# ACTIVITY OF *MARTICARIA CHAMOMILLA* CRUDE AND TOTAL FLAVONOID EXTRACTS AS ANTI-VIRULENCE FACTOR FOR CLINICALLY ISOLATED *PSEUDOMONAS AERUGINOSA*

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**ABSTRACT**

**This study was aimed to examine and detect the total flavonoid presence from the crude extract use in phytochemical test and HPLC device, and detecting on both extract by UV-spectrophotometer. The objective of this study for inhibition the pyocyanin pigment that produce by *pseudomonas aeruginosa* by use *Marticaria chamomilla* crude and flavonoid extract. The extraction of chamomile was accomplished by methanol 80%, where different concentrations and dilutions of the crude and flavonoid extract and used as anti-virulence factor. 130 clinical samples of burns and wounds where collected from patients in the medical city hospital. The result showed that 44 isolates where identified as *P.aeruginosa* using the vitik 2 test, and 19 isolates that can ability to produce pyocyanin pigment, when they were sub-culturing on king A medium. Isolate No. 33 was selected are the largest produce of pyocyanin and most resistance bacteria to antibiotics, as the anti-bacterial sensitivity test was compacted using five type antibiotics (Levofloxacin, Polymyxin, Piperacillin, Imipenem, Tetracycline). the result also slowed that the flavonoid extract inhibited pyocyanin pigment better than the crude extract and the concentration of 950 mg/ml in the first dilution where the flavonoid give 28mm in diameter of inhibition zone when compare with crude extract that give 25mm in diameter inhibition zone for pyocyanin.**

**Key words: pyocyanin, flavonoid, HPLC, inhibition, pigment, isolatesl.**



**فعالية مستخلصات البابونج الخام والفلافونويدية الكلية كعامل مضاد لضراوة بكتيريا الزوائف الزنجارية المعزولة سريرياً**

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**المستخلص**

**تهدف هذه الدراسة الى الكشف عن وجود الفلافونويد الكلي باستخدام المستخلص الخام في الاختبار الكيميائي النباتي وجهاز HPLC، والكشف على المستخلصين بواسطة مقياس الطيف الضوئي بالأشعة فوق البنفسجية. الهدف من هذه الدراسة هو تثبيط صبغة البيوسيانين التي تنتجها الزائفة الزنجارية باستخدام مستخلص البابونج الخام ومستخلص الفلافونويد. تم استخلاص البابونج بواسطة الميثانول 80٪، حيث تم استخدام تراكيز وتخافيف مختلفة من المستخلص الخام والفلافونويد واستخدامها كعامل مضاد للضراوة. 130 عينة سريرية من الحروق والجروح تم جمعها من المرضى بمستشفى مدينة الطب. أظهرت النتيجة أن 44 عزلة تم تحديدها على أنها زوائف زنجارية باستخدام اختبار vitik 2 ، و 19 عزلة يمكن أن تكون قادرة على إنتاج صبغة البيوسيانين، عندما تم استزراعها على وسط King A. اختيرت العزلة رقم 33 باعتباره أكثر منتج للبيوسيانين والبكتيريا الأكثر مقاومة للمضادات الحيوية، حيث تم اختبار الحساسية المضادة للبكتيريا باستخدام خمسة أنواع من المضادات الحيوية (ليفوفلوكساسين، بوليميكسين، بيبراسيلين، إيميبينيم، تتراسيكلين). أدت النتيجة أيضًا إلى تثبيط مستخلص الفلافونويد لصبغة البيوسيانين بشكل أفضل من المستخلص الخام وتركيز 950 مجم / مل في التخفيف الأول حيث اعطى الفلافونويد قطر 28 مم من منطقة التثبيط عند مقارنته بالمستخلص الخام الذي اعطى 25 مم في منطقة تثبيط البايوسيانين.**

**كلمات مفتاحية: بايوسيانين، فلافونويد، الطيف الضوئي، الميثانول, HPLC**

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## INTRODUCTION

A proteobacteria belonging to the family Pseudomonadaceae is *Pseudomonas aeruginosa*. This bacteria was first identified in 1882 by a French chemist and bacteriologist named Carle Gessard (10). opportunistic pathogen with a wide list of real and sometimes life-threatening illnesses (17). in healthy people, the chance of infection with *P.aeruginosa* is extremely low. But for a subset of patients with compromised immune systems, particularly patients with cystic fibrosis (CF), severely burned or weakened immune systems among HIV-infected patients, or cancer patients undergoing chemotherapy, there is a slightly increased risk of *P.aeruginosa* infection (28). *P.aeruginosa* infections: the pathogenesis varies depending on the multitude of virulence factors, such as elastase, hydrogen cyanide, exotoxin A, alkaline protease, phospholipase C, exoenzyme S, pigments, rhamnolipid, and lipopolysaccharide, there are several cell-associated factors, such as flagella, lipopolysaccharide and pili that contribute to this disease as well (5). because of its color and pigment synthesis, *Pseudomonas aeruginosa* gains attention. There are many clear indicators of a newly discovered colony of *P. aeruginosa* that one may identify, including the pungent, fruity, and sometimes even musty smell produced by the action of 2-aminoacetophenone. *P.aeruginosa* frequently produces green-tinted colonies on sheep blood agar plates because of its ability to produce pigment. Without this particular non-fermenting type of Gram-negative bacteria, no other type of Gram-negative non fermenting bacteria produce pyocyanin and thus the presence of pyocyanin serves as a useful identifier (22). Pyocyanin is the first phenazine chemical found in nature which was discovered in *P.aeroginosa* at (1890), that contribute to persistence of *P.aeruginosa* cause the broad range of cellular harm that it causes, such as the suppression of cell respiration, ciliary function, epidermal cell development, and even cancer induction (23). Pyocyanin has the ability to halt the electron transport chain of fungi, including fungi that cause human disease, and demonstrates antifungal action. It is used in the textile industry to color cotton and flax textiles, in addition to its usage in agriculture (24). Several pyocyanin inhibitors have been extracted and discovered from various plants (20). Medicinal plants are vital to the health of people and communities all around the globe. They include phytoactive components with therapeutic as well as preventative effects (12). For example, Chamomile (*Matricaria chamomilla*) is a common herb used in traditional medicine; it has beneficial effects on digestive and respiratory systems, reduces oxidative stress, and has antimicrobial properties (3). The *Matricaria chamomilla recutita* in the Asteraceae family is an annual plant native to Europe and Asia. the flower of *M.chamomilla* have a number of pharmacologically use active. They include chamazulene (an anti-inflammatory), bisabolol (an antioxidant with oil, antimicrobial properties and anti-inflammatory), apigenin (a phytonutrient that acts as a potent anti-inflammatory, antioxidant, antibacterial and anti-viral) and luteolin (a phytonutrient with potential antioxidant, anti-inflammatory and anticancer activity) (25). According to those mentioned above, this study aims to study the Inhibition of pyocyanin produced from clinical isolates *P. aeruginosa* by active compounds from a plant source and this has been achieved by:

1. Extracting and separating crude and flavonoid extracts from chamomile
2. Determining the activity of crude and flavonoid extracts for the inhibition of pyocyanin virulence

## MATERIALS AND METHODS

**Collection of bacterial isolates:** A total of 130 clinical samples were collected from patients suffering from various clinical infections, including wounds and burns. Samples were collected from the Medical City Hospital for the period from october 2020 to january 2021. In order to prevent any potential contamination, all samples were carefully collected as follows:

1. Burn samples were obtained using a sterile swab from patients with first-degree burns
2. Wound sample were collected from patients by using sterile swab medium transporter

Both samples were grown on MacConkey agar and incubated for 24 hours at 37ºC, under aerobic conditions.

**Detection of bacteria**

For detection of bacteria Some biochemical tests have been done on bacteria, including catalase, oxidase, gram staim, citrate, Methyl Red Test, Voges-Proskauer H- Test and indol test. All bacterial isolate were confirmed by using the Vitek 2 system to ensure correct diagnosis of bacteria. In this identification, newly activated bacteria were cultured on MacConkey ager and incubated at 37c for 14h, after incubation period a single colony of bacteria was transferred by using a plastic loop to 3 ml normal saline. Turbidity was measured by using McFarland spectrophotometer provided as parts of the device after the turbidity of the bacteria was standardize to 0.5 McFarland. The kit was added to every single tube and placed in the device. The results were obtained after 18 h of incubation in the device.

**Preparation of Culture Media**

**MacConkey agar:** MacConkey Agar is a differential and a selective medium for the isolation and separation of gram-negative bacteria based on their lactose fermentation potential. The growth of Gram-positive species inhibited by bile salts and crystal violet. The media was prepared by suspended 51.5g in 1 liter of DW and the mixture was heated to ensure it complete dissolvation, then sterilized by autoclave at (121°C) for 15 minutes.

**Muller – Hinton agar**

This media was used for determining the antimicrobial susceptibility by using the disk diffusion method. It was prepared by suspending 38g in one liter of DW. After sterilization, medium was left to cool to 45-50°c, and then poured into petri-dishes. After solidification, plates were stored in a refrigerator at 4°C until use.

**Cetrimide agar**

This media was used for the determination of *Pseudomonas spp*. It was prepared by suspending 46.7g in one liter of D.W with the addition of 100 ml glycerol. after sterilization, medium was left to cool to 45-50°, and then poured into petri-dishes. After solidification, plates were stored in a refrigerator at 4°C until use.

**King A media:** King A medium was prepared as described in King *et al*., (18) for the detection of pyocyanin, this media prepare by disolve gelatin peptone 20.0 gm, magnesium chloride1.4 gm, potassium sulfate 10.0 gm and agar 15.0 gm in liter of distill water, and the pH was set to 7.2-7.4. the media was autoclaved at 121°C for 15 minutes to detect pyocianin pigment formed by bacteria.

**Determination of antimicrobial activity**

This test was achieved by the disk diffusion method according to the CLSI guideline. Bacteria colony was reactivated and transferred to 5ml of normal saline, and the turbidity of the bacteria was compared with the McFarland (Tube 0.5). The bacterial suspension was speared on Muller Hinton agar by using a sterile cotton swab. The antibiotic discs listed in the Table (1) where placed on the agar by a sterilized forceps, (5 discs in each plate). Plates were incubated at 37ºC for 18hr.

**Table 1. Antibiotic disc and diameter zone according to the CLSI (2020).**

| Antibiotic | Concentration (mg/disc) | Sensitive | Intermediate | Resistance |
|---|---|---|---|---|
| Levofloxacin | 5 | ≥ 22 | 15-21 | ≤ 14 |
| Polymyxin | 100 | _ | _ | _ |
| Piperacillin | 100 | ≥ 21 | 18-20 | ≤ 17 |
| Imipenem | 10 | ≥ 22 | 19- 21 | ≤ 18 |
| Tetracycline | 30 | ≥ 15 | 12-- 14 | ≤ 11 |

**Collection and drying of chamomile flowers**

The flowers of chamomile were collected from local markets between December 2020 to February 2021. The plants were authenticated by Herbarium of Biology, at the Department of Biology College of Science, University of Baghdad, and plant herbivores. The flower were washed with tap water the dried at room temperture (22-25°C) for the week, after that they were grind using an electronic miller to gain coarse powder.

**Estimation of the extraction ratio of chamomile flowers:** Using the following equation, the extraction ratio (ER) was calculated (2).

$$E.R. = \frac{\text{Concentr. of the extract \{yield\} (g)}}{\text{Initial weight of fresh leaves (g)}} \times 100$$

**Crude extract of chamomile**

soaking method was used for the extraction of chamomile dried flowers using 80% of methanol. 200 gm of dried flowers were soaking in 800 ml of methanol mix with 200 ml of D.W(13), after 36 hours the extract was filtered using whatman No.41filter paper and the filtrate was concentrated by rotary evaporator. The crude extract was kept refrigerated in a dark glass jar at -4°C(4).

**Flavonoid extraction for *M.chamomilla***

Methanol is frequently used for flavonoid extraction (29). In a grinder, the dried flowers were homogenized for 4 minutes. Then twenty-five grams of homogenized was mixed with 150 ml of methanol at 30 °C for two hours using magnetic stirrer. to obtain particle free extract, the extract was filtered using Whatman No.41 filter paper. The process was repeated for three times on the extract residuce. Finally the extracts were combined, condensed, and vacuum dried (7).

**Total flavonoid content (TFC) for chamomile:** To create the calibration curve, a methanol stock solution of quercetin (0.5 mg/ml), then the standard solutions of quercetin were prepared by serial dilutions using methanol (20, 30 and 40 µl respectively), 20 µl of the stock solution was withdrawn and then supplemented with methanol to a volume of 1 ml then the following dilutions are prepared . Preparation of the measuring solution for the extract, to a 5 ml volumetric flask containing 2 ml of 80 percent ethanol and 0.5 ml crude extract were transferred separately, 0.1 ml of 10% (w/v) ALCL3 and 0.1 ml of 1 M potassium acetate, diluted with distilled water to the mark and mixed. the absorbance was estimated at 415 nm after 30 minutes (11).

**Flavonoids Identification test**

**Ferric Chloride test:** Plant extract was mixed with Ferric chloride solution- Dark green color formed.=

**Shinoda test:** The plant extract was mixed with few drops of concentrated HCI to this mixture, pieces of magnesium were added- Pink , red, yellow, or magenta color developed.

**Zinc-HCI reduction test**: Plant extract was mixed with concentrated HCl. To this mixture, zinc dust was added A magenta color developed.

**Lead acetate test:** Plant extract was mixed with 10% lead acetate solution- Yellow precipitate obtained

**Sodium hydroxide test**: Plant extract was mixed with 10% NaOH solution- Yellow precipitate was formed.

**Alkaline reagent test**: addition of increasing amount of sodium hydroxide to the residue show yellow coloration, which discoloration after addition of acid.

**Flavones test:** The test was done by adding 10 ml of ethyl alcohol ( 50%) to 10 ml of KOH solution (50%) and then mixed with 5 ml of plant extract after filtration. The appearance of yellow color indicated the presence of flavones in plant.

**General extraction procedure for HPLC**

1 gram of dry sample was crushed into small pieces in pastel-mortar, then suspended in 200 ml of HPLC grade methanol and deionized water (80:20) M, v/v. The flavonoid and glycoside extraction was carried out using an ultra-sonicator (Branson Sonifier, USA) at 60 percent service cycles for 25 minutes at 25 degrees Celsius, followed by centrifugation at 7500 rpm for 15 minutes. Every sample's clear supernatant was subjected to vacuum evaporation (buchirotaVapor Re type). The dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing, the mixture was passed through a 2.5 µm disposable filter and stored at 4°C for further study, and then 20um of the sample was injected into the HPLC system according to the best separation conditions. (30).

**Method work**

The experiment was performed using five different concentrations (150, 350, 550, 750, and 950 mg) of plant extracts and dissolved in 1 ml DMSO solution separately, then 4 dilutions were made for each concentration by placing 9 ml of DMSO solution in each test tube, after which the dilution process occurs. The well diffusion method was used on nutrient agar cultured with the bacteria. the three replications were made for each dilution, and the results were taken after 24 hours from cultue. after the results of inhibition are

obtained, a swab is taken from the inhibition area and re-culture on the cetromid medium to ensure that only pyocyanin was inhibited instead of killing bacteria.

**Statistical analysis**

Analysis of data was done using IBM-SPSS 26 for windows in which the data was analyzed based on the comparison between means and standard deviation. ONE WAY ANOVA was used to compare between more than three groups which give the analysis in the form of P-value.

## RESULTS AND DISCUSSION

**Isolation and Identification of *P.aeruginosa***

**Isolation:** For the isolation of *Pseudomonas species* 130 clinical samples were collected from burn and wound of infected patients at the Medical City Hospital between October 2020 and January 2021, were 44 isolates beared *P.aeruginosa* .

**Phenotypic test of *P. aeruginosa***

This research includes 44 *P.aeruginosa* isolates that were identified using morphological and biochemical characterization methods. on king A media, 19 *P.aeruginosa* isolates developed green pigment and the distinctive dour. *P.aeruginosa* responded favorably to the oxidase and catalase tests, as well as the Simmon Citrate test, but not to the methyl red, indole, or Voges Proskauer tests. and using the vitik 2 system also confirmed the result which revealed that all 44 tested isolates belonged to the *P.aeruginosa*.

**Screening of *pseudomonas aeruginosa* that produces pyocyanin:** To detection on pyocyanin production by *P.aeruginosa* use king A media, were pyocyanin production was carried out using King's A Medium as described by King in 1954 (18). 19 isolated *P.aeruginosa* produced pyocyanin pigment on the media as shows in Figure 1.

**Antibiotic susceptibility test (AST) of *P.aeruginosa* :** The isolates of *P. aeruginosa* (n=44) showed different susceptibility towards 5 antimicrobial agents used, there were about 42 (95%) resist to tetracycline, 18 (40%) resist to polymyxin, 13 (30%) resist to piperacillin and 9 (20%) resist to imipenem . There was less resistance to Levofloxacin 1 (2%) than other antibiotics, 24(55%) isolates showed Multi drug resistance(MDR) to ≥ 1 agent in ≥ 4 antimicrobial categories (15), all the results of AST shows in Table 2 and Figure 2.

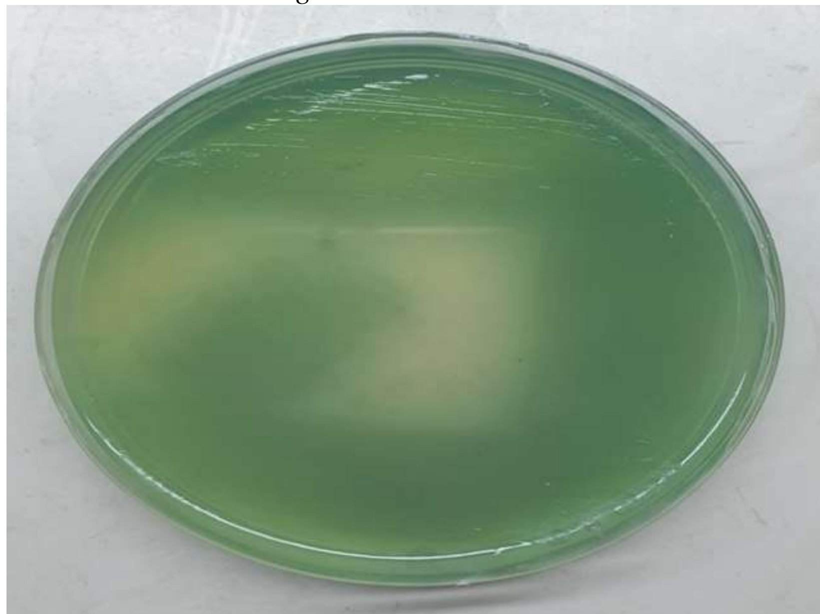

**Figure 1. *P.aeruginosa* growth on King A medium**

**Table 2. The Ratio of Antibiotics susceptibility test of *P. aeruginosa* isolates**

| Antibiotic | Resistance No.( %) | Intermediate No. (%) | Sensitive No. (%) |
|---|---|---|---|
| Levofloxacin | 1(2%) | 2(5%) | 41(93%) |
| Polymyxin | 18(40%) | 4(10%) | 22(50%) |
| Piperacillin | 13 (30%) | 6(13%) | 25(57%) |
| Imipenem | 9 (20%) | 7(16%) | 28(64%) |
| Tetracycline | 42(95%) | 2(5%) | 0(0%) |

**Figure 2. Antibiotic susceptibility test of *P. aeruginosa* isolates**

In (2009) by Vladimir Chachanidze (27) showed similar results when levofloxacin seems to have a better activity against *P.aeruginosa* than Piperacillin. The current study indicates that *P.aeruginosa* is becoming resistance to commonly used of antibiotic due to excessing consumption of antibiotics exerting selected present bacteria. The result confirmed the occurrence of MRD strains of *P.aeruginosa*, which agree with Berendonk *et al* (6) who found that the *P. aeruginosa* multi-resistance to most antibiotic. The prevalence of MDR of *P.aeruginosa* isolates was increased in Baghdad Province (1). on other hand Tumbarello and Cauda (26) determined that *P. aeruginosa* strains (66%) were identified as MDR.

**Extraction and estimation of extraction ratio for crude and flavonoid flowers extracts:** Methanol 80 percent was chosen as the extraction solvent for *M.chamomilla* since it had a high extraction capacity and excellent polarity. Raul agreed with this outcome (21). Furthermore, while the polarity of the water is strong, not all plant material can be dissolved and removed with it. As a result, 80% of methanol is made up of methanol and water at some point. The result of extraction showed that concentration of crude extracts reached 27.5 % and the concentration of flavonoid extracts reached 20.6%.

**The detection of total flavonoid content**

The detection of total flavonoid content was determined by a UV-spectrophotometer at wavelength 415 nm after calibrate it on quercetin, the result obtained is 7.66 which indicates the presence of flavonoids.

**detection on flavonoids by phytochemical test:** To detect the presence of flavonoids in chamomile extract, several botanical tests were conducted, namely Shinoda test, Ferric Chloride test, Zinc-HCI reduction test, Lead acetate test, Sodium hydroxide test, Alkaline reagent test and Flavones test, all of which gave positive results as evidence of the presence of flavonoids.

**Analysis of flavonoid by HPLC technique**

After evidence that was available for characterization of isolated chamomile and clove flavonoids, The high performance liquid chromatography (HPLC) technique was used also for characterization of isolate chamomile flavonoid sample comparing with chamomile standard according to Janmejai and Sanjay, 2009 (14) Concentration of sample μg/ml=(area of sample/area of standard)×100

The result of this characterization reveal the appearance of eight peaks of the isolated flavonoid sample as shown Figure (3-A,B). The first, second, third, fifth, seventh and eighth peaks was at retenation time (1.32, 1.83, 2.75, 5.67, 8.34 and 10.49) min respectively , which were smaller than chamomile standards , the fourth and sixth peaks were larger and at retenation time (4.73 and 7.52) min respectively, the The appearance of eight peaks of partial purified flavonoid referes to a good puring of isolated flavonoid . on the other hand , the presence of some smaller peaks of isolated flavonoid referes to presence of secondary compounds

derived from the main flavonoid , also indicates to the efficiency of extraction method Comparison of various techniques for the extraction *Matricaria chamomilla* processing (19). Table (3-A,B) illustrates the relation time for standard and chamomile

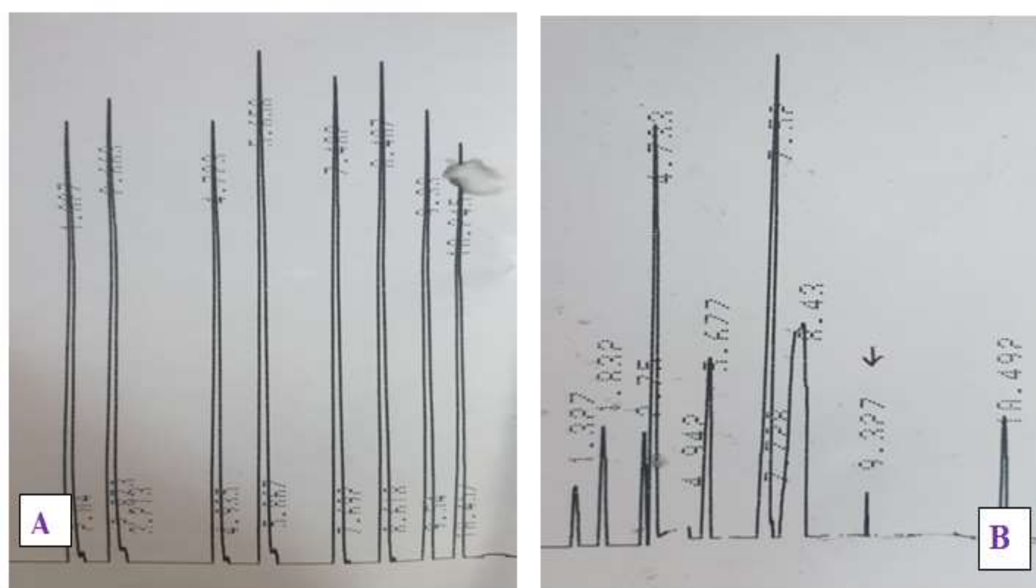


**Figure 3. HPLC for total flavonoid for chamomile extract. A: Standard total flavonoid. B:**

| Seq. | Subject | Retention time | Area uv |
|---|---|---|---|
| 1 | Apigenin-7-O-(4-acetyl) glycoside | 1.82 | 223470 |
| 2 | Apigenin-7-O-(3-acetyl) glycoside | 2.66 | 246292 |
| 3 | Apigenin-7-O-(6-acetyl) glycoside | 4.72 | 222799 |
| 4 | Luteolin | 5.65 | 261441 |
| 5 | Apigenin | 7.48 | 234143 |
| 6 | Eupatolitin | 8.40 | 232619 |
| 7 | Chrysosplenol D | 9.33 | 216639 |
| 8 | Chrysosplentin | 10.25 | 198853 |

**Separated total flavonoid samples**

**Table 3-A. the Relation time for standard HPLC for total flavonoid for chamomile**

**Table 3-B. the Relation time for HPLC for Separated total flavonoid chamomile extract**

| Seq. | Subject | Retention time | Area uv |
|---|---|---|---|
| 1 | Apigenin-7-O-(4-acetyl) glycoside | 1.327 | 23573 |
| 2 | Apigenin-7-O-(3-acetyl) glycoside | 1.832 | 58183 |
| 3 | Apigenin-7-O-(6-acetyl) glycoside | 2.75 | 53128 |
| 4 | Luteolin | 4.733 | 227123 |
| 5 | Apigenin | 5.677 | 90211 |
| 6 | Eupatolitin | 7.52 | 267699 |
| 7 | Chrysosplenol D | 8.43 | 107157 |
| 8 | Chrysosplentin | 10.492 | 55821 |

**Effect of chamomile crude and flavonoid extract on produce pyocyanin:** The activity of crude and flavonoid extracts tested against pyocyanin production was accomplished by using different concentration and dilutions of both extracts (Figure 4-A,B). This was done by identifying the range of the inhibition response of pyocyanin to both of the extracts as shows in Table (4-A) and (4-B). the first table shows that the first dilution of the 950mg/ml concentration of the crude extract give the highest inhibition zone reaching 25mm in diameter while the rest of the concentrations and dilution decreased the inhibition zone also decreased reaching zero mm in diameter at the fourth dilution of the fifth concentration. The explanation above shows in Table (4-A).

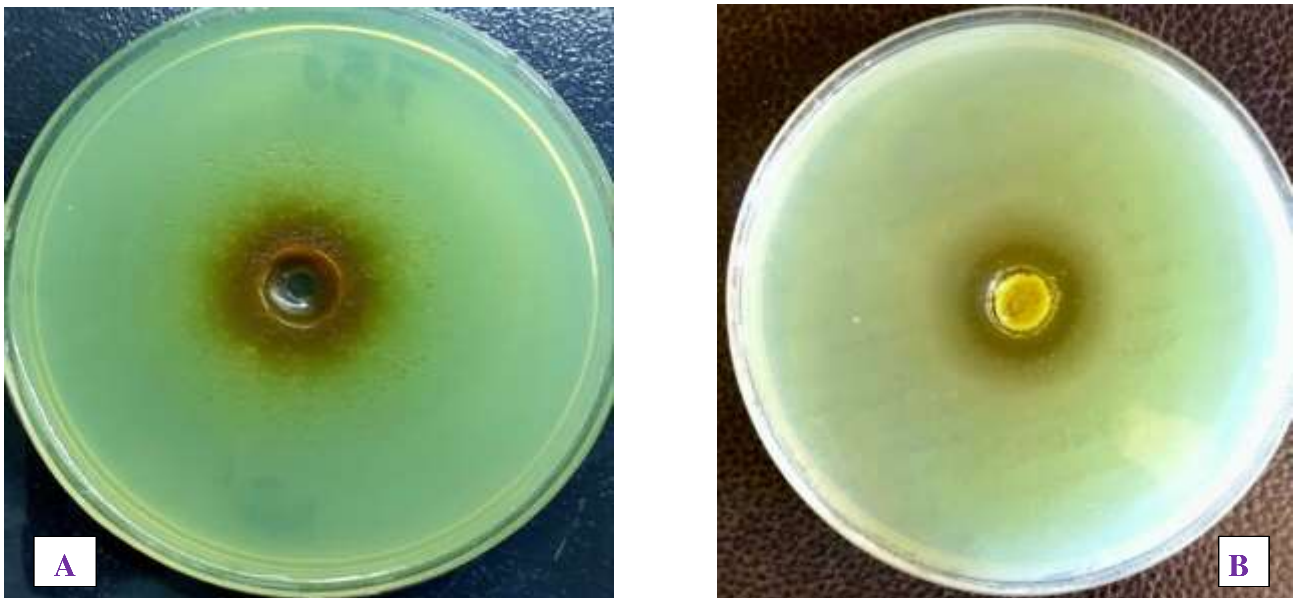


**Figure 4. Effect of chamomile extract on pyocyanin production in the 1st dilution of the 750mg/ml of concentration (A) Flavonoid extract., (B) Crude extract.**

**Table 4-A. Effect of chamomile crude extract on pyocyanin production**

| concentration of chamomile crude | Dulition 1 | Dulition 2 | Dulition 3 | Dulition 4 |
|---|---|---|---|---|
| 950 mg/ml | 25 mm | 21 mm | 17 mm | 13 mm |
| 750 mg/ml | 23 mm | 19 mm | 15 mm | 0 |
| 550 mg/ml | 20 mm | 18 mm | 14 mm | 0 |
| 350 mg/ml | 17 mm | 15 mm | 0 | 0 |
| 150 mg/ml | 15 mm | 14 mm | 0 | 0 |

the second table shows that the first dilution of the 950 mg/ml concentration of the flavonoid extract give the highest inhibition zone reaching 28mm in diameter while the rest of the concentration and dilution decreased the inhibition zone also decreased reaching zero mm in diameter at the fourth dilution of the fifth concentration. The explanation above shows in the Table (4-B). After the inhibition was done, a swab was taken from the inhibition area and replanted on citromid medium to ensure that only pyocyanin was inhibited instead of killing bacteria, and bacterial growth was observed on the medium after 24 hours shown in Figure (5).=

**Figure 5.** ***P.aeruginosa*** **growth on Cetromide**

**Table 4-B. Effect of chamomile flavonoid extract on pyocyanin production**

| oncentration of chamomile flavonoid | Dulition 1 | Dulition 2 | Dulition 3 | Dulition 4 |
|---|---|---|---|---|
| 950 mg/ml | 28 mm | 22 mm | 18 mm | 14 mm |
| 750 mg/ml | 25 mm | 20 mm | 16 mm | 0 |
| 550 mg/ml | 22 mm | 19 mm | 15 mm | 0 |
| 350 mg/ml | 20 mm | 17 mm | 14 mm | 0 |
| 150 mg/ml | 17 mm | 15 mm | 0 | 0 |

## Statistical analysis

The results of this study were analyzed by one-way Anova test to find out if there were any significant effect of crude and flavonoid extract. Moreover, to test whether the difference in means is statistically significant and if the ANOVA F-test shows there is a significant difference between the groups (8). One-way analysis of variance (abbreviated one-way ANOVA) is a statistical technique for comparing the means of two or more samples (using the F distribution). This can only be used with numerical data. (9). The values of F and p define the significance of the parameters, and the higher the value of F and the lower the value of p, the more significant (16). In all the ANOVA statistical tests, the level of significance was $p \leq 0.05$ or $p \leq 0.01$ that suggesting that the test is considered to be statistically significant .According to the Table (5), F-value was 1.082 while P- value was 0.300 for the significant differences among ability of crude and flavonoid extract for pyocyanin inhibition, This mean there is significant differences between crude and flavonoid extract for pyocyanin inhibition, because P-value is more than 0.05, This may be due to the effect of the flavonoid extract on the gene expression of lecA gene, which caused the inhibition of the pyocyanin pigment.

**Table 5. The Significant differences among ability of crude and flavonoid extract to pyocyanin inhibition**

| Type of Extract | No. of subject | Mean | Standard deviation | F - value | P- value |
|---|---|---|---|---|---|
| Flavonoid of chamomile | 60 | 14 | 8.79962 | 1.082 | 0.300 |
| Chamomile crude extract | 60 | 12.35 | 8.57676 | | |

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