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Study of the antibacterial and anti-biofilm activity of _Calendula officinalis_ extracts against antibiotic-resistant bacteria contaminated for burns and wounds in the hospitalized patients in the Medical City in Baghdad / Iraq

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دراسة النشاط المضاد للبكتيريا والأغشية الحيوية لمستخلصات الآذريون ضد البكتيريا المقاومة للمضادات الحيوية الملوثة للحروق والجروح لدى المرضى الراقدين في مدينة الطب في بغداد / العراق

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الملخص

: تعد عدوى الجروح والحروق من أهم المضاعفات السريرية التي تصيب المرضى المصابين، وقد أصبحت ظاهرة انتشار العدوى البكتيرية شائعة بين مرضى الحروق والجروح بسبب ظهور سلالات مقاومة للمعقمات والمضادات الحيوية. **الهدف:** هدفت الدراسة إلى دراسة الفعالية المضادة للبكتيريا المضادة للأغشية الحيوية لمستخلصات الآذريون ضد البكتيريا المقاومة للمضادات الحيوية الملوثة للحروق والجروح لدى المرضى الراقدين في مدينة الطب في بغداد / العراق. الطرق: تم جمع ١٠٠ عينة من مرضى الحروق والجروح في مستشفيات مدينة الطب ببغداد. أظهرت نتائج العزل الميكروبي وجود بكتيريا *P. aeruginosa* بنسبة (٢٦٪) وبكتيريا *S. aureus* بنسبة عزل (٢٥٪) وبكتيريا *Acinetobacter baumannii* بنسبة عزل (١٧٪) وبكتيريا *Enterococcus faecalis* بنسبة عزل (١٦٪). وقد خضعت جميع العزلات لاختبار حساسية المضادات الحيوية. **النتائج:** أظهرت النتائج وجود ١٤ عزلة مقاومة للمضادات الحيوية المتعددة وهي (*A. , S. aureus , P. aeruginosa, E. faecalis baumannii*). وقد خضعت هذه العزلات المقاومة لاختبار إنتاج الأغشية الحيوية. وكانت جميع مستخلصات نبات *الأذريون* فعالة في تثبيط نمو جميع العزلات البكتيرية. وتراوحت قيم التركيز المثبط الأدنى للنبات بين ٤٠٠ ملغم/مل و ١٢.٥ ملغم/مل. **المناقشة:** أظهرت المستخلصات الكحولية والأسيتونية فعالية أكبر من المستخلص المائي في تثبيط نمو العزلات البكتيرية. كما أظهرت مستخلصات نبات *الأذريون* نسبة عالية من تثبيط الأغشية الحيوية في بكتيريا *A. baumannii*، حيث بلغت نسبة التثبيط ٨١٪ عند 25) MIC ملغ / مل) في المستخلص الكحولي و (٦٧٪) عند 12.5) MIC ملغ / مل) في مستخلص الأسيتون. **الاستنتاجات:** إن المستخلصات النباتية لنبات الأذريون لها تأثير مثبط على نمو بعض البكتيريا المسببة للأمراض التي تلوث الحروق والجروح وهي (*A. ,P. aeruginosa , E. faecalis , S. aureus baumannii*) ويمكن دراسة تأثير المستخلصات النباتية على البكتيريا المسببة للأمراض الأخرى.الكلمات المفتاحية: المكورات العنقودية الذهبية، الزائفة الزنجارية، الراكدة البومانية، المكورات المعوية البرازية، *الأذريون* ، عدوى الحروق والجروح.

Abstract

Background: Infection of wounds and burns is one of the main clinical complications of infected patients. The phenomenon of the spread of bacterial infection has become common among patients with burns and wounds due to the emergence of strains that are resistant to sterilizers and antibiotics. **Aim:** The study aimed to study the antibacterial and anti-biofilm activity of calendula officinalis extracts against antibiotic-resistant bacteria contaminated for burns and wounds in hospitalized Baghdad/iraq. **Methods:** 100 samples were collected from burns and wounds patients in Baghdad Medical City hospitals. The results of the microbial isolation showed *P. aeruginosa* bacteria with a percentage of (26%), *S. aureus* bacteria with an isolation rate of (25%), and *Acinetobacter baumannii* bacteria with an isolated percentage of (17%), and *Enterococcus faecalis* bacteria with an isolation percentage of (16%). All isolates were subjected to an antibiotic sensitivity test. **Results:** The results showed 14 multidrug-resistant isolates, which are (*P. aeruginosa*, *S. aureus*, *A. baumannii*, and *E. faecalis*). These resistant isolates were subjected to a biofilm production test. All plant extracts of *Calendula officinalis L* were effective in inhibiting the growth of all bacterial isolates. The minimum inhibitory concentration values of the plant ranged between 400 mg/mL and 12.5 mg/mL. **Discussion:** The alcoholic and acetone extracts were more efficient than the aqueous extract in inhibiting the growth of bacterial isolates. Also, *C. officinalis* extracts showed a high percentage of biofilm inhibition in *A. baumannii* bacteria, where the rate of inhibition was 81% at MIC of (25 mg/mL) in the alcoholic extract and (67%) at MIC (12.5 mg/mL) of acetone extract. **Conclusions:** Plant extracts of *C. officinalis* have an inhibitory effect on the growth of some pathogenic bacteria that contaminate burns and wounds, namely *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *A. baumannii*. The effect of plant extracts on other pathogenic bacteria can be studied.Keywords: *Staphylococcus aureus, Pseudomonas aeruginosa, Acinetobacter baumannii, Enterococcus faecalis*, *Calendula officinalis*, burns and wounds infections

Introduction

Excessive use of common antibiotics contributed to hospital-acquired infections, caused by MRSA, vancomycin-resistant *S. aureus*, and *P. aruginosa*. Vancomycin-resistance enterococci VRE. Coagulase-negative Staphylococci, and *A. baumanni*, these strains resistant to most known antibiotics have contributed to the difficulty of treating diseases (Bereket *et al*. 2012). Therefore, researchers' attention turned to finding natural therapeutic alternatives by diagnosing some plant extracts and studying their effect on resistant bacteria and virulence factors, including biofilms. Biofilm is an important factor in the virulence of pathogenic and antibiotic-resistant bacteria, and it protects bacterial cells from the body's defense mechanisms, including phagocytosis by macrophages (Soto 2013).Bacteria use biofilms as a means of survival. Biofilms are composed of stable colonies surrounded by an exogenous polysaccharide matrix and affect many medical devices, facilitating their formation and causing persistent chronic infections. In order to reduce the harmful effects of pathogenic bacteria, the world turned to plant extracts because they have medicinal benefits. Plants produce secondary metabolic products such as phenols, whose broad biological effects have been proven. They are classified into several types: flavonoids, alkaloids, tannins, glycosides, saponins, coumarins, terpenes, and steroids (Campos 2018).These products were used as antibacterials and antioxidants and therefore contributed to the treatment of many diseases (Ragavan and Monisha 2015), for example. The effect of *Calendula Officinalis* extracts on the growth of Gram-positive bacteria was also shown (Çetin *et al*. 2017). *Calendula Officinalis* is considered one of the most famous medicinal plants. The part used of the *Calendula* is its orange flowers. The original home of this plant is southern Europe. The *C. officinalis* plant contains terpenes, resinous materials, bitter glucosides, volatile oils, sterols, phenols, gels, and carotenoids.The search aimed to study the pathogenic bacteria contaminated for burns and wounds in the hospitalised patients in the Medical City in Baghdad / Iraq (Burns Specialist Hospital and Martyr Ghazi Hariri Hospital for Specialized Surgery) and their resistance to antibiotics, and study the antibacterial and, anti-biofilm activity of *C. Officinalis* medicinal plant extracts against antibiotic-resistant bacteria isolates.

Materials and Methods

Isolation of bacteria The samples were transferred to the laboratory after being collected with a cotton swab from patients with burns and wounds. After 24 hours of incubation in Nutrient Broth, the samples were plated on selective media.

Cultural characteristics Using selective media, colonies were identified based on their phenotypic traits, including shape, color, texture, and odor.

Microscopic examination

After Gram staining, bacteria were studied under a compound light microscope to determine their morphology and discriminate between positive and negative bacteria. The layout, form, and Gram interaction of the separation cells were reported (MacFaddin 2000).

Biochemical tests:

Gram stain This dye was used to separate bacteria into two groups: positive bacteria (purple color) and negative bacteria (red color). This difference is caused by differences in the cell wall's composition and chemical content.

Oxidase test Oxidase is an oxidant enzyme found in the chain of respiratory enzymes responsible for oxidative phosphorus reactions that have the ability to oxidize some aromatic amines to form colored finishes. A little amount of bacteria is transferred with wooden stick and mixed on filter paper saturated with a solution of 1 percent Tetramethyl-1-pphenylene diamine. If a purple or blue tint appears soon after mixing, the bacteria have the enzyme, and the result is taken into account. And if it is not colored, the bacteria do not have it, resulting in a negative outcome (Grundmann *et al*. 1995).

Catalase test A sample of the bacterial colony is placed on top of a drop of a solution of hydrogen peroxide (H2O2 3 %). If bubbles appear, the test result is positive, and the bacteria have this enzyme; if bubbles do not appear, the test result is negative, and the bacteria do not have this enzyme.

Coagulase test This test was carried out using the slide method (Slid Coagulase test). A drop of sterile normal saline solution was applied on one side of the glass slide, according to what was indicated (Forbes *et al*. 2007). A drop of human blood plasma was obtained on the other side, and 1-2 colonies of bacteria growing on a nutrient medium were taken for 24 hours. And was added to a drop of saline solution, and the same quantity was added to a drop of human plasma at 37 °C. The result was judged positive when a clot formed within 5-10 seconds with the plasma, while the mixture remained homogeneous and devoid of lumps from the saline solution side.

Identification of bacterial isolates The bacterial isolates were identified using morphological criteria, biochemical assays, and the VITEC 2 system.

Antibiotic Sensitivity Test According to the manual on antimicrobial susceptibility testing, the antibiotic susceptibility test was performed using Kirby Bauer's disc diffusion method. Their findings were compared to those of the Clinical Laboratory Standards Institute (CLSI 2022). Bacterial isolate inoculum was generated by processing a new culture of each bacterial isolate in a saline solution adjusted to match the 0.5 McFarland turbidity standard. The bacterial suspension was streaked on Muller-Hinton agar plates using a sterile cotton swab. The plates were then left at room temperature for 3 to 5 minutes to allow excess moisture to be absorbed. Following that, antibiotic discs (Penicillin 10 µg , Gentamicin 10 µg , Tetracyclin 30 µg , Doxycyclin 30 µg , Ciprofloxacin 5µg , Levofloxacin 5 µg , Ofloxacin 5 µg , Rifampicin 5µg))were placed on the surface of the infected plate and incubated at 37°C for 18-24 hours. Following incubation, the widths of the inhibitory zones were measured in millimeters and compared to CLSI standards.

Biofilm formation test All isolates were grown in brain heart infusion broth for 24 hours at 37°C. Following that, 100 µL of bacterial growth was transferred into a tube containing 2 mL of normal saline, and the turbidity was adjusted to McFarland 0.5. Then,180 µL of brain heart infusion broth containing 1% glucose was poured into all wells, the first three wells were considered as a negative control. A sterile, flat-bottomed 96-well polystyrene microtiter plate in the rest of the wells was filled with a volume of 20 µL of bacterial suspension (made from normal saline). The plates were sealed with their lids and incubated for 24 hours at 37°C under aerobic conditions without shaking. All plates were gently cleaned with distilled water three times after incubation, then dried. Then, 200 µL of methanol was added to each well for the fixation of the biofilms, which was done after 15 min at room temperature, washing, and air drying, then, 200 µL of a 0.1% crystal violet solution was used to stain the plates for 15 minutes at room temperature. In addition, wells were cleaned and dried for about 30 minutes at 37 degrees Celsius. Then, 200 µL of absolute ethanol and 1:1 glacial acetic acid were used to resolubilize the dye for 10 minutes. Using a microtiter plate reader, the optical density (OD) of each well was determined at 600 nm. Three standard deviations more than the mean OD of the negative control were designated as the cutoff OD (ODc). All isolates were classified into four groups Table(1) based on the ODC value: non-productive, weak biofilm producer, moderate biofilm producer, and strong biofilm producer (Jaffar *et al*. 2016).**Table 1. Interpretation of biofilm production**

OD value	Biofilm intensity
OD* ≤ ODc*	Non-producer
ODc < OD ≤ 2X ODc	Weak

2X ODc < OD ≤ 4X ODc	Moderate
OD > 4X ODc	Strong

*** OD = Optical Density**

***ODc = Three standard deviations above the mean OD of the negative control**

Preparation of Plant Extraction

Calendula officinalis flowers and leaves were purchased from local markets, washed with distilled water, dried in the dark, and ground into fine powders using an electric household blender. Then, 40 g was weighed, transferred separately into conical flasks containing 160 mL of distilled water, 95% ethanol, and acetone for the aqueous, alcoholic, and acetone extracts, and then soaked for 72 hours in a shaking incubator. A muslin cloth was used to filter the extracts from each solvent before using Whatman No. 1 filter paper. In order to obtain a crude extract, which was kept at 4 °C in flasks, the solvents were first removed from the filters by air drying them in an oven for three days at 40 °C. Extraction during the preparation of concentrated alcoholic and acetone extracts. It was dissolved in dimethyl sulfoxide (DMSO) (Atya *et al*. 2018). Subsequently, the concentrates (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 mg/mL) were prepared.

Determination minimum inhibitory concentration of plant extract using Microtiter Plate Assay (MTP)

The antibacterial activity of plant extracts (aqueous, alcohol, and acetone) was tested using minimum inhibitory concentration (MIC) against bacteria (Elshikh *et al*. 2016). MIC was determined on a 96-well microtiter plate using the resazurin microdilution technique in Muller-Hinton broth. (MHB) as follows:

1- Medium (MHB) was prepared at double concentration. 2- Preparing the test material: a. Aqueous extract of *C. officinalis L* b. Alcoholic extract of *C. officinalis* L c. Acetone extract from *C. officinalis* L. d. Positive control (bacterial suspension only). e. Negative control (MHB only). 3- A100 µL of double-strength Muller-Hinton broth was distributed from the first to the 12th well in each raw sample in a 96-well round-bottom microtiter plate. 4-A 100 µL of raw plant extracts were pipetted into the test wells of each microtiter plate, and they were thoroughly mixed with the broth. Concentrations were (400, 200, 100, 50, 25, 12, 5, 6, 25, 3.12, 1.56, 0.78 mg/mL). A sterile pipette was used to transfer 100 µL of the first well's mixture to the second well, where it was thoroughly combined. Again, 100 µL was transferred from the second to the third well and thoroughly stirred to ensure uniformity. All the way up to the eleventh well, this process of repeated dilution was adhered. 5- 100 µL of the mixture was transferred from the second well to the third well and thoroughly mixed. This serial dilution was carried on to the 11th well. Finally, 100 µL was removed from each of the eleven wells and discarded. The final concentration of plant extract in each well was now one-half of the initial concentration. The 12th well in each raw was left as a control positive (plant extracts -free control well). 6- All wells were inoculated with 100 µL of bacterial suspension equivalent to McFarland standard no 0. 5 ($1{,}5 \times 10^8$ Cfu/ml) .The microtiter plate underwent 24 hours of incubation at 37 C and a further 4 hours of incubation at 37 C. Resazurin (0.015%) was added to all wells in the amount of 30 µl and the color change was then observed after another 2-4 h of incubation. Color alterations (from blue to pink) were seen and noted. The Minimum Inhibitory Concentration was the lowest value prior to the change in color (MIC).

Anti-biofilm effect of plant extracts

All isolates were cultured for 24 hours at 37°C in brain heart infusion broth. A tube containing 2 mL of ordinary saline was then filled with 100 µL of bacterial growth, and the turbidity was adjusted to McFarland 0.5. A180 µL of brain heart infusion broth containing 1% glucose was then poured into each well, in the first three wells serving as a negative control. In a sterile microtiter plate containing 96-well flat-bottomed, then 20 µL of the bacterial suspension of the isolates adjusted to 0.5 McFarland was added to the wells (4, 5, 6), which was cosidered as a positive control. Then the rest wells were filled with 10 µL bacterial suspension after being adjusted to 0.5 McFarland and 10 µL of aqueous, alcohol, and acetone plant extracts were added. The plates were covered with a lid and incubated for 24 hours at 37°C. After the incubation, non-adherent cells were removed by dipping each plate in distal water three times. Then 200 µL of 99% methanol was added to each well to stabilize the biofilms, after 15 min of washing and air drying after that stain the plate with 200µL of 0.1% crystal violet solution for 15 min, then the wells were washed with distilled water and dried at 37 °C for 30 min. then added 200 µL of ethanol, after 10 min measured at 600 nm using ELISA reader (Jaffar *et al*. 2016).The percentage of biofilm inhibition was determined by the formula: (Shinde *et al*. 2021).

Biofilm reduction % = (OD control - sample OD / control OD) x 100%

Statistics Analysis

Software called SPSS V.23 was used to do the data analysis. The significant differences between values, the standard error and one Sample T-Test.

RESULTS AND DISCUSSION

Identification of Bacterial Isolates

A total of 100 clinical samples of burns and wounds were collected for both male and female patients of different age groups. From June 2022 to August 2022 , samples were collected from burns and wounds patients at hospitals in Baghdad (Burns Specialist Hospital and Martyr Ghazi Hariri Hospital for Specialized Surgery). Samples were taken by passing and rotating a cotton swab, and then the samples were taken to the laboratory, to isolate and grow bacteria, they were cultured on plates of nutrient agar (NA) and MaCconkey agar to stimulate bacterial growth. The plates were then incubated at 37°C for 24–48 hours under aerobic conditions. The different colonies were then reseeded on fresh plates to obtain pure isolates.After the samples were incubated in a nutrient broth medium for 24 hours at 37 °C, the initial diagnosis of developing bacterial isolates depending on the phenotype and staining with Gram stain and biochemical tests Was performed to allow the growth of bacterial colonies. Thirty samples were free of bacterial growth, while 70 samples gave bacterial growth. . Fifty-six bacterial isolates were obtained from them, (15) bacterial isolates (*P. aeruginosa*) with a percentage of (26%), (14) bacterial isolates (*S. aureus*) with an isolation rate of (25%), and (10) bacterial isolates (*A. baumannii*) with an isolated percentage of (17%), and (9) bacterial isolates (*E. faecalis*) with an isolation percentage (of 16%), and (5) bacterial isolates (*Proteus* spp) with an isolated percentage(8%), and (3) bacterial isolates (*Klebsiella* spp) with an isolated percentage (5%).

Biochemical Tests

In the Oxidase test Bacterial isolates The change of color after mixing to purple or blue indicates a positive result and that the bacteria possess this enzyme, but the lack of color change indicates a negative result. This test was conducted to distinguish *P. aeruginosa* bacteria that were positive as shown in Table (2) for this test from other races belonging to Enterobacteriaceae. In the Catalase test that directly formed the air bubbles indicating positive testing and not being bubbles indicative of the negative test this test was used to distinguish the positive *Staphylococcus* as shown in Table (2) from negative carotids for this test. In the Coagulase test was conducted to identify the ability of bacteria to release the enzyme coagulation occurred,the thrombosis during 5-10 seconds with plasma while the mixture remains homogeneous and free of lumps on the side of the saline solution this test was conducted to distinguish the pathogenic S. aureus as shown in Table (2) from the rest of the other non *P. aeruginosa* . The results of biochemical tests for all bacterial isolates are clarified in table (2).

Table 2. Biochemical Tests of bacterial isolates

NO.	Bacterial Isolates	Oxidase test	Catalase test	Coagulase test
1	***Staphylococcus aureus***	-	+	+
2	***Enterococcus faecalis***	+	-	-
3	***Pseudomonas aeruginosa***	+	+	-
4	***Acinetobacter baumannii***	-	+	-

(+) Positive result, (-)Negative result

Identification of Bacterial Isolates Using the Vitek-2 Compact System

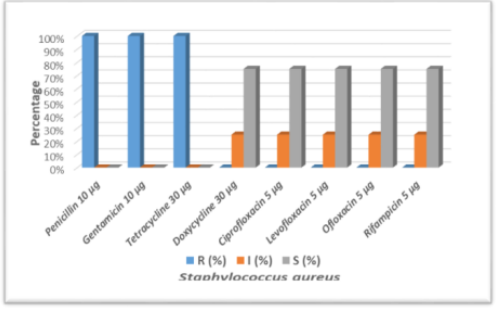
The Gram-negative and Gram-positive cards of the VITEK 2 system were utilized to guarantee the identification of the bacterial species. 56 bacterial isolates were determined to belong to four distinct species using this approach of identification. The results of the VITEK 2 system concur with those of the biochemical tests conducted on the pathogenic isolates and displayed in the results of the Vitek 2 examination showed different types of pathogenic bacteria . The results of VITEK 2 system agree with the obtained results of the biochemical tests applied for the pathogenic isolates.

Antibiotic Sensitivity of Bacterial Isolates

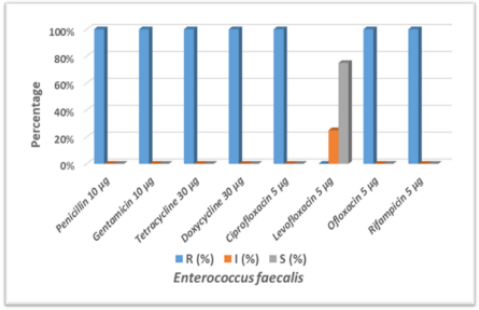
Fifty-six bacterial isolates underwent sensitivity tests for different classes of antibiotics by disc diffusion method (Kirby-Bauer method) according to the recommendations (Bauer et al. 1966) and Clinical Laboratory Standards Institute (CLSI) 2022. Pathogenic bacterial isolates showed different levels of resistance. and sensitivity pattern to eight different antibiotics. The most resistant (multi-drug resistant) bacterial isolates were selected , (4)isolates from P. aeruginosa, (4) isolates from S. aureus , (3) isolates from E. fecalis, and (3) isolates from A. baumannii, and antibiotic sensitivity test results were obtained for these resistant bacterial isolates as shown in

Figure(1). MDR (multi-drug resistance) in bacteria is associated with bacterial enzymes as well as AmpC-lactamases (Chakraborty et al. 2011). Gram negative pathogens have an increased capacity to produce altered receptors for antimicrobial agents and enzymes to destroy antibiotics, as well as increased resistance to metabolic pathways (Parajuli et al. 2017). Bacteria that have become resistant to the antibiotics to which they were previously sensitive is known as antimicrobial resistance (AMR). Antimicrobial resistance is a natural phenomenon that is exacerbated by frequent and unnecessary exposure to antimicrobials (WHO 2016).The result of this study showed that isolates of S. aureus had a rate of resistant to (Penicillin, Gentamicin, and Tetracycline) 100% as shown in Figure (1,a). These antibiotics belong to different groups, where penicillin belongs to the group of penicillins that contain the β-lactam ring. S. aureus resistance to penicillins is due to its secretion of the enzyme penicillinase or beta-lactamase, which is encoded by the gene (blaZ). β-lactam converts it to the inactive form (beta-lactam inactive).Finkel et al. (2009) reported that there are other causes of resistance, for example, the occurrence of spontaneous mutation in deoxynucleic acid (DNA) as a result of insertion, deletion, or substitution of one or more nucleotides within the genome of the microorganism, or decreased permeability of the antibiotic through the cell membrane Penicillin-binding proteins (PBPS) are enzymes involved in building the bacterial cell wall in addition to maintaining their appearance. Therefore, it is recommended here that it is necessary to use clavulanic acid, which acts as an inhibitor of the beta-lactamase enzyme, as this acid binds to these enzymes, turning them into the inactive form, thus protecting the antibiotics, which are the basis for these enzymes. Staphylococcus aureus resistance to gentamicin was an approach to study (Manyahi 2022).The reason for resistance to tetracyclines is due to the bacteria's possession of the R factor (R factor), which reflects their inability to synthesize this antibiotic. While the results of tetracyclin resistance do not agree with the study (Raheem et al. 2022). It recorded a sensitivity rate of 65% for tetracyclines, which indicates the emergence of resistant strains to this antibiotic. S. aureus isolates were intermediate by 25% and sensitive to the antibiotic (Doxycycline, Ciprofloxacin, Levofloxacin, Ofloxacin, and Rifampicin) by 75%.

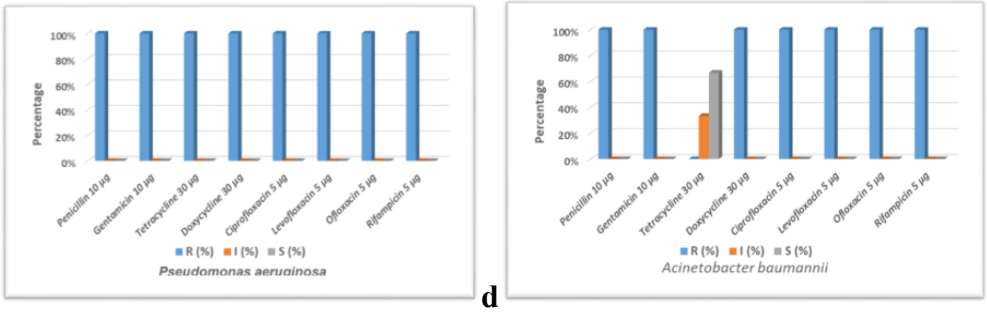
E. faecalis isolates were %100 resistant to antibiotics (Penicillin, Gentamicin, Tetracycline, Doxycycline, Ciprofloxacin, Ofloxacin, Rifampicin) as shown in Figure (1.b), while they were intermediate by 25% and sensitive to the antibiotic Levofloxacin by 75%. According to our findings, the resistance of E. faecalis was at its peak. However, broad-spectrum antibiotics like tetracycline are not recommended for treating E. faecalis infections. Many factors contribute to antibiotic resistance in developing countries, including poor antibiotic usage strategies and inappropriate antibiotic use, particularly in the treatment of E. faecalis infections (Del Rosario et al. 2008).The results proved that the resistance rates of P. aeruginosa bacteria were high to most antibiotics, as the percentage of their resistance was 100% to the antibiotic (Penicillin, Gentamicin, Tetracycline, Doxycycline, Ciprofloxacin, Levofloxacin, Ofloxacin, Rifampicin) as shown in figure (1.c). P. aeruginosa possesses multiple resistance mechanisms, including permeability of the outer membrane, Efflux System, Alteration in the target site, and the presence of some virulence factors that increase the virulence of P. aeruginosa.Acinetobacter baumannii isolates were100% resistant to antibiotics (Penicillin, Gentamicin, Doxycycline, Ciprofloxacin, Ofloxacin, and Rifampicin) as shown in Figure (1.d), while they were 25% intermediate to the antibiotic Tetracycline and sensitive by75% . Due to its incredible capacity to develop antimicrobial resistance, A. baumannii has emerged as one of the pathogens with the greatest impact on contemporary healthcare. Many clinically available antibiotics are ineffective against a number of strains of A. baumannii. The resistance mechanisms used by A. baumannii include β-lactamases, enzymes that modify aminoglycosides, efflux pumps, permeability flaws, and modifications to target sites. The number of antibiotic classes that can be used in clinical practice to treat A. baumannii infections has gradually decreased as a result of the accumulation of numerous resistance mechanisms in this organism (Lin and Lan 2014).



a



b



c d

Figure 1. The Percentage Antibiotic susceptibility test results of the bacterial isolates, a. S. aureus, b. Enterococcus faecalis, c. Pseudomonas aeruginosa, d. Acinetobacter baumannii

Test the Ability of Bacteria Isolates to Form A Biofilm The results of testing the ability of the fourteen bacterial isolates mentioned in Table (3) to form biofilm ranged from moderate to strong. According to the results shown in Figure (2), the isolates of S. aureus consist of a moderate biofilm ratio of (75%), while those that form a strong biofilm were proportional to (25 %).The isolates of E. faecalis that formed moderate biofilms had a percentage of (75%), while the isolates that formed strong biofilms had a percentage of (25%).For isolates of P. aeruginosa bacteria, the percentage of isolates that formed moderate biofilms was (66%) and formed strong biofilms by (25%). A. baumannii isolates that form moderate biofilms appeared in the proportion (66%) and strong biofilms in the proportion (25%).

Table 3. Test the production of biofilm for bacterial isolates

NO.	Bacterial Isolates	Moderate OD mean ± SE	Strong OD mean ± SE	P-value
1	*Staphylococcus aureus*	0.846±0.846	1.178±0.018	0.000*
2	*Enterococcus faecalis*	0.526±0.035	1.169±.071	0.000*
3	*Pseudomonas aeruginosa*	0.656±0.021	1.528±0.106	0.000*
4	*Acinetobacter baumannii*	0.771±0.031	2.332±0.056	0.000*

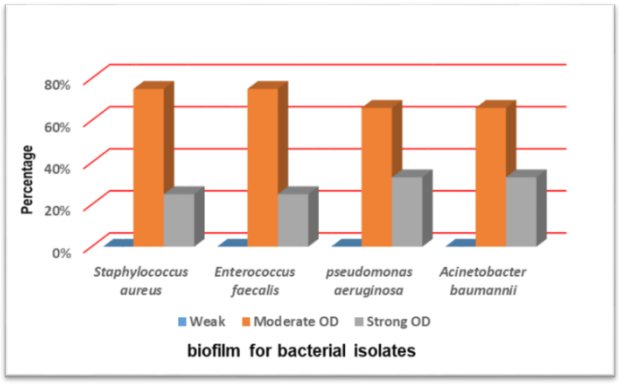


Figure 2. The Percentage of the production biofilm for bacterial isolates

To screen biofilm formation, a microtiter plate (MTP) was used. Bacterial adherence to abiotic surfaces can be observed quantitatively using formation only on the outside. A 96-well flat microtiter plate is used to incubate bacteria for examination. Following incubation, plankton bacteria are removed by washing and clinging. The color crystal violet is used to stain bacterial biofilms (Coffey and Anderson 2014).Such variation may be a result of the genetic diversity of these isolates as well as the expression of their quorum- sensing and biofilm genes. One of the most crucial factors determining bacterial isolates virulence is the production of biofilms, which aid in bacterial survival. Once formed, biofilms are very challenging to get rid of and effectively act as barriers to antimicrobial treatments (Bahador *et al*. 2019). Four of the strongest biofilm-producing bacterial isolates were selected. The ability to form biofilms is classified according to OD values (600 nm) according to the following equations as the Table (3).

Antibacterial Activity of Plant Extracts *Calendula officinalis*

The microtiter plate colorimetric method was used to test the antibacterial activity of four isolates belonging to four different species including two strains of Gram-positive bacteria (S. *aureus* and *E. faecalis*) and two strains of Gram-negative bacteria (*P. aeruginosa* and *A. baumannii*). Finding the minimum inhibitory concentration of bacteria using aqueous, alcohol, and acetone extracts of *C. officinalis*. Using the following concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78) mg/mL.

Resazurin's 96-well-based microdilution method was used to determine the minimum inhibitory concentration of surfactants to test for antibacterial activity. The term minimum inhibitory concentration (MIC) is defined, which is a critical measure of the effectiveness of an antimicrobial agent. This method was used because it is standardized, accurate, economical, and easy to use. The micro-dilution method is standard, accurate, economical in, performancee and easy to use (Reller *et al*. 2009). It is better than the disc dispensing method for obtaining normal product test results because the disc dispensing method can only give indicative results for the inhibition area. In addition, some technical issues, such as the polarity of the natural compound, which may affect the degree of diffusion, may be a factor in the inaccuracy of these methods (Sánchez and Kouznetsov 2010).In the case of lactating sophorolipids with resazurin dye added as a redox indicator, the active bacteria convert non-fluorescent resazurin (blue) to fluorescent resorufin (pink), which can then be converted to hydroresorufin, providing an accurate indicator of the metabolic activity of the bacteria. The color change can be seen visually.

The antibacterial activity of *Calendula officinalis* for *Staphylococcus aureus* bacteria

The results showed in Table (4) that all aqueous, alcoholic, and Estonian of the plant extracts were effective in inhibiting the microbial growth of *S. aureus*. In *C. officinalis* alcoholic extract was superior to the aqueous and acetone extract of the *C. Officinalis* plant, where the value of MIC in the alcoholic extract was at a concentration of (25 mg/mL), while the MIC for aqueous and acetone extract was at a concentration of (50 mg/mL). This result is in agreement with (Çetin *et al*. 2017). The discrepancy in the effectiveness of plant extracts in the *C. Officinalis* plant against bacteria is due to the different polarity of the solvents used in the extraction (Ak *et al*. 2021), and this difference affects the solubility of some active substances present in the plant such as flavonoids, phenols, saponins and alkaloids with nucleo-inhibiting effects bacteria. The superiority of the alcoholic extract over the aqueous and acetone extract in inhibiting bacterial growth may be due to the ability of ethyl alcohol to dissolve many active substances dissolved in alcohol and in other polar and non-polar solvents, especially the essential oils found in the *C. officinalis* plant (Janssen *et al*. 1986)**Table 4. Determination of Minimum Inhibitory Concentration (Microtiter Plate Method -MTP) For *Staphylococcus aureus* bacteria**

NO.	Concentration mg/mL	aqueous	alcohol	acetone
1	400			
2	200			
3	100			
4	50	MIC		MIC
5	25		MIC	
6	12.5			
7	6.25			
8	3.12			
9	1.56			
10	0.78			

The antibacterial activity of *Calendula officinalis* for *Enterococcus faecalis* bacteria The results showed lowest inhibitory concentration against bacteria using plant extracts of *C. officinalis* as shown in Table (5), the MIC of the aqueous extract was (100 mg/mL) but in the alcoholic extract it was (50 mg/mL) while the MIC of the acetone extract was (400mg/mL). Previous results showed that the alcoholic extract had the best effect on *E. faecalis*. These results agree with (Efstratiou *et al*. 2012). The values of the minimum growth inhibitory concentrations for bacteria vary, and this may be due to the possibility of precipitation of the largest possible amount of active compounds during the extraction process, or because of the presence of these active compounds in plants, but at low concentrations.**Table 5. Determination of Minimum Inhibitory Concentration (Microtiter Plate Method -MTP) For *Enterococcus faecalis***

NO.	Concentration mg/mL	aqueous	alcohol	acetone
1	**400**			**MIC**
2	**200**			
3	**100**	**MIC**		
4	**50**		**MIC**	
5	**25**			

6	12.5			
7	6.25			
8	3.12			
9	1.56			
10	o.78			

The antibacterial activity of Calendula officinalis for Pseudomonas aeruginosa bacteria

The results in Table (6) showed that the MIC of the aqueous, alcoholic, and acetone extract of C. officinalis of the bacterium p. aeruginosa was (25 mg/mL), and this means that the three extracts had the same effect on the bacteria p. aeruginosa, confirming that the active compounds have activity on bacteria, the antibacterial activities are related to the total phenols and total flavonoids present in the C. officinalis plant, where there is a significant correlation between the phenolic structure and antibacterial activity (Cushnie and Lamb 2005).

Table 6. Determination of Minimum Inhibitory Concentration (Microtiter Plate Method -MTP) For pseudomonas aeruginosa

NO.	Concentration mg/mL	aqueous	alcohol	acetone
1	400			
2	200			
3	100			
4	50			
5	25	MIC	MIC	MIC
6	12.5			
7	6.25			
8	3.12			
9	1.56			
10	0.78			

The antibacterial activity of Calendula officinalis for Acinetobacter baumannii The results in Table (7) indicate that the effect of water and acetone extract of C. officinalis on A. baumannii is better than that of alcohol extract. The MIC was for the aqueous extract and acetone at a concentration of (12.5 mg/mL), while the MIC for the alcoholic extract was at a concentration of (25 mg/mL). The solubility of the active compounds in C. officinalis such as phenols is characterized by their ability to form hydrogen bonds with water molecules, which increases their water solubility. Acetone is an organic solvent that can dissolve many organic compounds, including phenols, which bind to the active sites of cellular enzymes via hydroxyl groups that can form hydrogen bonds with those sites (Vuolo et al. 2019), inhibiting important metabolic activities carried out by these enzymes such as growth, reproduction, and protein synthesis These results are in agreement with previous studies. The resistance between Gram negative and Gram-positive bacteria can be attributed to differences in cell membranes. The outer membrane of Gram-negative bacteria creates highly moist surfaces, while the lipophilic ends of fatty acids in the cell membrane of Gram-positive bacteria may facilitate the penetration of hydrophobic compounds, and the group of aromatic compounds is known to have significant antibacterial activity. It was discovered that the latter inhibits the synthesis of amylase and proteases, destroys the cell wall, and causes cell lysis (Shukla et al. 2017).(Mustafa et al. 2018) report that terpenoids, alkaloids, and phenolic chemicals, which are components of an antimicrobial plant extract, interact with microbial cell membrane enzymes and proteins to inactivate them, allowing protons to flow out of the cell and possibly killing microbes or suppressing essential enzymes.**Table 7. Determination of Minimum Inhibitory Concentration (Microtiter Plate Method -MTP) For Acinetobacter baumanni**

NO.	Concentration mg/mL	aqueous	alcohol	acetone
1	400			
2	200			
3	100			
4	50			
5	25		MIC	
6	12.5	MIC		MIC
7	6.25			

8	**3.12**			
9	**1.56**			
10	**0.78**			

The Anti-biofilm Activity of *Calendula officinalis* Extracts The biofilm activity of the four biofilm-forming bacterial isolates was vigorously tested using MIC concentrations of aqueous, alcohol, and acetone extracts of *C. officinalis*, and the percentage of biofilm inhibition was calculated using the equation. Bacterial growth was determined after a 24 hours incubation period at 37 °C by measuring absorbance at OD 600 nm using a microplate (ELIZA) reader. Minimum inhibitory concentration (MIC), is the minimum concentration of extracts that inhibit visible bacterial growth (optical method), and significant change in absorbance compared to the positive control (Shinde *et al*. 2021).Anti-biofilm *Calendula officinalis* Extracts Testing for *Staphylococcus aureus* The effect of inhibitory concentrations on biofilm formation was evaluated based on the MIC of *S. aureus*. The results were as shown in Table (8). In *C. officinalis* extracts, alcoholic extract recorded the highest percentage of biofilm inhibition by 64% at a concentration (50 mg/mL), while the percentage of biofilm inhibition in acetone extract was 54% at a concentration (50 mg/mL), and the inhibition rate in the aqueous extract was 42% at concentration (25 mg/mL). These results are consistent with previous studies (El-Ganiny *et al*. 2017).The presence of these differences in the effect of the extracts may be due to the solubility of the active substances in the plant in the three solvents of water, alcohol, and acetone, where flavonoids have an effect in making the plant extract effective in its anti-biofilm activity for the treatment of *S. aureus* because these compounds can inhibit the adhesion and transport of proteins and damage the bacterial cell membrane. Several studies have shown that phenolic acids have effects on the cell wall and cell membrane breakdown in Gram-positive bacteria.Teethaisong *et al*. (2018) reported the extract of *Boesenbergia rotunda* L. (Chinese keys/Chinese ginger) can damage the cell membrane and the peptidoglycan layer of the cell wall, causing lactam-resistant *S. aureus.* to leak out intracellular substances.Table 8. Anti -biofilm activity of the MIC *Staphylococcus aureus*

NO.	extract	MIC mg/mL	Inhibition Percentage(%) M±SE	P-value
1	**aqueous**	**50**	**0.630±0.132 (42%)**	**0.070**
2	**alcohol**	**25**	**0.392±0.029 (64%)**	**0.002***
3	**acetone**	**50**	**0.505±0.065 (54%)**	**0.012***

*** ODC= 1.105 If * (P≤0.05), the results are significant**

Anti-Biofilm Activity of *Calendula Officinalis* Extracts Testing for *Enterococcus Faecalis*

Table (9) shows that the acetone extract gave the highest percentage (33%) in biofilm inhibition n *E. faecalis* at (400 mg/mL) concentration, while the biofilm inhibition rate in the aqueous extract was (7%) at concentration (100 mg/mL) and in the alcoholic extract (16%) at a concentration (50 mg/mL).The acetone extract of *C. officinalis* had a rate of biofilm inhibition in *E. faecalis* and it was higher for the rest of the aqueous and alcoholic extracts because the concentration was high which is (400 mg/mL). Algburi *et al*. (2017) showed that concentrated volatiles known as essential oils are inherently hydrophobic, this has become a popular tool used in combating biofilm formation, along with nanoparticles and antibiotics there is a significant relationship between phenolic composition and antibacterial activity. Among the active compounds in *C. officinalis* are quercetin, an antibacterial molecule that can inhibit lipase production, and d-alanine ligase which occurs in peptidoglycan production. In addition, there are antibacterial phenolic compounds such as rutin epicatechin and procyanidin B2.**Table 9. The anti-biofilm activity of the MIC *Enterococcus faecalis***

NO.	extract	MIC mg/mL	Inhibition Percentage(%) M±SE	P-value
1	**aqueous**	**100**	**1.079±0.043 (7%)**	**0.176**
2	**alcohol**	**50**	**0.979±0.053 (16%)**	**0.071**

3	acetone	400	0.774±0.078 (33%)	0.037*

***ODC = 1.169 If * (P≤0.05), the results are significant**

Anti-Biofilm Activity of *Calendula Officinalis* Extract Testing for *Pseudomonas Aeruginosa*

The results in Table (10) in the extracts of *C. officinalis* showed that the percentage of inhibition of the alcoholic extract (46%) at the concentration (25 mg/mL) of the biofilm of *P. aeruginosa* was the highest between the aqueous extract and acetone, while the percentage of inhibition was the biofilm of bacteria in the aqueous extract (15%) at concentration (25 mg/mL) and in acetone extract (20%) at concentration (25 mg/mL). These results are consistent with previous studies (ElGaniny *et al*. 2017). Mierziak *et al*. (2014) indicated The presence of saponins in *C. officinalis* plant have the ability to break down the plasma membrane of bacterial cells.**Table 10. The anti-biofilm activity of the MIC of *Pseudomonas aeruginosa***

NO.	extract	MIC mg/mL	Inhibition Percentage(%) M±SE	P-value
1	aqueous	25	0.559±0.225 (15%)	0.688
2	alcohol	25	0.352±0.082 (46%)	0.064
3	acetone	25	0.528±0.082 (20%)	0.244

ODC 0.664 If * (P≤0.05), the results are significantAnti-biofilm Activity of Calendula officinalis Plant Extract Testing for Acinetobacter baumannii The results in Table (11) in C. officinalis extracts showed that the alcoholic extract had the highest biofilm inhibition rate in A. baumannii (81%) at a concentration (25 mg/mL), while the biofilm inhibition rate in the aqueous extract was (28%) at a concentration (12.5 mg/mL) and in acetone extract the rate of biofilm inhibition was (67%) at a concentration (12.5 mg/mL).It is clear from the above that the extracts of C. officinalis show the best effect on A.baumannii among other types of bacteria (S. aureus, E. faecalis, P.aeruginosa). This difference is due to the mechanism of resistance of the types of bacteria studied to these extracts.**Table 11. The anti-biofilm activity of the MIC of Acinetobacter baumannii**

NO.	extract	MIC mg/mL	Inhibition Percentage(%) M±SE	P-value
1	aqueous	12.5	0.2470±0.028 (28%)	0.073
2	alcohol	25	0.064±0.012 (81%)	0.002*
3	acetone	12.5	0.113±0.057 (67%)	0.056

***ODC 0.346 If * (P≤0.05), the results are significant**

ConclusionsIt can be concluded from the previous results in this study that the aqueous and alcoholic extracts of *Calendula officinalis* have an inhibitory effect on the growth of some types of pathogenic bacteria (multi-drug resistant) that contaminate burns and wounds, namely *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa* and *Acinetobacter baumannii*. It can be concluded that the aqueous, alcoholic and acetone extracts of *C. officinalis* have an inhibitory effect on the growth of the biofilm of some types of pathogenic bacteria (multi-drug resistant) contaminating burns and wounds, namely *S. aureus, E. faecalis, P. aeruginosa* and *A. baumannii*. Alcoholic extract was the most efficient in inhibiting the biofilm of bacterial isolates (*S. aureus, P. aeruginosa*, and *A. baumanni*), followed by acetone, while the aqueous extract was the least efficient in inhibiting the biofilm.

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