

# Inhibition of *Staphylococcus epidermidis* Biofilm Formation in Response to Some Purified of Phytochemical Antimicrobial Materials.



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## ABSTRACT

*Staphylococcus epidermidis* was a Gram-positive bacteria, belong to the Micrococcus family, non-motile and non-spore forming. The single cells show under the microscope in circular (cocci) shapes with a diameter ranging from 0.5-1.5 µm. Generally. To evaluate the effect of some phytochemical substances as antibiofilm agents. Isolation and identification of *S. epidermidis* from different clinical sources using conventional bacteriological tests and automated system, Antimicrobial susceptibility test using disk diffusion according to Clinical Laboratory Standards Institute (CLSI), Detection of biofilm formation using phenotypic method and testing the ability of some phytochemical substances on biofilm production using modified crystal violet and MTT stains, and The combination of phytochemical compounds with antibiotic using Checkerboard technique against *S. epidermidis* could improve the susceptibility of this bacteria toward these antibiotics to treat infections resulted from multidrug-resistant bacteria, A total of 164 clinical specimens were collected from AL-Ramadi city during the period from August 2021 to December 2021 and were taken from surgical wounds, blood, stones of kidney, eyes, and skin. Specimens Patients' ages ranged from 15 to 60 years old, with both male and female patients participating. Specimens were cultured on blood and manitol salt agar for isolation and identification of bacteria. By using biochemical tests revealed that the bacteria was positive for the gram stain, and Catalase test, but negative for the Coagulase, Fermentation of manitol, and Hemolysin tests and by using Vitek compact system GP-2. In this study there are 24 (14.63%) positive samples and 140 (85.37%) negative cultures for *S. epidermidis* isolates. Biofilm measured by microtiter plate assay (MtP), 24 isolates (100%) are capable of producing biofilm, 12(50%) of the isolates formed strong biofilm, 10(41.66%) formed moderate biofilm, while 2(8.34%) formed weak biofilm. The inhibitory effects of Gallic acid, Cinnamic acid, Salicin, Linoleic acid, and Erythromycin, and were tested against biofilms of *S. epidermidis*. The phytochemical compounds and antibiotics solution significantly inhibited the initial cell attachment of the *S. epidermidis* but were less inhibitory at 8 h incubation in preformed biofilms formed on polystyrene surface, the inhibition of *S. epidermidis* preformed biofilm was increased with increasing incubation time except for efficacy of Erythromycin. However, there was a synergistic effect between Erythromycin with Salicin, Gallic acid by using Checkerboard technique.

## Introduction

*Staphylococcus epidermidis* was a regular element of the common human microbiota and frequently found on the skin and mucous membranes. *S. epidermidis* was able to establish a lifelong commensal relationship with the host by connecting to tissue surface molecules via specific adhesions. Starting early in human existence [1].

The coagulase-negative staphylococci group's most frequently isolated member was *S. epidermidis*. By lacking the ability to manufacture coagulase, this group can be distinguished from *S. epidermidis*. Due to its prominence as the leading cause of nosocomial infections. *S. epidermidis* has attracted a lot of attention recently *S. epidermidis* was long thought to be comparatively harmless, however it is now widely acknowledged to be a pathogen. *S. epidermidis* must be categorized as opportunistic since it requires a susceptible host in order to change from a usual resident of human skin to an infectious agent [2]. *S. epidermidis*

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propensity to build biofilms and pathogenicity are what contribute most to its long-term persistence [3]. Biofilm is an extracellular polymer-encased, non-transient microbial aggregation that adheres to surfaces. In developed biofilms, proteins, polysaccharides, and extracellular DNA are frequently present [4]. The creation of biofilms is a crucial component of staphylococcal pathogenicity and proliferation. *Staphylococcus* can persist as a commensal bacterium because it adheres firmly to host proteins in the skin [5]. Phytochemical Compounds are chemical compounds created by plants, typically to enhance their resistance to diseases from fungus, bacteria, and plant viruses. They have been employed as poisons and traditional medicines; it is a large group of biologically active natural compounds with important pharmaceutical and nutritional characteristics [6]. These entire compounds act on biofilm by six main mechanisms like substrate deprivation, membrane disruption, and binding to adhesion complex and cell wall; bind to proteins; interact with eukaryotic DNA, and blocking viral fusion[7].

## Experimental

### Sample collection

From August 2020 to December 2021, 164 Samples were collected from 2 hospitals in Al- Anbar: Al-Ramadi Teaching hospital, Al-Ramadi Maternity and Children hospital and outpatients from private clinics, these samples included: Eye, skin, Kidney stone, Surgery operation swab.

### Ethics and Approval Committee of Specimens collection

All study techniques that involve Specimens collection were approved by the Ethical Approval Committee, University of Anbar, Al-Ramadi, Iraq.

### Isolation and identification of *S. epidermidis*

Each specimen was cultured on 5% human blood agar, then it was incubated at 37°C with aerobic conditions. After 24 hours, the bacteria that grew on blood agar were transferred to manitol salt agar to detect *S. epidermidis*. Morphological identification of *S. epidermidis*, including the Gram Stain, Catalase Test, Coagulase test, producing hemolysis, the isolates were recognized as *S. epidermidis*. As well as using

automated identification by (VITEK-2 system).

### Antibiotics Susceptibility Test

The disk diffusion testing was used in this study. Eleven antibiotic agents (disks) were tested against *S. epidermidis* they are: Erythromycin, Cefoxitin, Ciprofloxacin, Clindamycin, Gentamicin, Rifampin, Chloramphenicol, Nitrofurantoin, Penicillin, Tetracycline and Trimethoprim- Sulfamethoxazole. For basic procedural steps, the Kirby-Bauer method was adopted with takes all variables into account [8]. The Clinical and Laboratory Institute's standards were followed for interpreting the results (Clinical and Laboratory Institute Standards 2021).

### Detection of Biofilm Production by *S. epidermidis*

In this study, the Microtiter plate (MtP) assay was used to determine biofilm production by a microplate reader using 96-well sterile flat-bottomed polystyrene microtiter biofilm method, which is a quantitative method described by [9].

### Determination of Minimum Inhibitor Concentration (MIC)

Minimum inhibitory concentration (MIC) of antimicrobial solutions (Erythromycin) and phytochemical compounds (Cinnamic acid, Gallic acid, Linoleic acid, Salicin) were evaluated by Resazurin Microtiter-plate Assay (REMA).

### Synergism between Erythromycin and Salicin, Gallic acid

Checkerboard assay was used to see if two test materials together in microtiterplates had an antibacterial effect. The use of the Checkerboard approach to combine Erythromycin and Salicin, Galic acid.

### Determination of Biofilm Inhibitory Activity of Phytochemical Compounds and Some Antibiotics Solution

#### A-Inhibition of Initial Cell Attachment

The effect of phytochemical compounds and some antibiotics solution on initial cell attachment of biofilm formation was estimated according to [10]. In two separated microtiter plates, solutions of test materials (equivalent to 0.25 × MIC, 0.5× MIC, 1 × MIC, and 2 ×

MIC) were prepared. Biofilm production was measured using the modified crystal violet assay (CV) and metabolic activity was measured using the MTT assay.

### B- Inhibition of Preformed Biofilm

The effect of phytochemical compounds and some antibiotics solution on biofilm maturity and formation was estimated according to [10]. Biofilms were allowed to form for 24 hours before the addition of test materials. The plates were incubated for 8 hours, 12 hours, 16 hours, 20 hours, and 24 hours. After incubation, biofilms were measured for biomass attachment using the modified CV assay, and MTT assays.

### Biofilm Biomass Assay

Assessment of cell attachment for *S. epidermidis* isolates was achieved by using the modified crystal violet (CV) assay described by [11]. The absorbance was measured at 595 nm using a microplate reader. The mean absorbance (OD<sub>595</sub> nm) was used to calculate the biomass formation inhibition percentage for each concentration of the test materials based on the following equation:

$$\text{Percentage inhibition} = 100 - \left[ \frac{\text{OD}_{595 \text{ nm experimental well with test material}}}{\text{OD}_{595 \text{ nm control well without test material}}} \times 100 \right]$$

### Biofilm Metabolic Activity Assay

The metabolic activity of the biofilms development by *S. epidermidis* was assessed using MTT assay according to [12]. The absorbance was then measured at 570 nm using the microplate reader [13].

## Results and discussion

### Isolation of *S. epidermidis*

Among 164 samples, only 24 (14.63%) were related to *S. epidermidis*, and 140 (85.37%) were related to other types of microorganisms, no growth was seen in the other samples. The distribution of *S. epidermidis* isolates was: wounds 2(8.3%), stones of kidney 3(12.5%), eyes 5(20.8%), skin 14(58.4%). The result of this study inconsistent with result obtained by [14]. Who found the prevalence of *S. epidermidis* was 29.4% in wound. However, other studies have indicated that, the prevalence rate of *S. epidermidis* in eye and wounds may reach 17.2%, 27.5% respectively [15].

### Identification of *S. epidermidis*

The isolates could belong to *S. epidermidis* and specific biochemical tests, were performed for additional verification. All isolates were showed a positive outcome for catalase, and gram stain positive, negative results appear in the following biochemical tests, coagulase and hemolysin. To confirm the isolates belong to *S. epidermidis* was used automated Vitek 2 system use GP-ID cards which contained 64 biochemical tests. The results demonstrated that all 24 (100%) isolates were confirmed, this technique was characterized by fast detection of bacteria.

### Susceptibility to Antibiotics

The result of this study showed the highest resistance to Penicillin (100%), Erythromycin (100%), Cefoxitin (100%), Tetracycline (100%), Trimethoprim-sulfamethoxazole (75%), Gentamycin (41.68%), Rifampin (58.32%), Nitrofurantoin (25%), Clindamycin (54.16%), Ciprofloxacin (12.51%), and Chloramphenicol (0%). In this study, results showed high resistance (100%) to each of the Penicillin, Cefoxitin, Erythromycin, and Tetracycline, which is higher than the results recorded by the study in Iran where the resistance of *S. epidermidis* to each of Penicillin (95.6%), Cefoxitin (85%), Erythromycin (82.6%), and Tetracycline (91.3%) [16]. Three significant mechanisms for resistance to  $\beta$ -lactam antibiotics in Gram-positive bacteria are primarily caused by: bacterial  $\beta$ -lactamase enzymes, change of main target of antibiotics with Penicillin-Binding Protein (PBP2a) and permeability modification [17]. Resistance to tetracycline occurs due to alteration of the *tetM* gene or by the efflux pump that is encoded with the *tetK* gene [18]. The Erythromycin resistance in *Staphylococcus* is caused due to the presence of particular genes: *ermA*, B and C or by efflux pump that is encoded with *msrA* gene [19].

### Detection of Biofilm Production by *S. epidermidis*

Biofilm formation was tested in 24 isolates; results showed that some isolates produced strong, moderate, and weak biofilms. The characterization of *S. epidermidis* isolates varied between strong 12(50%), moderate 10(41.66%), and weak 2(8.34%). These results

are consistent with those of Harris *et al.* (2016) found that isolated *S. epidermidis* (100%) produced biofilms due to their ability to adhere to any surface. These adherent cells are frequently embedded within a self-produced matrix of extracellular polymeric substance. Bacterial biofilms are an essential virulence factor with a vital role in the pathogenesis of bacteria; it is essential due to increased resistance to host defenses, which promotes microbial survival and growth [20].

### Determination of Minimum Inhibitor Concentration by Using (REMA) Method

The MIC results of antibiotics solution and Phytochemical Compounds are shown in table 1.

Table 1: MIC of antibiotics solution, and Phytochemical Compounds

inhibitors	MIC
Cinnamic acid	0.312 mg/ml
Erythromycin	2.5 mg/ml
Gallic acid	2.5 mg/ml
Linoleic acid	5 mg/ml
Salicin	0,625 mg/ml

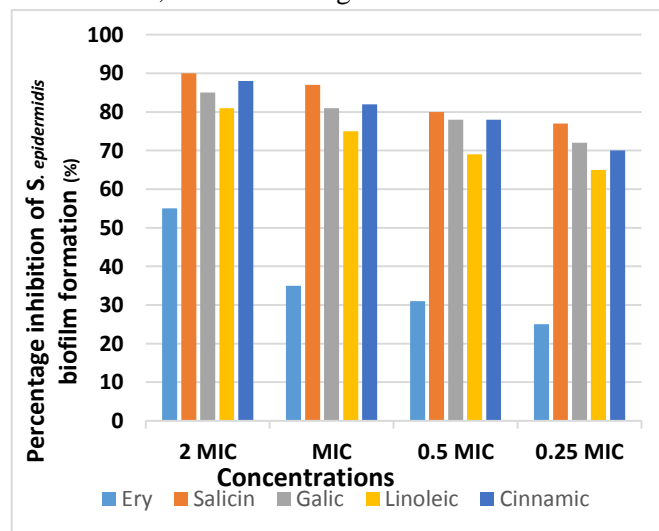
### Evaluation the Effect of the Combination of Phytochemical Compounds and Antibiotic Solution Using Checkerboard Technique

The emergence of resistant bacteria has reduced the efficacy of conventional antibiotics resulting in a need to find alternative strategies to cope with infections resulting from drug-resistant bacteria [21]. Checkerboard assays of *S. epidermidis* gave synergistic profiles when Erythromycin was combined with Salicin, and Gallic acid. The MIC values for Erythromycin (2.5 mg/ml), Salicin (0.625 mg/ml), and Gallic acid (2.5 mg/ml). FICI values when Erythromycin was combined with Salicin (0.0468), and FICI values when Erythromycin was combined with Gallic acid was (0.0117). FICI values less than 0.5 indicate a synergistic effect between the tested materials. Synergistic interaction of the natural compounds with the already available antibiotics is an effective way to deal with the resistance phenomenon. Synergism is a term applied if the combined therapeutic effect of two compounds is more than their individual effects. Previous findings demonstrated from this study are that the combination between Erythromycin and other materials exerts synergistic effects evaluated as metabolic activity reduction and restores sensitivity to

Erythromycin in Erythromycin-resistant strains of *S. epidermidis*. So Synergistic interactions of herbal drugs and phytochemicals with antibiotics and other clinically important drugs is a recent and effective tool for the management of resistant bacteria. The development of alternative antibiotics and the discovery or development of adjuvants are among the potential strategies proposed to increase or restore antimicrobial efficacy against multidrug-resistant bacteria [22]. Due to the very difficult to discover new antibiotics with the pace with which microbes are developing strategies to combat antibiotics was used checkerboard technique is one approach to tackle the resistance capability of the microbes and make them susceptible to already available antibiotics [23].

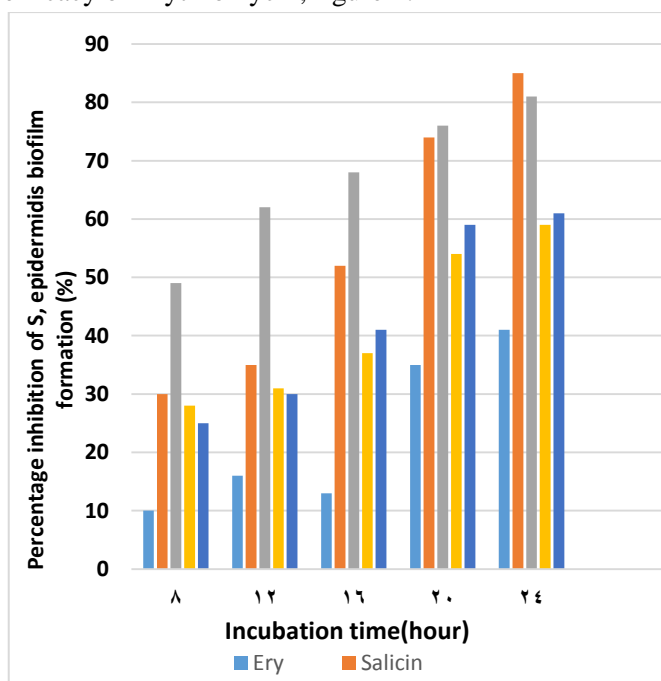
### Antimicrobial Activity Against Sessile Cells Determination of the Antibiofilm Effect Against Biomass in *S. epidermidis* Biofilms

In order to anti-biofilm activity of some antibiotics, phytochemical compounds and effects were tested on both the initial cell attachment and performed (24h) biofilms. Modified CV assay indicated that the effect of the Phytochemical compounds and antibiotic solutions on biomass attachment exceeds 70% (percentage inhibition) 2 MIC for all test materials except Erythromycin, which was 55% due to high resistance of *S. epidermidis* isolates to Erythromycin, also at the MIC and 0.5 MIC the inhibition was above 50% except for Erythromycin was under 50%. Even at 0.25 MIC, initial cell attachment was reduced but not like inhibition of 2 MIC or MIC, as shown in figure 1.



**Figure 1: Result of various concentrations of antibiotics, Phytochemical Compounds on initial cell attachment of *S. epidermidis*, shown as Percentage inhibition of *S. epidermidis* biofilm formation (%).**

However, it does not achieved complete inhibition of cell attachment despite using 2 MIC of antibiofilms. Overall, the use of Phytochemical Compounds to modify biofilm formation sites makes them unsuitable for attachment and appears to be a useful method of dealing with microbial adherence [24]. The antibiotic solution and phytochemical compounds were tested against a preformed biofilm. Biofilm formation involves an initial reversible (weak) attachment phase followed by an irreversible (strong) attachment phase [25]. The results showed that the MIC of inhibitors was used against *S. epidermidis* preformed biofilm (24 h) and tested for 8h, 12 h, 16 h, 20 h, and 24 h incubation, which was estimated by the CV assay, we observed that Percentage inhibition of *S. epidermidis* preformed biofilm was increased with increasing incubation time except for efficacy of Erythromycin, figure 2.



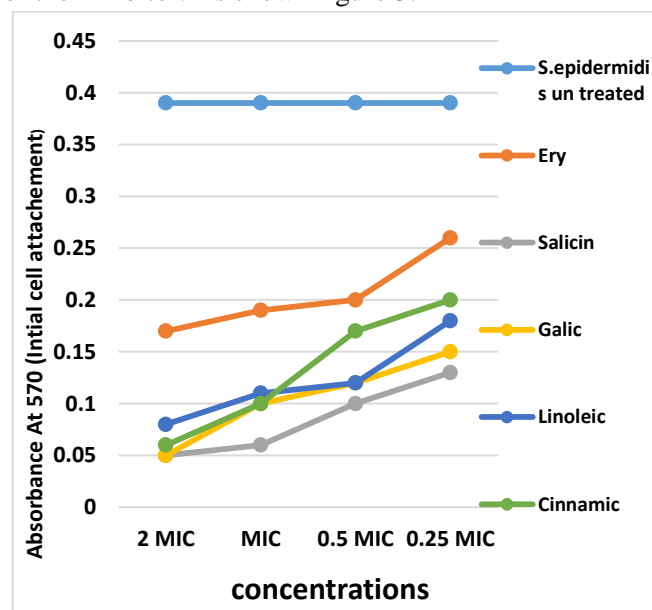
**Figure 2: Percentage inhibition of *S. epidermidis* biofilm formation (%) on 24h preformed biofilm of *S. epidermidis*.**

This observed resistance of a preformed biofilm can be associated with the presence of extracellular

polysaccharide layer in the biofilm, which may repel the entry of antimicrobials, or to the compact three-dimensional arrangement of a mature biofilm that may hinder the entry of these compounds into the biofilm. Another factor that may contribute to this increased resistance is that most antimicrobial compounds are more effective against actively growing cells. The cells in a biofilm have a poor growth rate due to a lack of nutrients and oxygen, which may reduce the antimicrobial effects of compounds against them [10].

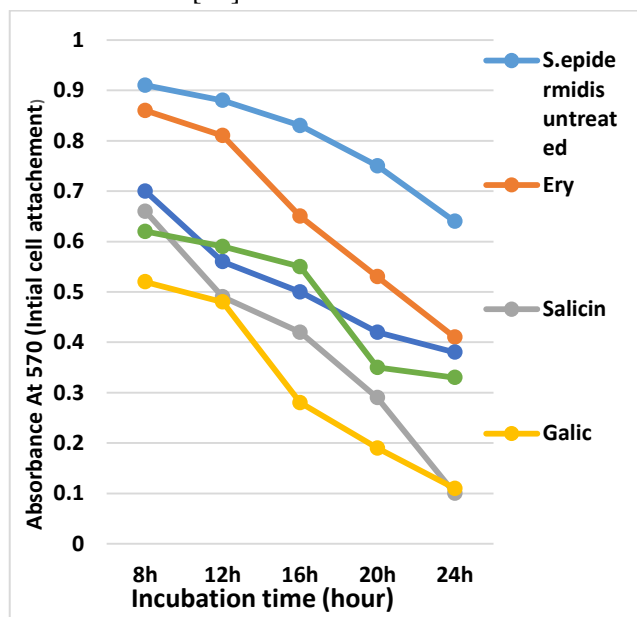
### Determination of the Antibiofilm Effect Against the Metabolic Activity of *S. epidermidis* Biofilms

MTT assay was used as an indicator of attached viable cells, while CV stains both viable and non-viable cells that may be attached. Only living cells can reduce MTT (thiazolyl blue tetrazolium bromide) into a colorful compound that can be calorimetrically measured. So, the MTT assay indicates only the living cells based on their metabolic activity [26]. The results of the MTT assay confirmed that the antibiotic solution and phytochemical compounds significantly inhibited metabolic activity of the biofilms formed by *S. epidermidis*. MTT test results showed the highest anti-adhesion activity at 2 MIC, inhibition begins to decrease as the concentration of each antibiofilm decreases, least inhibition was at 0.25 MIC. Due to the low concentration of the inhibitor, so to inhibit biofilm formation we need a high concentration of the inhibitor. As shown figure 3.



**Figure 3: Effect of antibiotics, Phytochemical Compounds on the metabolic activity of *S. epidermidis* initial cell attachment at different concentration of test materials.**

However, in the case of preformed biofilms the antibiotics, and Phytochemical compounds inhibited the metabolic activity of *S. epidermidis* at MIC. The metabolic activity suppression was found to increase with increased time of exposure, thus the activity being highest at 24 h exposure. As shown figure 4. Natural products, mainly phytochemicals, as anti-biofilm agents have been studied more in in vitro and in vivo conditions, but not a single FDA-approved drug was developed despite huge efforts. Most of them failed in phase II and phase III clinical trials [7]. The possible reason for this failure may be the availability of the compound in humans after administration which decreases the efficacy of the compounds. One possible solution to this problem is a combination of strategies like antibiotics, along with natural anti-biofilm agents for better results [27].



**Figure (4): Effect of antibiotics, Phytochemical Compounds on the metabolic activity of preformed biofilm cells of *S. epidermidis* incubated for 8h, 12h, 16h 20h, and 24h, as determined by the MTT assay. Metabolic activity of the antibiotics, Phytochemical Compounds treated cells was inhibited considerably as compared to the untreated control cells.**

### Conclusion

This work reports *S. epidermidis* prevalence rate

(14.37%) from 164 clinical specimens in Al-Ramadi city. The current research found that all 24 *S. epidermidis* isolates formed biofilm ranging between weak, moderate, and strong. The data showed that *S. epidermidis* strains were high resistant to Penicilin and Cefoxitin. This increases the possibility prevalence of the *mecA* gene responsible for resistance to these antibiotics between *Staphylococcus* strains. The Phytochemical compounds have antibacterial activity against the sessile phase of biofilm in antibiotic-resistant *S. epidermidis*. The combination of Phytochemical compounds with antibiotic using Checkerboard technique against *S. epidermidis* could improve the susceptibility of this bacteria toward these antibiotics to treat infections resulted from multidrug-resistant bacteria.

### Funding Sources

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## تثبيط تكوين الأغشية الحيوية للمكورات العنقودية البشرية في الاستجابة لبعض المواد الكيميائية النباتية المضادة للميكروبات

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### الخلاصة:

المكورات العنقودية البشرية هي بكتيريا موجبة الجرام، تنتمي إلى عائلة المكورات الدقيقة، غير متحركة وغير مكونة للأبواغ. تظهر الخلايا المفردة تحت المجهر بأشكال دائرية (مكورات) يتراوح قطرها بين 0.5-1.5 مايكرومتر. عموماً، تقييم تأثير بعض المواد الكيميائية النباتية كعوامل مضادة للأغشية الحيوية. عزل وتحديد المكورات العنقودية البشرية من مصادر سريرية مختلفة باستخدام الاختبارات البكتريولوجية التقليدية والنظام الآلي، واختبار الحساسية المضادة للميكروبات باستخدام الانتشار القرصي وفقاً لمعهد معايير المختبرات السريرية (CLSI)، والكشف عن تكوين الأغشية الحيوية باستخدام الطريقة المظهرية واختبار قدرة بعض المواد الكيميائية النباتية. على إنتاج الأغشية الحيوية باستخدام صبغات البنفسج البلوري المعدلة وصبغات MTT، كما أن مزج المركبات الكيميائية النباتية مع المضادات الحيوية باستخدام تقنية الشطرنج ضد المكورات العنقودية البشرية يمكن أن يحسن قابلية هذه البكتيريا تجاه هذه المضادات الحيوية لعلاج الالتهابات الناتجة عن البكتيريا المقاومة للأدوية المتعددة. تم جمع 164 عينة سريرية من مدينة الرمادي خلال الفترة من آب 2021 إلى كانون الأول 2021 وتم أخذها من الجروح الجراحية والدم وحصوات الكلى والعين والجلد. تراوحت أعمار المرضى في العينات من 15 إلى 60 عاماً، بمشاركة المرضى من الذكور والإناث. تم زراعة العينات على أوساط الدم وملح المانيتول لعزل وتشخيص البكتيريا. وباستخدام الاختبارات الكيموحيوية أظهرت أن البكتيريا موجبة لصبغة الجرام واختبار الكاتليز، ولكنها سلبية لاختبار التخثر وتخمر المانيتول والهيمولايسين وبواسطة استخدام نظام Vitek المضغوط GP-2. يوجد في هذه الدراسة 24 (14.63%) عينة إيجابية و140 (85.37%) مزرعة سلبية لعزلات المكورات العنقودية البشرية. تم قياس الأغشية الحيوية بواسطة مقياس لوحة العيار الميكروي (MTP)، 24 عزلة (100%) قادرة على إنتاج الأغشية الحيوية، 12 (50%) من العزلات شكلت بيوفيلم قوياً، 10 (41.66%) شكلت بيوفيلم معتدلاً، بينما شكلت 2 (8.34%) بيوفيلم ضعيف. تم اختبار التأثيرات المثبطة لحمض الجاليك وحمض السيناميك والساليسين وحمض اللينوليك والاريترومايسين ضد الأغشية الحيوية لبكتيريا المكورات العنقودية البشرية. تمنع المركبات الكيميائية النباتية ومحلول المضادات الحيوية بشكل كبير الارتباط الخلوي الأولي للمكورات العنقودية البشرية ولكنها كانت أقل تثبيطاً عند حضانة 8 ساعات في الأغشية الحيوية المشكلة مسبقاً والمتكونة على سطح البوليسترين، وتم زيادة تثبيط الأغشية الحيوية المشكلة مسبقاً للمكورات العنقودية البشرية مع زيادة وقت الحضانة باستثناء الفعالية من الاريثرومايسين. ومع ذلك، كان هناك تأثير تآزري بين الاريثرومايسين مع الساليسين وحمض الغاليك باستخدام تقنية الشطرنج.

**الكلمات المفتاحية:** المكورات العنقودية البشرية، الأغشية الحيوية، التثبيط، المواد الكيميائية النباتية.