeruginosa* isolates cultured from sputa of adults with cystic fibrosis. J. Clin. Microbiol. 40:4172–4179.
10. Leigh R. and Alfred L.1973). Production and Characterization of the SlimePolysaccharide of *Pseudomonas aeruginosa*. Journal Of Bacteriology,v ol. 116, No. 2., p. 915-924.
11. Aaby K; Skrede G. and Wrolstad RE (2005): Phenolic composition and antioxidant activities in flsh and achenes of strawberries (*Fragraria ananassa*). *J Agric Food Chem* 3: 4032–4040.
12. Singleton V L and Rossi J A. (1965). Colorunetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Amer. J. Enol. Viticult. 16:144-58.
13. Nabajit Deka. (2014). Comparison of Tissue Culture plate method, Tube Method and Congo Red Agar Method for the detection of biofilm formation by Coagulase Negative *Staphylococcus* isolated from Non-clinical Isolates. *nt.J.Curr .Microbiol.App.Sci* 3(10) 810-815.
14. Rompicherla, V; Dhandapani, K.and Meenakshisundar, J. (2014). Study of biofilm production and antimicrobial resistance pattern of the bacterial isolates from invasive devices. *Int J Res Health Sci*. [Online] 2 (1). p. 274-81.
15. Shivani Saxena, Gopa Banerjee Rajiv Garg, and Mastan Singh(2014) Comparative Study of Biofilm Formation in *Pseudomonas aeruginosa*Isolates from Patients of Lower Respiratory Tract Infection.J Clin Diagn Resv.8 (5); 2014 May.
16. Cristina A.,Sara C.,José O., ElisiárioT.,Fernanda M.F,NatérciaT and GeorginaC.(2013) Development of a new gas chromatography–mass spectrometry (GC–MS) methodology for the evaluation of 5α-reductase activity. TalantaVolume 107, 30 March, Pages 154-161.
17. Mothershed, E. A. and Whitney A. M. (2006). Nucleic acid-based methods for the detection of bacterial pathogens:Present and future considerations for the clinical laboratory, *Clinica Chimica Acta*., 363:206 – 220.
18. Rodney M. and J. William C(2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. Clin Microbiol Revv.15(2).
19. Zahra Ghanbarzadeh Corehtash, Ahmad Khorshidi, ,*Farzaneh Firoozeh, Hosein Akbari, and Azam Mahmoudi Aznaveh.( 2015). Biofilm Formation and

Virulence Factors Among *Pseudomonas aeruginosa*Isolated From Burn Patients J Microbiol. Oct; 8(10): e22345.

20. Jabalameli F, Mirsalehian A, Khoramian B, Aligholi M, Khoramrooz SS, Asadollahi P, *et al*.(2012) Evaluation of biofilm production and characterization of genes encoding type III secretion system among Pseudomonas aeruginosa isolated from burn patients. Burns. ;38(8):1192–7. doi: 10.1016/j.burns.2012.07.030.
21. A bidi SH, Sherwani SK, Siddiqui TR, Bashir A, Kazmi SU.(2013). Drug resistance profile and biofilm forming potential of Pseudomonas aeruginosa isolated from contact lenses in Karachi-Pakistan. BMC Ophthalmol.;13:57. doi: 10.1186/1471-2415-13-57. [PMC free article] [PubMed] [Cross Ref].
22. Andrade, D., Gil, C., Breitenfeld, L., Domingues, F., Duarte, A.P., (2009). Bioactive extracts from Citrus ladanifer and Arbutus unedo L. Ind. Crops Prod. 30, 165–167.
23. García-Viguera C., Zafrilla P., Artés F., Romero F., Abellán P. and Tomás-Barberán F., (1998). Colour and anthocyanin stability of red raspberry jam. In: J Sci Food Agric, 78, p. 565-573.
24. Sangita Rebdewala,(2012). characterization of bacterial etiologic agents of biofilm formation in medical devices in critical care setup research article, Hindawi publishing corporation, Critical care research and practise. Volume Article ID 945805 6 pages. DOI: 10.1155/2012/945805
25. Ruzicka F *et al*. (2004). Biofilm detection and clinical significance of *Staphylococcus epidermidis* isolates. Folia Microbiol; 49(5):596-600
26. Koehn FE, Carter GT.( 2005). The evolving role of natural products in drug discovery. Nature Reviews Drug Discovery 5;4:206.
27. Naïma Guendouze-Bouchefa ; Khodir Madani ; Mohamed Chibane ;Lila Boulekbache-Makhlouf ; Didier Hauchard ; Martin Kiendrebeogo ; Caroline Stévigny ; Philippe Ndjolo Okusa and Pierre Duez(2015) Phenolic compounds, antioxidant and antibacterial activities of three Ericaceae from Algeria. Industrial and croups product.70(459-466).
28. Tiwari BK; Valdramidis VP; O'Donnell CP; Muthukumarappan K;Bourke P and Cullen P.(2009). Application of natural antimicrobials for food preservation. Journal of Agricultural and Food Chemistry 57:5987.
29. Tetz G. V., N. K. Artemenko, and V. V. Tetz, "Effect of DNase and antibiotics on biofilm characteristics,(2009).Antimicrobial Agents and Chemotherapy, vol. 53, No. 3, pp. 1204–1209.
30. Blackledge M. S.; R. J. Worthington, and C. Melander,(2013) "Biologically inspired strategies for combating bacterial biofilms," Current Opinion in Pharmacology, vol. 13, no. 5, pp. 699–706, 2013.
31. Kam A.; Razmovshi-Naumovshi.V; Nammi S.; Shi J Chan K ;and . L G.i (2013). A comparative study on the inhibitory effects of different parts and chemical constituents of pomegranate on α-amylase and α-glucosidase Phytother. Res., 27 pp. 1614-1620.
32. Zhang, B.: Deng, D.; Ramdath, Y.; Tang, P. ;Chen, R; Liu, Q. andLiu, R. (2015)Phenolic profiles of 20 Canadian lentil cultivars and their contribution to antioxidant activity and inhibitory effects on á-glucosidase and pancreatic lipase Food Chem., 172 , pp. 675-684