Original Article

Effect Of Shikimic Acid And Acetylsalicylic Acid On Biofilm Formation In Staphylococcus Epidermidis

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Abstract

The aim of the current study was to evaluate the activity of Shikimic acid (SA) and Acetylsalicylic acid (ASA) against S. epidermidis biofilms formation in different conditions. A total of (231) samples were collected from patients who attended different hospitals in Ramadi and Faluja cities during the period from December(2020) to the end of July (2021). Samples were cultured on blood, and manitol salt agar and identification was performed by the VITEK 2 system. Antibiofilm activity of SA was evaluated by two methods, modified crystal violet, and MTT assay. The SA was tested in five MIC (0.25 MIC, 0.5 MIC, MIC2, MIC, and 4 MIC) and was tested during different incubation periods (2 hr, 4 hr, 6 hr, 12 hr, and 24 hr), Shikimic acid gave highest percentage inhibition of biofilm formation was 93% at 12hr and ,94% at 24hr , while Acetyl salicylic acid was gave 85% at 12hr and 91% at 24hr incubation period . Then evaluated the synergistic activity of SA and ASA acid with ceftriaxone and azithromycin against biofilm formation also then determination of the antibiofilm activity of SA and ASA acid against bacteria adherence to Foley balloon latex catheter (FBLC) pieces by REMA assay.In addition, determine the efficacy of SA and ASA acid against cell adherence using murine mammary adenocarcinoma cell line (amn3)cells. The results showed high percentage inhibition of biofilm formation as the concentration of SA and ASA increased . In conclusion, SA and ASA have the potential to be further developed as a new antitumor agent due to their impressive antibiofilm action against bacteria. shikimic acid , acetyle salicylic acid were gave up regulation of atlE gene , 2 (−ΔΔCT) was (2)for shikimic acid while (3)for acetyl salicylic acid , aap,icaA genes, all antibiofilm agents were gave down regulation result 2 (−ΔΔCT) was (0.2)for shikimic acid while (0.1)for acetyl salicylic acid.

1-INTRODUCTION

A vital part of the normal human flora, Staphylococcus epidermidis aids in preventing the invasion and colonization of potentially harmful microbial infections. The great incidence of S epidermidis on epithelial surfaces and its capacity to colonize prosthetic medical devices, however, have made it a major opportunistic pathogen[1]. It is a facultative anaerobic, non-spore-forming, Gram-positive, non-motile, catalase-positive, and coagulase-negative organism. Antibiotic classes such tetracyclines, aminoglycosides, cephalosporins, fluoroquinolones, penicillins, and macrolides are only a few that S. epidermidis strains often show resistance to[2,3].

The impact of biofilms on human health and medicine is enormous. A significant contributor to the contamination of medical equipment and the development of infectious and chronic diseases in the body is bacterial biofilm. In reality, since they induce severe infections and are resistant to antimicrobial medications, biofilms are the cause of a number of human disorders. Antibiotics and disinfectants are substantially less effective against the microbial cells that live within biofilms, making it particularly challenging to treat biofilm infections.

The capacity of S.epidermidis to build a biofilm is principally responsible for its pathogenicity and chronic persistence. Biofilm-forming microorganisms have been linked to between 60 and 80 percent of all human illnesses. Drug resistance has often been linked to pathogen-produced biofilms. [3] Due to its high prevalence on epithelial surfaces, capacity to colonize prosthetic medical devices, and association with other coagulase-negative relatives, Staphylococcus epidermidis is a typical opportunistic pathogen that causes significant nosocomial infections and has a significant impact on human life and health. It is also the main cause of bacteremia. Additionally, several severe infections were caused by the contamination of indwelling medical equipment such dentures, artificial joints, and prosthetic heart valves [4].

Staphylococcal infections typically call for intrusive treatment techniques, increase the risk of chronic morbidity and death, and raise the expense of healthcare. Additionally, these organisms have been linked to other hospital acquired illnesses, including the newly discovered Methicillin Resistant S. epidermidis (MRSE) [5] . The majority of S. epidermidis infections are non-aggressive, although they are very resistant to antibiotic treatment. .A continuing search for non-antibiotic medications with potential antimicrobial and antibiofilm properties is thus necessary. [6]. NSAIDs nonsteroidal anti-inflammatory medications, are widely used to treat fever, discomfort, and inflammation.[3]. NSAIDS have been proven in certain cases to have antibacterial effect against bacteria. [7], [8]. Shikimic acid can be used to synthesis (6S)-6-fluoroSA [9], an antibiotic which inhibits the aromatic biosynthetic pathway [10]. The aim of current study to revealing the effect of some inhibitors on biofilm formation using shikimic acid, cranberry, acetyle salisilylic acid,

. A significant contributor to the contamination of medical equipment and the development of infectious and chronic diseases in the body is bacterial biofilm. In reality, since they induce severe infections and are resistant to antimicrobial medications, biofilms are the cause of a number of human disorders. Because the microorganisms that make up biofilms are substantially more resistant to antibiotics and disinfectants, biofilm infections are more challenging to treat [11]. The ability of staphylococcus epidermidis to adhere to biotic and abiotic surfaces, as well as the presence of the genes necessary for biofilm formation. These genes include Biofilm Associated Protein (Bap), Extracellular Matrix Binding Protein, Polysaccharide Intercellular Adhesion (PIA), Accumulation-Associated Protein (Aap), the atlE gene, Phenol-Soluble Modulins (PSMs) proteins, and Fibri [43]. Shikimic acid, a key biochemical metabolite in plants and microorganisms, has recently been identified as a new anti-biofilm agent. Shikimic acid use as a precursor in the synthesis of the antiviral Tamiflu.[11].Acetyle salisilic acid is effective at removing biofilms formed by Staphylococcus epidermidis [12].

2- MATERIALS AND METHODS

2.1 Bacteria sample and identification

Atotal of (231) samples were collected from patients who attended different hospitals of Ramadi and Falluja cities during the period from December (2020) to the end of July (2021).

Clinical specimens were collected from skin, urine, wounds, eye, blood, surgery operations ,Kidney stones and plaque of teeth.Bacteria was identified by biochemical tests and VITEK 2 system [14].

2.2A Biofilm assay (modified crystal violet assay).

Utilizing the modified crystal violet (MCV) test developed by [15], evaluation of biofilm growth for Staphylococcus epidermidis isolates in the first stages of biofilm formation was conducted. In order to quantify the percentage of inhibition of biomass growth caused by each concentration of the test materials, the following equation was used in conjunction with the mean absorbance (OD630 nm).

$$\text{Percentage Inhibition } \% \ = 1 - [\frac{\text{OD experimental well with test material}}{\text{OD control well without test material}}] \times 100$$

2.2B Biofilm metabolic activity assay using (MTT) assay

A Thiazolyl Blue Tetrazolium Bromide reduction (MTT) assay, as described by [16], was used to measure the metabolic activity of the S. epidermidis-developed biofilms in the second stage of biofilm production. Using a microplate reader, measure the absorbance at 570 nm. The following equation was used to compute the percentage of inhibition.

Viability % = [(OD experimental well with test material – OD control well without test material / OD experimental well with test material] ×100 .

2.3 Effect of antibiofilms against on bacterial adherence before and after adding anti-biofilm agents

According to[17], murine mammary adenocarcinoma cells are seeded on 24-well polystyrene plates and used in the adhesion experiment, which is carried out in quadruplicate. Bacterial inoculation was carried out by adding 10 ml of BHI broth to a screw tube and incubating the mixture for 18 hours. By using Giemsa staining and light microscopy, three wells were used to count the

number of adhering bacteria, and one well was used to see the adherence phenotypes. Briefly, 107 bacteria from overnight LB media or DMEM cultures are added to cell monolayers at 80% confluence, and they are then incubated for 3 hours at 37°C in a humidified environment with 5% CO2. The technique for a ten-fold dilution of a sample to a dilution factor of 10-6 was carried out in accordance with [18]. The wells were cleaned twice with PBS (pH 7.4), treated with 0.1% Triton X-100, and then serially diluted ten times. A replica sample is preserved in 300 L of methanol, stained with Giemsa, and mounted on glass slides. The stained samples are then viewed using a Nikon Eclipse microscope T 300-E. These studies are carried out in triplicate on three distinct days, with mean values represented as adherent CFUs.

2.4 Effect of antibiofilm agent on Staphylococcus epidermidis adherence to Foley balloon latex catheter (FBLC)

According to this experiment, [19] A sterile disposable Foley balloon latex catheter (FBLC) is used for each experiment twenty-four hours beforehand. was sliced within a biological safety cabinet (ENTEPLIN size G16) (to maintain sterile conditions). The FBLC were trimmed to a length of 0.5 cm. After overnight cultures were diluted (1/104), 600 l of broth culture was obtained, and this amount was placed in each well of a 48-well plate with one piece of FBLC. The plates were then incubated at 37 °C for 24 hours to facilitate biofilm development (adhesion period). The FBLC pieces were incubated for 2, 4, 6, 8, 12 and 24 hours in fresh BHI broth at 37°C before being transferred to new wells and rinsed with sterile distilled water at room temperature. Following a 24-hour incubation period, FBLC pieces were rinsed in sterile, room-temperature distilled water before being placed in 2 cc of BHI broth. Sessile cells may be moved using the Miles Misra method[20] and [21].

2.5 Synergism between antibiofilm agents and antibiotic

The checkerboard assay was used to determine the antibacterial effect of two test materials shikimic acid and Acetyl salicylic acid in combination with two antibiotic ceftriaxone and erythromycin .[22]

2.6 Detection of Virulence factors genes by PCR

2.6.1 Primers

All primers were supplied macrogen Company as a lyophilized product of different picomols concentrations and resuspension using nuclease free water to reach a final concentration for 100 picomols /µl of suspension. (10 picomol / µl of primer, 90 microlitir of nuclease free water) Before usage, they were diluted with vortex and kept at -20°C. Table contains a list of the primers used in this investigation (2-1)

Table (2-1): The primers used in this study

No.	Gene	Sequence	Amplicon Product size	Source
1.	iacABCD	F: TTATCAATGCCGCAGTTGTC R: GTTTAACGCGGTGCGCTAT	516	(Freitas, A. I ,et al., 2018)
2.	macA	F: GTGAAGATATACCAAGTGATT R: ATGCGTATAGATTGAAAGGAT	147	(Freitas, A. I et al., 2018)
3.	Bhp	F: TGGACTCGTAGCTTCGTCCT R: CTGCAGATACCCAGACAACC	213	(Freitas, A. I , et al., 2018)
4.	Aap	F: GCACCAGCTGTTGTTGTCC R: GCATGCCTGCTGATAGTTCA	190	(Freitas, A. I, et al., 2018)
5.	atlE	F: CAACTGCTCAACCGAGAACA R: TTTGTAGTTGTGCCCCA	682	(Freitas, A. I, et al., 2018)
6.	Fbe	F:TAAACACCGACGATAATAACCAAA R: GGTCTAGCCTTATTTTCATATTCA	495	(Freitas, A. I, et al., 2018)

7.	16srRNA	F: GGCGACTTTCTGGTCTGTAACT R: CTAGAGGGGTCAGAGGATGTCA	285	(Freitas, A. I, et al., 2018)
8.	Is256	F: TGAAAGCGAAGAGTTCAAAGC R: TGTAGGTCCATAAGAACGGC	1102	(Freitas, A. I ,et al., 2018)

* F: Forward sequences, R: Reverse sequences

2.6.2 Monoplex PCR mixture and PCR program conditions:

Under sterile circumstances, PCR reactions were carried out in 25 µl volumes in PCR tubes. The whole volume of the reaction mixture was diluted to 25 µl with sterile DDH2O. A negative control blank containing all PCR material other than the target DNA was used in each amplification test. The master mix, which had been lypholyzed, had the ideal concentrations of the necessary reaction ingredients. (Primer F 1µ, primer R 1µ, Green PCR Master Mix 12.5, Nuclease-free water 9.5µ, Template DNA 1µ) The program of PCR thermo cycling conditions used in the amplification of genes' targets DNA, Initial denaturation(5min) at(94-98 C˚),Denaturation(30sec)at95 C˚ ,Annealling (1min)at(50-65 C˚) ,Extension(1min)at(75-80 C˚) ,Final extension (5min)at(70-74 C˚) ,Hold.

2.7 Effect of shikimic acid and acetyl salicylic acid on expression of biofilm formation

Gene expression for target genes (icaA, atlE, and aap gene) compared with 16SrRNA as a housekeeping gene (normalized gene). This experiment appied according promega kit 2018.The bacterial isolates were treated with sub minimum inhibitory concentration for antibiotic and anti-biofilm agents of bacteria (ceftriaxone, erythromycin, Shikimic acid, cranberry, and Acetyl salicylic acid). Then incubated tubes for 24 hrs at 37°C, and extraction RNA nucleic acid by trizol method. [46] .

2.7.1 Analysis of Relative Gene Expression Data Using Real-Time Applied Quantitative PCR and the 2 (−ΔΔCT) Method (Kenneth J. Livak* and Thomas D. Schmittgen ,2001).

The 2 (−ΔΔCT) Livak technique is efficient in analyzing the relative time quantitative PCR experiments changes in gene expression from real-time

ΔCt treatment = Ct gene – Ct House Keeping

ΔCt control = Ct gene – Ct H.K

ΔΔCT = ΔCt treatment - ΔCt control

Gene expression = 2(−ΔΔCT)

3-RESULTS AND DISCUSSION

3.1. Bacterial identification

Out of 231 samples, only 20(8.5%) were related to Staphylococcus epidermidis, while 211(91.5%) were related to other types of microorganisms Figure (3-1).The distribution of Staphylococcus epidermidis isolates was : surgical wounds(5%), stones of kidney (10%) , eyes (25%) , skin(acne (20%), folliculitis(15%) . This result consistent with results according to Keshari et al., (2019) who found that, (12%) of S. epidermidis for skin lesion was isolated from (61%) and (35%) from surgical wound.

3.2 Evaluation antibiofilm activity of shikimic acid and acetylsalicylic acid

The results as shows in Table (3-1), Figure (3-1) the higher Percentage inhibition of shikimic acid was 93% at 12hr, this give meaning shikimic acid was good inhibitor of biofilm.

Table (3-1): Percentage inhibition of biofilm inhibitory activity of Shikimic acid

Percentage inhibition

Time(hr)	0.25 MIC	0.5 MIC	MICµg/ml	2 MIC	4 MIC
2 hrs	27.0%	19.3%	9.0%	0.0%	15%
4 hrs	68.4%	62.0%	42.7%	32.1%	15.4%
6 hrs	77.1%	72.5%	63.8%	47.6%	54.2%
12 hrs	93.3%	90.0%	85.9%	83.5%	78.7%
24 hrs	10.0%	17.0%	48.8%	40.1%	12.1%

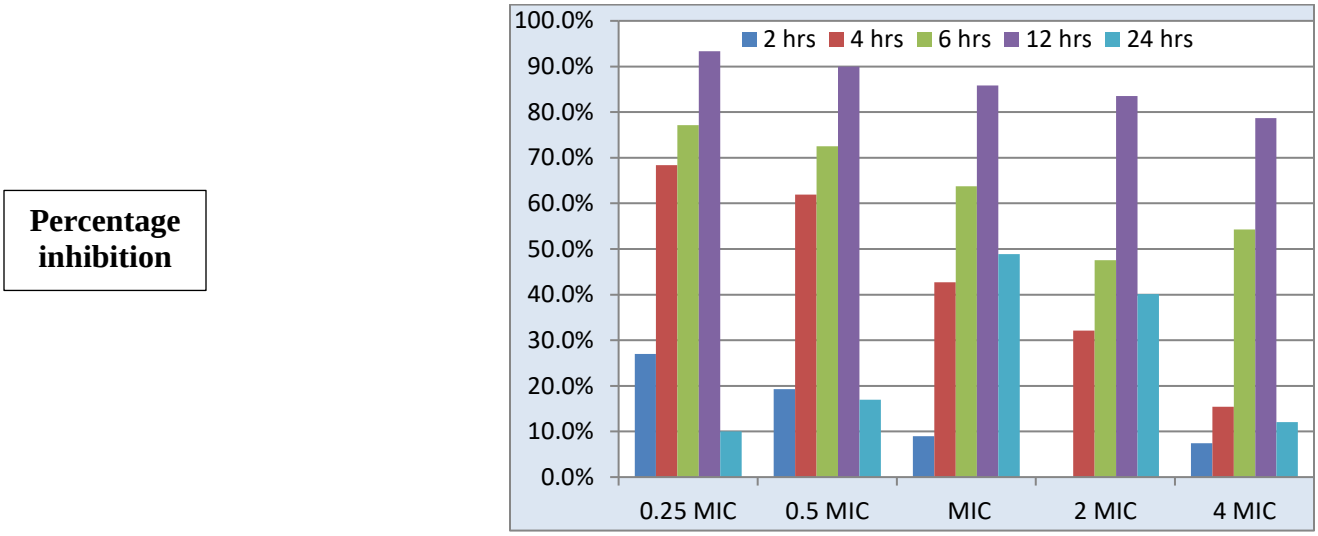


Figure (3-1): Biofilm inhibition activity of Shikimic acid on S. epidermidis for 2 h, 4 h, 6 h, 12 h, and 24 h incubation as determined by the microtiter plate (MTP) assay.

So, the effect of Acetylsalicylic acid, on initial cell attachment were studied, the results showed that, more effect of Acetylsalicylic acid MIC at 12 hrs were appeared, the results were shown in Table (3-2), Figure (3-2). The higher Percentage inhibition was 85% at 12hr, this give meaning acetyle salicylic acid was good inhibitor.

Table (3-2): Percentage inhibition of biofilm inhibitory activity of Acetylsalicylic acid

Percentage inhibition					
Time(hr)	0.25 MIC	0.5 MIC	MICµg/ml	2 MIC	4 MIC
2 hrs	24.4%	26.2%	26.0%	11.6%	7.5%
4 hrs	30%	30.7%	35.0%	10.0%	12.1%
6 hrs	44.5%	74.6%	70.4%	65.6%	56.0%
12 hrs	79.4%	85.3%	85.9%	82.0%	76.9%
24 hrs	21.3%	55.5%	34.2%	42.4%	30.6%

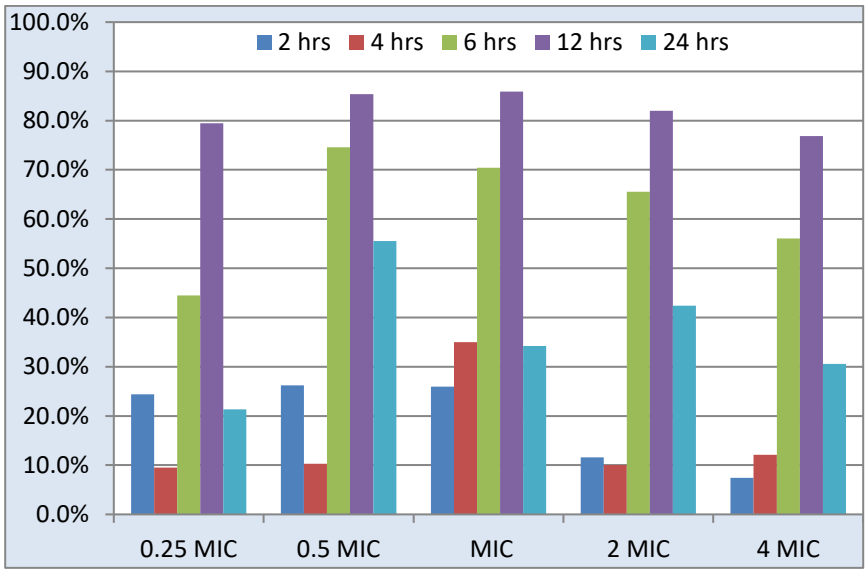


Figure (3-2): Effect of Acetylesalisilic acid acid on S. epidermidis (first stage of biofilm formation) for 2 h, 4 h, 6 h, 12 h, and 24 h incubation as determined by the microtiter plate (MTP) assay.

The findings of this investigation are consistent with those of [23], who discovered that neither the growth mode nor the initial bacterial concentration had any effect on the development of biofilms, but that after 12 hours, cell viability reduced. Communities of microorganisms known as biofilms, which are affixed to a surface, are crucial to the bacterial infections' ability to survive. In comparison to planktonic bacteria, microorganisms inside a biofilm are many orders of magnitude more resistant to antibiotics. No medications that particularly target bacterial biofilms are currently being used in clinical trials [24].

The first stage of biofilm development for the human pathogen S. epidermidis is adhesion to human matrix proteins like fibronectin (Fn) and fibrinogen (Fg). The peptidoglycan on the cell wall is covalently coupled to microbial surface components that recognize sticky matrix molecules dependent adhesions. More than 12 microbial surface components recognizing sticky matrix molecule genes are present in S. epidermidis. The initial attachment of biofilms is facilitated by noncovalent adhesions, such as those mediated by autolysins [25]. Analysis may be hampered by the interaction of a number of factors due to the dynamic nature of biofilm physiology, including the kind of substrate, the strain being employed and how domesticated it is, the carbon supply, etc. For instance, research has shown that the initial colonization and adhesion of cells to the surface are influenced by the surface hydrophobicity and charge [26].

The effect of Shikimic acid, on second stage of biofilm formation was shown in Table (3-3), Figure (3-3). The higher Percentage inhibition was 94% at 24hr, this give meaning shikimic acid was good inhibitor.

Table (3-3): Percentage inhibition of biofilm inhibitory activity of Shikimic acid

Percentage inhibition					
Time(hr)	**0.25 MIC**	**0.5 MIC**	**MICµg/ml**	**2 MIC**	**4 MIC**
2 hrs	**10.3%**	**64.5%**	**87.0%**	**83.1%**	**85.8%**
4 hrs	**33.8%**	**87.1%**	**91.6%**	**91.8%**	**92.4%**
6 hrs	**4.6%**	**83.8%**	**87.9%**	**89.4%**	**88.1%**
12 hrs	**43.5%**	**88.4%**	**90.1%**	**87.4%**	**90.7%**

24 hrs	**94.3%**	**94.1%**	**91.2%**	**90.2%**	**91.5%**

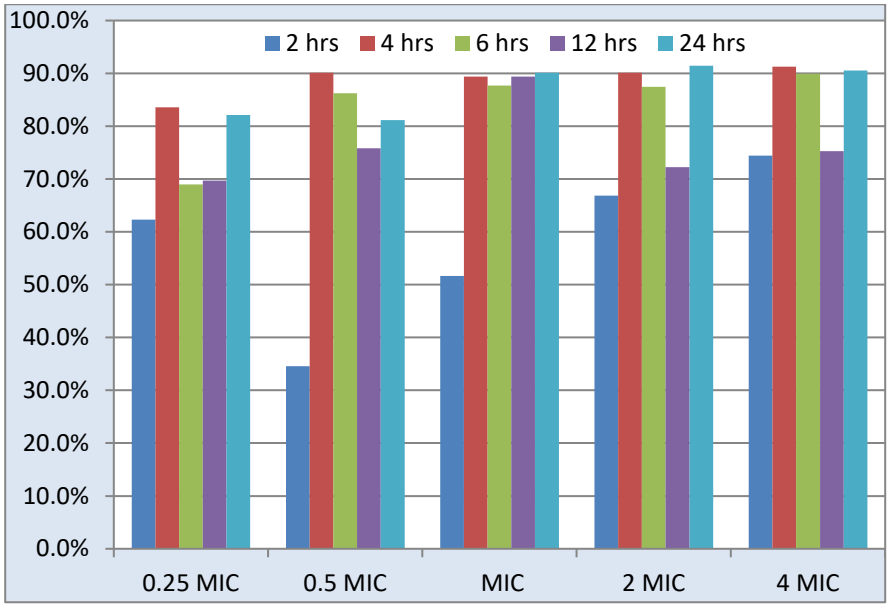


Figure (3-3): Effect of Shikimic acid on S. epidermidis (second stage of biofilm formation) for 2 h, 4 h, 6 h, 12 h, and 24 h incubation as determined by the microtiter plate (MTT) assay.

The effect of Acetyl salicylic acid, on second stage of biofilm formation was shown in Table (3-20), Figure (3-21). The higher Percentage inhibition was 91% at 24hr, this give meaning acetyle salicylic acid was good inhibitor because its acidity property.

Table (3-4): Percentage inhibition of biofilm inhibitory activity of Acetyl salicylic acid

Percentage inhibition					
Time(hr)	**0.25 MIC**	**0.5 MIC**	**MICµg/ml**	**2 MIC**	**4 MIC**
2 hrs	**35.0%**	**6.3%**	**73.5%**	**85.7%**	**89.7%**
4 hrs	**38.1%**	**46.1%**	**84.3%**	**90.9%**	**92.1%**
6 hrs	**42.5%**	**45.5%**	**68.5%**	**81.3%**	**87.5%**
12 hrs	**30.2%**	**28.3%**	**47.5%**	**78.9%**	**80.6%**
24 hrs	**43.5%**	**38.8%**	**89.5%**	**90.2%**	**91.9%**

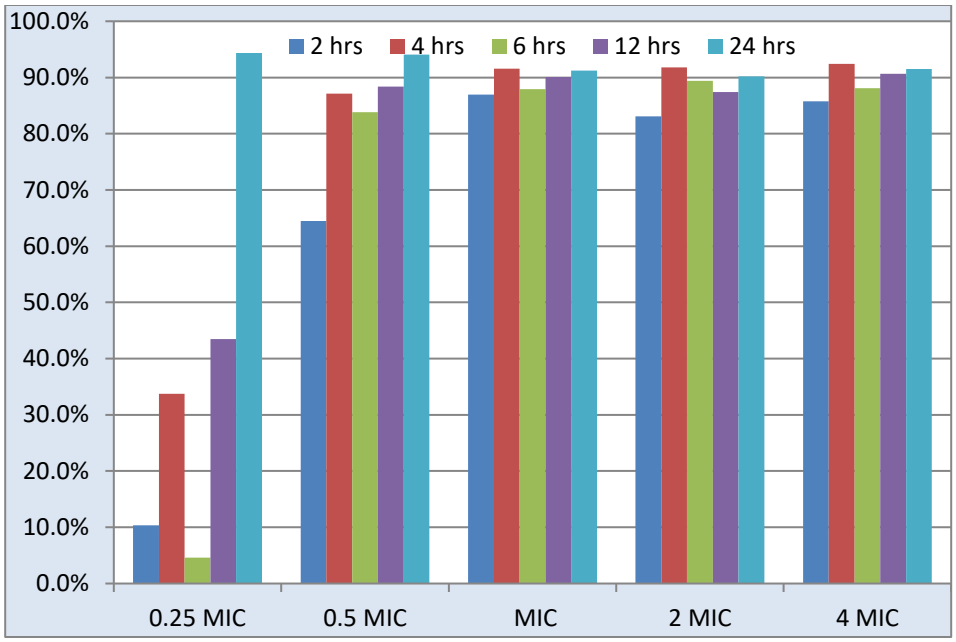


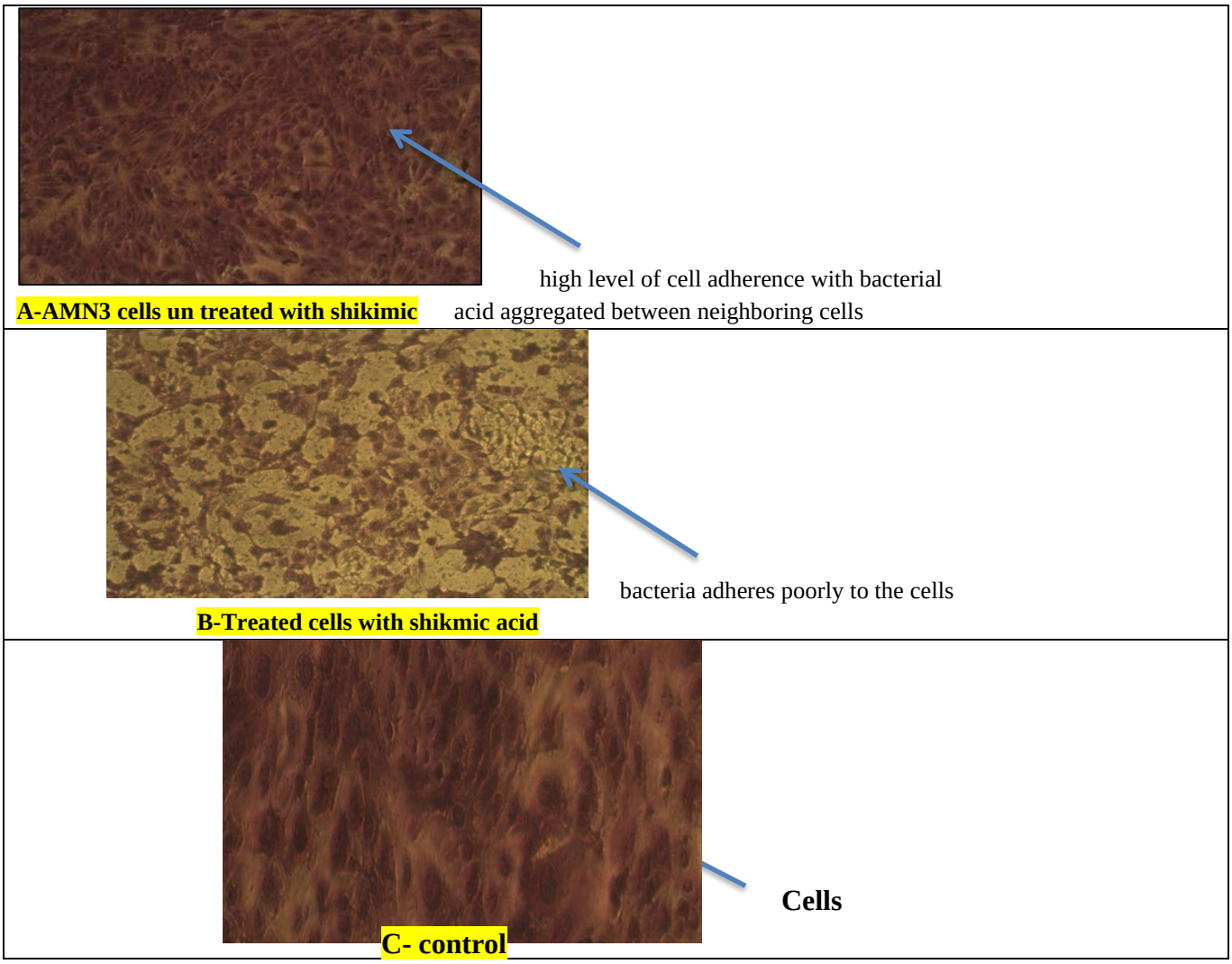
Figure (3-4): Effect of Acetyl salicylic acid on S. epidermidis (second stage of biofilm formation) for 2 h, 4 h, 6 h, 12 h, and 24 h incubation as determined by the microtiter plate (MTT) assay.

[27] have shown that antimicrobial drugs like erythromycin may successfully suppress biofilm development at sub-minimum inhibitory doses (sub-MICs). But according to another research [28], ceftriaxone antimicrobial drugs may cause the production of bacterial biofilms at sub-MIC levels. It has been documented how some Gram-negative bacteria produce biofilms by using antimicrobial drugs with sub-MICs. Gram-positive bacteria, however, have received very little research, most likely due to the complexity of the adjustment process. Some common antimicrobial substances have received a lot of attention recently due to their potential to fight illness by reducing the growth of biofilms [29].

3.3 Effect of antibiofilms against on bacterial adherence before and after adding anti-biofilm agents:

In this experiment, we tested whether there is bacterial biofilm growth on the biotic surfaces, which are human murine mammary adenocarcinoma cells used in this experiment.. In addition, we studied whether there is an effect of the anti-biofilm substance SA on the growth of bacterial biofilm, SA prevent bacteria adhesion and killed bacteria in the first three dilutions $1.0x10^{-1}$, $1.0x10^{-2}$, $1.0x10^{-3}$, and Treated with Shikimic acidCFU/ml was ($7.5x10^{2,}$ $5.0x10^{3,}$ $1.0x10^{4}$) ,while Control Untreated CFU/ml $2.75x10^{3}$, $1.5x10^{4}$, $7.5x10^{4}$, therefore number of bacterial cells were decreased .This result agreement with [17].

Another research looked at how SA affected two strains of Lacticaseibacillus bacteria, Lacticaseibacillus rhamnosus GG strain and Lacticaseibacillus paracasei ATCC334 strain, in their capacity to form biofilms and adhere to biotic and abiotic surfaces, including intestinal epithelial cells. These isolates have a well-documented ability to produce biofilms in a test tube [42], the findings of their research showed that there is a significant effect of SA on preventing the formation of biofilms on living surfaces, and this is consistent with the current study. The reason, as mentioned previously, may be due to the effect of SA on the genetic material of bacteria, preventing them from forming biofilms, or disrupting the pathways of the formation of sugars that need for biofilm formation.



Fig(3.8) Human murine mammary adenocarcinoma cell adherence phenotypes exhibited by staphylococcus epidermidis

3.4. Effect of antibiofilm agent on Staphylococcus epidermidis adherence to Foley balloon latex catheter (FBLC)

Staphylococcus epidermidis showed a growth pattern on pieces of FBCL Viable counts were isolated every 24 hours. Additionally, plating serial dilutions reach ten-fold. For 24 hours, Matrix was monitored. The lowest number of bacterial colonies when used acetyle salicylic acid was $1.75x10^6$ and $1x10^6$ in incubation period 24hr on shaking times (6min, 10min) respectively.While shikimic acid was gave best result on this times(8hr in shaking time (6,15) $1.5x10^7$) ($1.5x10^6$) respectivly, and in (12hr in shaking time 15($1.5x10^6$) (table 3.5) .Shikimic acid has inhibitor and acidity trait for biofilm formation.

Table (3.5) Development of biofilms on foley latex balloon pieces (0.5 cm) in logarithmic panel represented dislodging .sessile cells at 6, 10, and 15 minutes.

	Sonicating waterbath		
	Shik	Acetyl	Control

	6 min		
	CFU/ml		
2 hr	$2.75x10^6$	$4.25x10^5$	$3x10^7$
4 hr	$2.5x10^6$	$4.5x10^5$	$4.25x10^7$
6 hr	$2.75x10^6$	$5x10^5$	$6x10^7$
8 hr	$1.5x10^7$	$5.25x10^5$	$4.5x10^8$
12 hr	$2.5x10^7$	$6x10^5$	$5.75x10^8$
24 hr	$4.25x10^7$	$1.75x10^6$	$6.5x10^9$
	10 min		
2 hr	$3.5x10^5$	$3.5x10^5$	$3x10^7$
4 hr	$3.25x10^5$	$3.75x10^5$	$4.25x10^7$
6 hr	$3.75x10^5$	$3x10^5$	$6x10^7$
8 hr	$2.0x10^6$	$4.5x10^5$	$4.5x10^8$
12 hr	$2.25x10^6$	$5x10^5$	$5.75x10^8$
24 hr	$6.5x10^6$	$1.0x10^6$	$6.5x10^9$
	15 min		
2 hr	$2.25x10^5$	$2.5x10^4$	$3x10^7$
4 hr	$2.25x10^5$	$2.5x10^4$	$4.25x10^7$
6 hr	$2.0x10^5$	$1x10^5$	$6x10^7$
8 hr	$1.5x10^6$	$1x10^4$	$4.5x10^8$
12 hr	$1.5x10^6$	$1.5x10^5$	$5.75x10^8$
24 hr	$4.25x10^6$	$2x10^5$	$6.5x10^9$

Our findings concur with those of (32), which found that among clinical isolates from catheter infections, 49% of S. epidermidis and 61% of S. aureus are slime producers. Isolates that are ica-negative and do not produce slime are likely to represent strains that adhere to surfaces using alternative mechanisms, such as MMSCRAMS which are important in the development of catheter-related infections.

[33] study revealed that by suppressing the transcription of sarA, the SA hinders the creation and development of S. aureus biofilms at their earliest stages. Biofilm is created by the polymerization of sucrose glucosyl moieties and the cross-linking of carbohydrates. three genes called gtfB, gtfC, and gtfD, the GTF enzymes produce glucans utilizing sucrose, according to J. Kreth et al. (2008). discovered that SA might lower the amount of gtf gene expression, resulting in less GTF proteins and glucans produced by GTFs (35).

3.5. Synergism effect of (Ceftriaxone, Erythromycin), SA and ASA MIC concentrations .

The results showed in table 3.6 A, B that, synergistic effects between Acetylsalicylic acid , Cranberry, shikimic acid with Ceftriaxone have more effected on inhibit S. epidermidis biofilm formation, MIC value 0.182 , 0.176, 0.266 µg/ml respectively was necessary to dispersion of the biofilm .

Table (3.6 A) MIC of alone and combination of antibiotics and anbiofilm material

Conc. µg/ml	Ceftriaxone	Erythromycin	Shikimic acid	acetylsalicylic acid	Cranberry
MIC alone	**15.625**	**31.25**	**30.24**	**22.68**	**75**
MIC comb.	**0.25**	**1**	**7.56**	**3.78**	**12.5**

Table(3.6 B) Synergism between antibiofilm materials and antibiotics against Staphylococcus epidermidis

Antibiotic	MIC (µg/ml) antibiotic	FicA	Anti biofilm material	Anti biofilm mic	Fic B	FICI	Outcome
Ceftriaxone	**15.625**	**0.016**	**Shikimic acid**	**30.24**	**0.25**	**0.266**	**synergistic**
Ceftriaxone	**15.625**	**0.016**	**acetylsalicylic acid**	**22.68**	**0.166**	**0.182**	**synergistic**
Ceftriaxone	**15.625**	**0.016**	**Cranberry**	**75**	**0.16**	**0.176**	**synergistic**
Erythromycin	**31.25**	**0.032**	**Shikimic acid**	**30.24**	**0.25**	**0.282**	**synergistic**
Erythromycin	**31.25**	**0.032**	**acetylsalicylic acid**	**22.68**	**0.166**	**0.198**	**synergistic**
Erythromycin	**31.25**	**0.032**	**Cranberry**	**75**	**0.16**	**0.192**	**synergistic**

FicA MIC A in combination with antibiotic

FicB MIC B in combination with antibiofilm

Since none of the FICI values were more than 0.5, synergism was present. FICI index values below 0.5 were regarded as synergistic, and as the value decreases toward zero, the degree of synergy rises.

These antibiofilm drugs have a definite impact on enhancing the synergy between antibiotics and biofilm prevention. There have been claims that SA possesses antimicrobial properties [39]. In a water extract made from Cedrus deodara pine needles, SA was identified as a key antibacterial component that was effective against S. aureus in a prior work [40]. According to a prior research, SA may disrupt the lipid bilayer structure of cell membranes and prevent cells from changing the composition of the membrane, which reduces membrane fluidity, increases cell permeability, and makes the membrane partially soluble [41].

By specifically targeting cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) throughout the body, acetylsalicylic acid prevents the formation of prostaglandins everywhere [36]. Aspirin-treated biofilm cells are likely entirely incapable of cell division but yet maintain some metabolic activity, including that detected in the MTT experiment. ASA seems to have an even higher impact on increasing synergism MIC. The complex effects of COX inhibitors on biofilm formation, viability, and those seen here together with those on prostaglandin synthesis point to the possibility of one or more COX-dependent pathways in this organism [37,38]. Even though very little is known about the COX enzymes in S. epidermidis, these effects point to the possibility of one or more COX-dependent pathways in this organism.

2.6 Detection of Virulence factors genes by PCR

The findings of this study were in agreement with those of [45], who discovered that all 22 S. epidermidis isolates produced biofilm genes (icaC, icaD, icaA, icaB, and icaR) in PCR assay, in contrast to [44], who discovered that only ica A and ica D genes were expressed among S. epidermidis isolates. A major function for icaA and icaD in S. epidermidis biofilm formation has been reported[49]. It is crucial to note that both of these genes have been found in S. epidermidis strains that may produce

biofilms. New approaches for therapeutic intervention, as well as and physical electrical barriers and biomaterials to prevent bacterial colonization, are in need of further study[50].

3.7 Effect of shikimic acid and acetyl salicylic acid on genes involved in biofilm formation:

S epidermidis biofilm produced on culture containing chemical agents was employed to measure changes in gene expression using real-time reverse transcription-polymerase chain reaction. Results showed up regulation of atlE gene, $2^{-(\Delta\Delta Ct)}$ value was (2),(3)for shikimic and acetyl salicylic acid respevtively, while in aap,icaA genes all antibiofilm agents were gave down regulation result ,$2^{-(\Delta\Delta Ct)}$ value was (0.21),(0.10)for shikimic and acetyl salicylic acid respevtively, for aap gene, (0.10),(0.2) for shikimic acid and acetyle salicylic acid . According to [44], the findings provide evidence in favor of the hypothesis that S. epidermidis is a "accidental pathogen" and that the ica operon is the primary mechanism responsible for the production of biofilm in clinical and commensal isolates.

From two hours to twelve hours, the level of gene expression for the autolysin atlE protein was lower, but by the time 48 hours had passed, it had grown by 10 fold. In contrast, in vitro expression of the ica locus, which is involved in initial adhesion and intracellular aggregation, increased up to 100-fold during the time period of 2 to 48 hours. Additionally, there was no connection found between the surface chemistry of the biomaterial and S. epidermidis gene expression. These findings imply that the development of the S. epidermidis biofilm is influenced by autolysin atlE. Intercellular adhesions such PS/A and PIA have been found in S. epidermidis biofilm-forming strains. [45,46]. Fewer research have examined the expression of these genes and how it affects biofilm accumulation, despite the fact that several studies have linked biofilm formation to the presence of known biofilm-associated genes. RNA was taken at the early (12 h) and later (24 h) phases of biofilm development, and quantitative PCR was used to determine the degree to which each gene was expressed in mediating biofilm formation on the phenotypic variances seen across S. epidermidis isolates (qPCR). SECOM049A was unable to form a biofilm with the same thickness and biomass as the other PIA-dependent biofilms because SECOM049A did not increase icaA expression at later stages. However, icaA expression was sufficient to generate a smaller biofilm of the "mushroom-like" kind. Additionally, the reduced disruption by NaIO4 p reported by this isolation may be explained by the lower levels of icaA expression at the later time point. [44]

Conclusion

In conclusion, SA and ASA exhibit remarkable antibiofilm powerful activity against bacteria by prevent bacteria adherence and is capable of being further developed as antibiofilm agent. Shikimic acid gave highest percentage inhibition of biofilm formation was 93% at12hr incubation period , while Acetyl salicylic acid was gave 85% at 12hr . The highest percentage inhibition of biofilm formation in second stage(biofilm performed) by using antibiofilm , Shikimic acid 94% at 24hr incubation period and acetyl salicylic acid 91% at 24hr incubation period . Results showed up regulation of atlE gene, $2^{-(\Delta\Delta Ct)}$ value was (2),(3)for shikimic and acetyl salicylic acid respevtively, while in aap,icaA genes all antibiofilm agents were gave down regulation result ,$2^{-(\Delta\Delta Ct)}$ value was (0.21),(0.10)for shikimic and acetyl salicylic acid respevtively, for aap gene, (0.10),(0.2) for shikimic acid and acetyle salicylic acid

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