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ANTIBACTERIAL ACTIVITY OF ELLAGIC ACID AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* PLANKTONIC CELLS AND BIOFILM FORMATION

Layth Lateef Hamid[1], Safaa Abed Latef Al-Meani[2]
[1] *Biology department, College of science, University Of Anbar, Ramadi, Iraq*
[2] *Biotechnology department, College of science, University Of Anbar, Ramadi, Iraq*

ABSTRACT

The antibacterial activity of the ellagic acid, ciprofloxacin and gentamicin were estimated against planktonic and biofilm population of pathogenic methicillin-resistant *S. aureus* (MRSA) isolates. Ellagic acid developed high antibacterial activity against free cells with a significant increase in this activity when combined with ciprofloxacin and gentamicin separately using alamar stain as an indicator in the checkerboard assay.Ciprofloxacin, gentamicin and ellagic acid demonstrated a synergistic effect against both planktonic and biofilm populations. Repression of biofilm development and maturity after incubation with certain concentrations of ellagic acid, ciprofloxacin and gentamicin were estimated by the modified crystal violet (CV) and thiazolyl blue tetrazolium bromide reduction (MTT) assay. The two antibiotics and the ellagic acid significantly inhibited the initial cell attachment of the MRSAon the polystyrene surface of the microtitre plate, with a significant inhibitory effect observed from the 3 incubation hour towards 24 h preformed biofilms. Metabolic activity of the MRSAbiofilm reduced extremely after incubation with ellagic acid, ciprofloxacin and gentamicin. Ellagic acid may be considered as a helpful additive in the development of new bactericidal and sanitizer formularization for the medical and pharmaceutical industry applications.

Keywords: MRSA, Ellagic acid,Biofilm, Thiazolyl blue tetrazolium bromide, Synergistic activity.

1. Introduction

Staphylococcus aureus is one of the most common pathogenic bacteria which causes serious community and nosocomial infections and has for a long time been considered a major problem of public health. The pathogenicity of *Staphylococcus aureus* is due to their ability to produce many toxins, invasiveness and antibiotic resistance (Gnanamani, Hariharan, and Paul-Satyaseela 2017; Sultan and Al Meani 2019). Acute and chronic *S. aureus* diseases occur as a result of this bacteria's ability to adherence and colonization to the host tissue, interferes with host pathways, evading the immune system, kills host cells and spread to other sites. Moreover, *S. aureus* can develop biofilms that are considered insusceptible barriers against antibiotics and make the infections difficult to treat(Nowicka and Grywalska 2019).

S. aureus infection diseases have become more difficult because of the emergence of multidrug-resistant strains especially methicillin-resistant *Staphylococcus aureus* (MRSA) and that emerged as a serious problem for health care professionals worldwide(Gurung, Maharjan, and Chhetri 2020). MRSA includes all strains of *S. aureus* that have acquired resistance to methicillin and other beta-lactam antibiotics. It is responsible for several intractable infections in humans. Methicillin and other β-lactam antibiotics play a role in inhibiting penicillin-binding proteins (PBPs) that are involved in the synthesis of peptidoglycan. Methicillin resistance by *S. aureus* is due to the production of an altered form of penicillin-binding protein 2a (PBP2a) that has a lower affinity for all β-lactam antibiotics, thus enables staphylococci to survive and tolerate high concentrations of these agents(Akanbi et al. 2017).

Recently, there is a rising interest in understanding the role and mechanism of the phenolics compounds in various biological functions. Among all phenolics compounds, ellagic acid (EA) has been getting the greatest concern because ithave a variety of medical and biological activities, such as antibacterial, antiviral and antioxidant properties(Metsämuuronen and Sirén 2019; Park et al. 2014).

Ellagic acid ($C_{14}H_6O_8$) is a dimer of gallic acid, belongs to the polyphenol compounds, is generated by the hydrolysis of ellagitannins. Chemically it is (2,3,7,8-tetrahydroxy[1]benzopyranol[5,4,3-cde]benzopyran-5,10-dione), found in specific plant parts such as walnuts, pomegranates, strawberries, blackberries, cloudberries and raspberries (García-Niño and Zazueta 2015). EA activity is not changed, neither its potency weakened, by freezing, drying, or processing into a powder (Mekkawy 2013).

Antimicrobial activity of EA is described by various mechanisms including bind to substrates such as mineral elements, vitamins and polysaccharides making them unavailable for bacterial cells, prevention enterotoxin production and causing strong disturbing in the structural and functional cell membrane (Aldulaimi 2017; Takó et al. 2020).

The present study was offered to investigate the inhibitory activity of ellagic acid on pathogenic MRSA in the planktonic and sessile phase usingresazurin and thiazolyl blue tetrazolium bromide saltas an indicator, alone and in combination with selected antibiotics.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

From July 2020 to October 2020, 155 isolateswere collected from 2 hospitals in Al-Anbar, Iraq, these samples included: abscesses, blood, burn, ear, throat, urine, vaginal and wound swab.Also,*Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 15442) were obtained from the Al-Razi center, Ministry of Industry and Minerals, Baghdad, Iraq. *S. aureus* isolates were obtained following growth on selective media (mannitol salt agar) and were diagnosed based on colony morphology, mannitol fermentation, and other biochemical tests (indole, methyl red, voges-proskauer, citrate utilization, catalase, oxidase and coagulase test). Before each assay, *S. aureus* isolates were re-cultured on nutrient agar NA (Biomark labs) and incubated at 37 °C for 24 hours. A single colony from NA plate was inoculated in mueller hinton broth MHB (Himedia) and incubated at 37 °C for 24 hours. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard to obtain 1.5×10^8 CFU before used in experimental tests.

2.2. Antimicrobial susceptibility test

The disk diffusion testing was used in this study. Eight antibiotic agents (disks) were tested against *S. aureus* they are: azithromycin, cefoxitin, ciprofloxacin, clindamycin, gentamicin, rifampin, tetracycline and trimethoprim. For basic procedural steps, the Bauer- Kirby method was adopted as the reference method with some modification according to Matuschek et al., 2014; CLSI, 2020 (Matuschek, Brown, and Kahlmeter 2014).

2.3. Preparation of ellagic acid (EA)

Ellagic acid (EA) was purchased from the ministry of science and technology - department of materials research, Baghdad, Iraq. 10mg of EA was dissolved in 1ml D.W. contain 10% dimethyl sulfoxide (DMSO) to obtain 10mg/ml concentration of ellagic acid, then it was mixed well using a vortex, deposited by a centrifuge at a speed of 8000 rpm for 10 min, residual was discarded, the supernatant was taken and kept in vials at 4°C until use

2.4. Agar well diffusion method

The antibacterial activity of the EAwas screening using the agar well diffusion method according to Jafari-Saleset al. (2019) (Jafari-Sales, Hossein-Nezhad, and Bolouri 2019)with some modification. At first, 100 µl of *S. aureus* (ATCC 29213) and *P.aeruginosa* (ATCC 15442) broth cultures (as described in Section 2.1) was mixed with newly prepared mueller hinton agar (MHA,Biomark labs) at the cooling period (42°C) after autoclaving and poured into Petri dishes. After solidification, five wells (7 mm diameter) were filled with 30%, 50% and 100% of the EA, solvent solution 10% of dimethyl sulfoxide (DMSO, Applichem) as a negative control. The plates were incubated at 37 °C for 24 h before measuring the zone of inhibition (diameter in mm).

2.5. Minimum inhibitory concentration (MIC)

MIC ciprofloxacin, gentamicin and EAwere evaluated by Resazurin Microtitre-plate Assay (REMA) according to(Sarker, Nahar, and Kumarasamy 2007).with some modifications. The results were analyzed visually by observing the changes in the color of resazurin, changes from purple to pink, red, or colorless being recorded as positive. The lowest concentration with no change of resazurin color was taken as the MIC value

2.6.Checkerboard assays

The checkerboard assay was used to determine the antibacterial effect of two test materials in combination with each other in the microtiter-plates according to (Langeveld, Veldhuizen, and Burt 2014; Mutambuze 2014) by using resazurin stain as an indicator. The last well with no color change was considered as the point at which the MIC of the two antimicrobials in combination intersect. Then we determined the Fractional Inhibitory Concentration (FIC) of each antimicrobial in combination and then used this value to determine the Fractional Inhibitory Concentration Index (FICI) as below:

$FIC^{A} = (MIC^{A}$ in combination with (B))/MIC^{A}alone

$FIC^{B} = (MIC^{B}$ in combination with (A))/MIC^{B}alone

A: first test material, B: second test material.

The $\sum$ FIC (FICI) = $FIC^{A} + FIC^{B}$

A FICI number of ≤ 0.5 indicates a synergistic influence, > 0.5 but ≤ 4 indicates an additive effect, and >4indicates an antagonistic influence.

2.7.Inhibition of initial cell attachment

The activity of ciprofloxacin, gentamicin and ellagic acid on initial cell attachment was evaluatedaccording to Sandasi et al. (2010)(Sandasi, Leonard, and Viljoen 2010). The solutions of test materials (equivalent to 0.25×MIC, 0.5×MIC, 1×MIC, 2×MIC, and 4×MIC) were prepared. in triplicate,one hundred microlitres of each test materials were added to individual wellsof microtitre-plate (separately and in combination). Only media were added as negative controls while gentamicin (CN - 1 mg/ml) was added as a positive control. one hundred microlitres of bacterial culture (section 2.1) were added to the wells to yield a final measure of 200 µl in each well and incubated at 37°C for 24 h. Biofilm formation was evaluated using the crystal violet CV assay and the metabolic activity evaluated using thiazolyl blue tetrazolium bromide (MTT) assay (sections 2.9. and 2.10.).

2.8. Inhibition of preformed biofilm

The activity of ciprofloxacin, gentamicin and ellagic acid (separately and in combination) on preformed biofilm was evaluated according to Sandasi et al. (2010)(Sandasi, Leonard, and Viljoen 2010). Biofilms were permitted to be developed for 24 h before the addition of test materials as described in section 2.7. After the treatment of preformed biofilms with test materials, the plates were incubated for 1 h, 3 h, 6h, 12h and 24 h. After incubation, the biofilms were evaluated for biomass attachment using the CV and MTT assays were performed for the preformed biofilm cells (sections 2.9. and 2.10.).

2.9. Crystal violet (CV) assay

The modified crystal violet (CV) assay was described by Djordjevic et al. (2002) (Djordjevic, Wiedmann, and McLandsborough 2002). After the incubation period, the bacterial cultures were removed from all wells. To remove non-adhered bacterial cells, the wells were carefully washed 3 times with distilled water. The plates allow oven-dried at 60-65 ºC for 45-50 min. After that, the 1 %crystal violet stain (Thomas baker) was added (100 µl into each well); after incubation for 15 minutes at room temperature, the plate was washed with distilled water 3 times to remove execs stain. Before reading the results, ethanol (95 % v/v) was added (125 µl into each well) for 10 min and the absorbance was measured at 595 nm using a microplate reader(Humareader HS ELIZA). The mean absorbance (OD_{595} nm) was used for evaluating the percentage inhibition of biomass formation for each concentration of the test materials based on the following equation:

Percentage inhibition = 100 - [{OD_{595} nm experimental well with test material/OD_{595} nm control well without test material}x 100].

2.10. Biofilm metabolic activity assay

The metabolic activity of the biofilms developed by the *S. aureus* was evaluated using thethiazolyl blue tetrazolium bromide MTT assay as described by Schillaci et al., (2008)(Schillaci et al. 2008). The MTT solution was prepared by dissolving 100 mg of thiazolyl blue tetrazolium bromide (MTT, Sigma) in 20 ml of sterilized Phosphate buffer (PBS) under sterilized conditions to obtain 5mg/m1 concentration. After the incubation period, the bacterial cultures were removed from all wells and the plates were air-dried. 100 µl of PBS and 5 µl of MTT solution (5 mg/ml) were pipetted into each well and incubated for 3 h at 37 °C under sterile conditions. The insoluble purple formazan was further dissolved in DMSO. The absorbance was then measured at 570 nm using the microplate reader.

3. Results and discussion

All isolates were diagnosed by culturing methods (blood agar and Mannitol salt agar), phenotypic methods (colony morphology, mannitol fermentation and gram stain) and biochemical methods (all isolates were positive to catalase, coagulase, methyl red, voges-proskauer, citrate utilization, and negative to oxidase andindole). Among *S. aureus* isolates, the variant susceptibility for antimicrobial agents was detected (Table 1). 37 isolates (92.5%) were obtained as methicillin-resistant *S. aureus* (MRSA) depending on the cefoxitin resistant *S. aureus* by cefoxitin disk diffusion. Studies reported that the high percentage of methicillin resistance due to the presence of *mecA* gene complex which encodes to produce a penicillin-binding protein (PBP2a) that has a low affinity for binding β-lactam antibiotics including penicillins and cephalosporins(Miragaia 2018).

Table 1: Antimicrobial susceptibility results of the *S. aureus* in this study.

Anti-microbial agents (symbol)	Sensitive (%)	Intermediate Resistant (%)	Resistant (%)
Azithromycin (AZM)	29 (72.5%)	8 (20%)	3 (7.5%)
Cefoxitin (FOX)	3 (7.5%)	0 (0%)	37 (92.5%)
Ciprofloxacin (CIP)	30 (75%)	4 (10%)	6 (15%)
Clindamycin (CD)	8 (20%)	18 (45%)	14 (35%)
Gentamicin (CN)	34 (85%)	4 (10%)	2 (5%)
Rifampin (RA)	36 (90%)	2 (5%)	2 (5%)
Tetracycline (TE)	0 (0%)	11 (27.5%)	29 (72.5%)
Trimethoprim (TM)	17 (42.5%)	13 (32.5%) b	10 (25%)

S. aureus isolates were tested to produce some virulence factors (production of hemolysin, DNase, urease, gelatinase, and protease enzyme). As a result, beta hemolysis was detected in (62.5%) of isolates, 37.5% of isolates did not give any clear zone. On the other hand, all isolates (100%) produced DNase, urease and gelatinase enzymes. While 60% of *S. aureus* showed proteolytic activity against skim milk containing media.

In phenolics, multiple mechanisms of antibacterial activity have been described: they interact with bacterial proteins and cell wall structures, they may cause damage to cytoplasmic membranes, reduce membrane fluidity, inhibit nucleic acid synthesis, cell wall synthesis, or energy metabolism(Daglia 2012; Gyawali and Ibrahim 2014). In the present study, the ability of 40 clinical isolates of *S. aureus* to form biofilm and antibiotic susceptibility was performed. We chose the strongest biofilm-forming and multidrug-resistant MRSA isolate for the following experiments. So, the current knowledge estimates the inhibitory activity of ellagic acid against one isolate of MRSAin their planktonic and sessile phase, alone and in combination with ciprofloxacin and gentamicin.

The well diffusion assay was used to observe the antibacterial activity of ellagic acid, using the solvent solution (DMSO) as a negative control. after 24 h incubation with 100% v/v concentration of the ellagic acid, a 24-millimeter zone of inhibition was observed for the *S. aureus* (ATCC 29213) (Figure 1) and a 24-millimeter zone of inhibition was observed for the *P. aeruginosa* (ATCC 15442) (Table 2). Antimicrobial activity of EA is described by various mechanisms including disturbing in the cell membrane and bind to many materials such as mineral, vitamins and polysaccharides making them unavailable for bacterial cells (Aldulaimi 2017; Takó et al. 2020).

Table 2. Antibacterial inhibition zones (millimeter) of ellagic acid in various concentration.

Bacterial strain	Test materials			
	Ellagic acid Concentration			DMSO concentration
	30% (v/v)	50% (v/v)	100% (v/v)	10% (v/v)
	Inhibition zone in millimeter (mm)			
S. aureus (ATCC 29213)	18 mm	22 mm	24 mm	0 mm
P. aeruginosa (ATCC 15442)	17 mm	20 mm	24 mm	0 mm

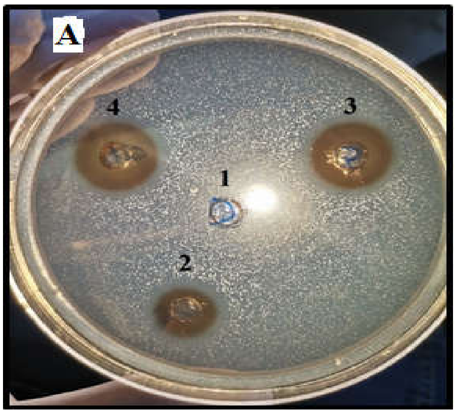


Figure 1: The well diffusion assay for (1) 10% of DMSO (2) 30% (3) 50% and (4) 100% v/v concentration of the ellagic acid that used to observe the antibacterial activity against *S. aureus* (ATCC 29213).

Using the REMA assay (Figure 2), the MIC of the ciprofloxacin, gentamicin and ellagic acid (separately) were determined for the clinical MRSA select isolate as (62.5 μg/ml), (125 μg/ml), and (1250 μg/ml), respectively (Table 3). The combination of ciprofloxacin (Cip.), gentamicin (Gent.), and ellagic acid (EA) with each other against MRSA select isolateshowed effectively (Table 3). Started with sub-MIC, checkerboard assay (Figure 2) results show decreases in the MIC for all test combine materials. the FICI values of the ciprofloxacin, gentamicin and ellagic acid (in combination) were determined for the select isolate as (0.0925 for Cip. with Gent.), (0.281 for Cip. with EA) and (0.156 for Gent. with EA) (Table 3). FICI values that less than 0.5 indicate a synergistic effect between the tested materials.

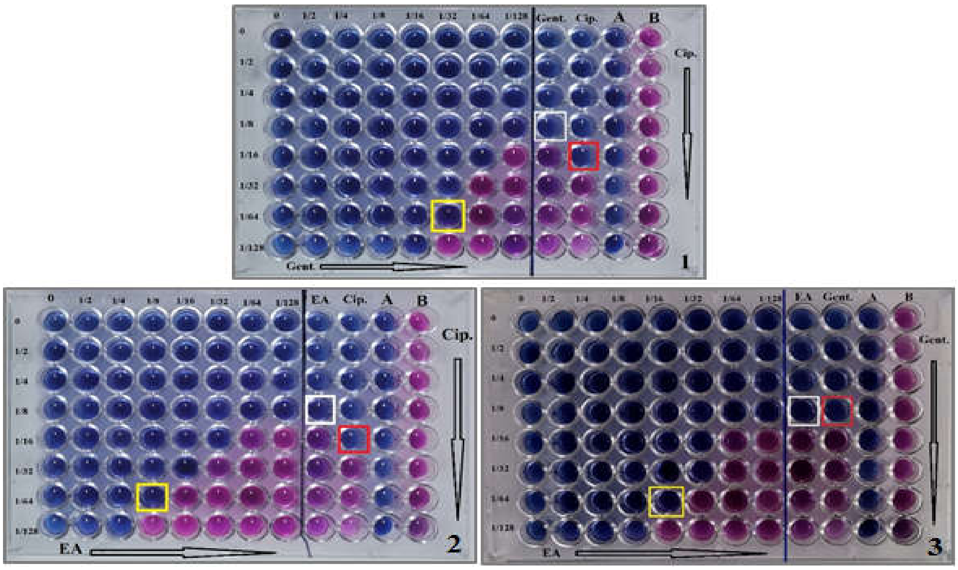
It is clear that the emergence of antibiotic resistance has seriously decreased antibiotic effectiveness and that a rising number of infections are therefore becoming challenging to treat. One approach to the recovery of antibiotic activity is to administer existing antibiotics in conjunction with non-antibiotic compounds that reduce bacterial resistance mechanisms. Ellagic acid is potential adjuvants to enhance the activity of antibiotics against resistant strains of bacteria(Abuelsaad et al. 2013). Chusri et al. (Chusri et al. 2009)observed that ellagic acid represents a promising antibiotic adjuvant lead compound, especially given its low cytotoxicity, and suggesting that ellagic act as efflux pump inhibitors.

Table 3. The MIC of the ciprofloxacin, gentamicin and ellagic acid (separately and in combination) for the clinical MRSA select isolate

Test materials	MIC value of (A)	MIC value of (B)	MIC value of (A) in combination with (B))	MIC value of (B) in combination with (A))	FICI value (ΣFIC)	Type of interaction
Cip. (A) + Gent. (B)	62.5 μg/ml	125 μg/ml	1.953 μg/ml	7.8 μg/ml	0.0925	S
Cip. (A) + EA (B)	62.5 μg/ml	125 μg/ml	1.953 μg/ml	312.5 μg/ml	0.281	S

Gent. (A) + EA (B)	125 µg/ml	125 µg/ml	3.9 µg/ml	156.25 µg/ml	0.156	S

*Cip., ciprofloxacin; Gent., gentamicin; EA., ellagic acid; S, Synergism; MIC, minimum inhibitory concentration; FIC, fractional inhibitory concentration; FICI, Fractional Inhibitory Concentration Index



***Cip., ciprofloxacin; Gent., gentamicin; EA, ellagic acid.**

Figure 2: The MIC and checkerboard assay for the antimicrobial combination of (1) ciprofloxacin with gentamicin, (2) ciprofloxacin with ellagic acid and (3) gentamicin with ellagic acid. The numbers on the left and at the top indicate dilution numbers from 1/2 to 1/128 for each test material as listed by the arrow. The wells represented by yellow squares indicate the point at which the MIC of combination materials. The wells represented by white squares indicate the point at which the MIC of the first test material. The wells represented by the red squares indicate the point at which the MIC of the second test materials. (A) negative control (media + bacterial growth), (B) positive control (media + antibiotic).

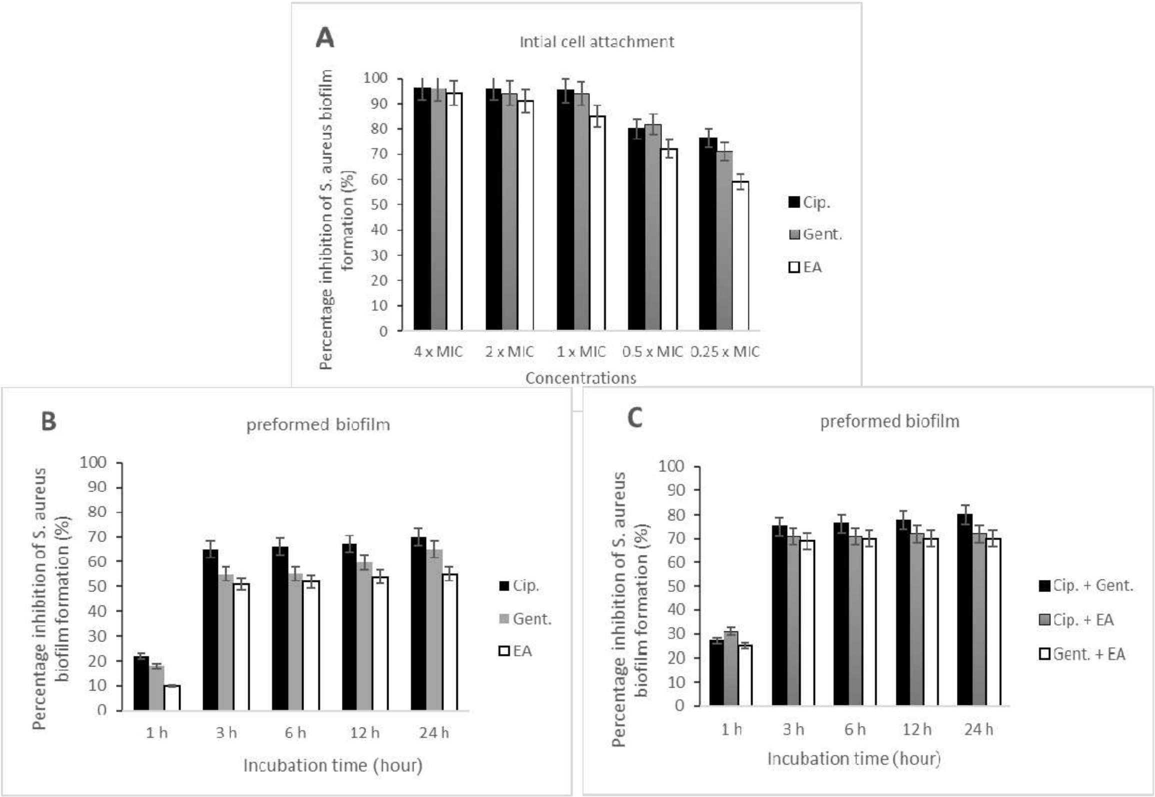
It is well known that a great number of chronic infectious diseases are associated with the formation of bacterial biofilms. Also, biofilm related infections often fail to respond to antimicrobial treatment. The bacterial ability to form biofilms is an important feature in the pathogenesis of medical device associated infections and offers a major therapeutic challenge. Although several methods to assess biofilm formation have been described, most of the studies were conducted using microtiter plate based method(Coenye and Nelis 2010; Kawamura et al. 2011).

To understand the anti-biofilm activity of ciprofloxacin, gentamicin and ellagic acid, its effects were tested on both the initial cell attachment as well as on development (24 h) biofilm. The modified CV assay indicated that the effect of the antibiotic solutions and the oil on biomass attachment exceeds 90% (percentage inhibition) in 4 × MIC and 2 × MIC for all test materials, although even at 0.25 × MIC initial cell attachment was reduced by 76.4%, 71% and 59% for the clinical MRSA isolate treated with ciprofloxacin, gentamicin and ellagic acid, respectively (Figure 3.A).Lin et al. (Lin et al. 2011)observed that plant polyphenols inhibited biofilm formation by *S. aureus* independently of growth mechanisms. It prevented the initial attachment to solid surfaces and the synthesis of polysaccharide intercellular adhesion compounds.

The MIC of ciprofloxacin, gentamicin and ellagic acid were one-fold higher (2 × MIC) than planktonic were used against MRSA preformed biofilm (24 h) and tested for 1h, 3 h, 6 h, 12 h and 24 h incubation. As estimated by the crystal violet assay, after a 1 h of incubation with ciprofloxacin, gentamicin and ellagic acid, separately with the preformed biofilm, only 22%, 18%, 12% and 10% inhibition occurred at 2 × MIC levels for the MRSA strain, respectively. Percentage inhibition of MRSA preformed biofilm was increased greatly after 3 h incubation until it reaches 70%, 65% and 55% inhibition for 24 h of incubation with ciprofloxacin, gentamicin and ellagic acid, respectively (Figure 3.18.A and A1). Also, inhibition of biofilm formation was increased significantly when exposure to test materials combined than separately in the same experimental conditions (Figure 3.B and C).

From our study we observed that, higher concentrations of antibiotic or natural substances were needed to inhibit the growth of MRSA sessile phase than the planktonic phase. Also, long-term exposure to antibiotics or ellagic acid plays a crucial role in the anti-biofilm activity. Dos Santos Rodrigues et al.(dos Santos Rodrigues et al. 2017)observed that higher amounts (sub-MIC) of phenolic compounds were needed to inhibit the growth of *S. aureus* in the sessile phase and besides that, an inductive effect was observed in all test isolates after a longer exposure time.

Our results show that a lower inhibitory effects against biofilm formation at the first hour of exposure to test materials (separately or in combination) as a result of the ability of mature biofilm to resist the antibiotics and ellagic acid at the onset of exposure. However, a significant increase in the percentage inhibition of MRSA biofilm formation was noticed in the third hour of incubation. The higher tolerance of mature biofilm to many antimicrobial substances could be related to the protective effect of the extracellular matrix (EPS). EPS has been associated with increased antibiotic resistance in *S. aureus* biofilms (Periasamy et al. 2012).



*Cip., ciprofloxacin; Gent., gentamicin; EA, ellagic acid.

Figure 3: Result of various concentrations of ciprofloxacin, gentamicin and ellagic acid (shown as Percentage inhibition of *S. aureus* biofilm formation (%)) on initial cell attachment and on 24 h development biofilm of *S. aureus*, (A) effect of test materials in different concentrations on initial cell attachment, (B) effect of test materials (separately) in 2 × MIC on preformed biofilm for 1 h, 3 h, 6 h, 12 h, and 24 h incubation, (C) effect of test materials (in combination) in 2 × MIC on

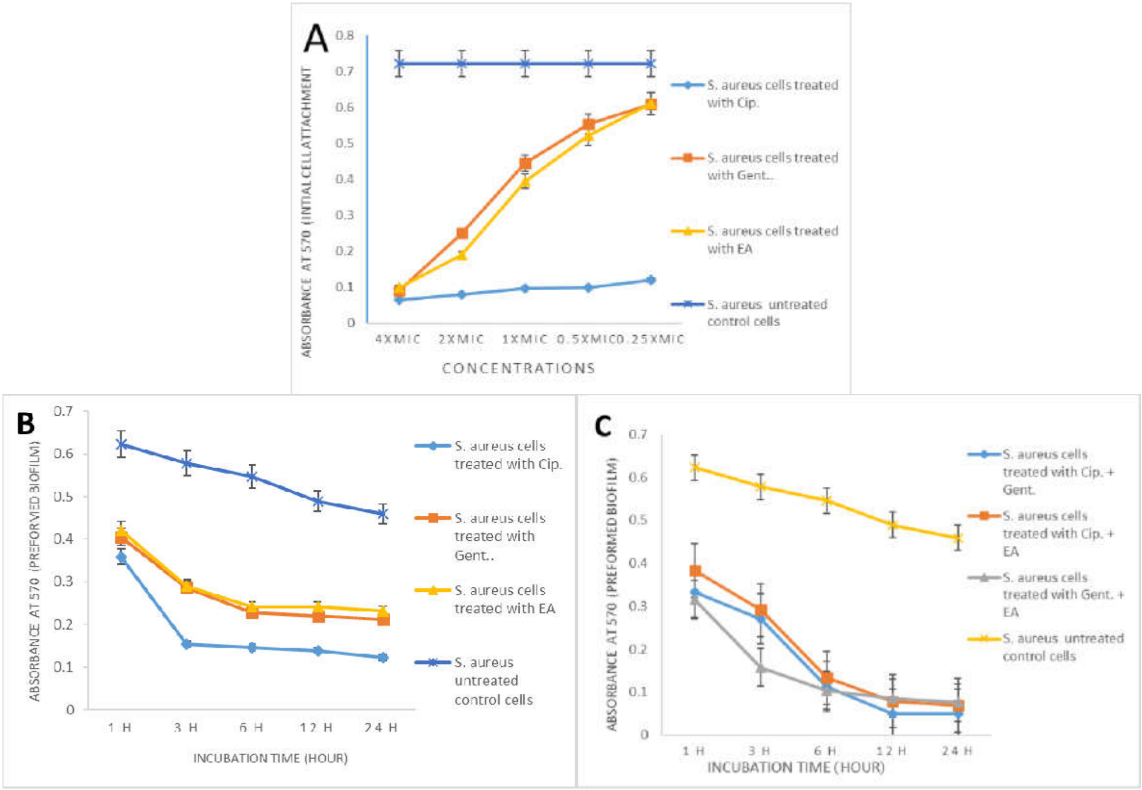
preformed biofilm for 1 h, 3 h, 6 h, 12 h, and 24 h incubation, As determined by the crystal violet assay.

The MTT assay results show the highest anti-adhesion activity for ciprofloxacin in the 4 × MIC, 2 × MIC and 1 × MIC with reducing the effect at 0.5 × MIC and 0.25 × MIC, respectively. The gentamicin and ellagic acid shows significant inhibition of cell attachment at 4 × MIC and 2 × MIC, the inhibition reduced in decrease the concentration from 1 × MIC to 0.25 × MIC (Figure 4.A).

In the situation of preformed biofilm, the two antibiotics and ellagic acid (separately) significantly inhibited the metabolic activity of the MRSA biofilm development at 2 × MIC. the metabolic activity repression was observed to rise from the third hour of incubation with the risen time of exposure until reach the highest effect at 24 h (Figure 4. B). At the same conditions, more increase in metabolic activity repression after exposer for test materials in combined than in the separate state and also the inhibition effect observed from the third hour of incubation until reaching the highest effect at 24 h (Figure 4.C).

In general, bacteria have the genetic capacity to both gain and transmit resistance to drugs used as therapeutics. Associations of antimicrobials are evaluated for their ability to suppress the emergence of resistant mutants, and to produce in vivo synergistic effects. Extending the useful life of current antimicrobials might be possible if they were used in combination with natural products. These combinations could represent therapeutic alternatives for the treatment of infections (Araújo Silva et al. 2016).

Recent research on ellagic acid showed good activity against *S. aureus* in planktonic and biofilm growth forms including polyresistant strains, and eradicated bacteria in already established 24-h biofilm. Ellagic acid and its derivatives can limit *S. aureus* biofilm formation to a degree that can be correlated with increased antibiotic susceptibility (Bakkiyaraj et al. 2013; Rendeková et al. 2016).



*Cip., ciprofloxacin; Gent., gentamicin; EA., ellagic acid.

Figure 4: Effect of ciprofloxacin, gentamicin and ellagic acid on the metabolic activity of MRSA (A) initial cell attachment at different concentration of test materials (separately), (B) preformed biofilm cells incubated with test materials (separately) at 2 × MIC for 1 h, 3 h, 6 h, 12 h and 20 h, (C) preformed biofilm cells incubated with test materials (combinedly) at 2 × MIC for 1 h, 3 h, 6 h, 12 h and 20 h, as determined by the MTT assay.

4. Conclusion

The ellagic acid has a significant activity against the freecells and biofilmpopulationof MRSA. Also, ellagic acid was observed to be further effective when it combined with ciprofloxacin and gentamicin against MRSAfreecells, developed biofilm and initial cell attachment. The MTT assay results show a hiegher activity of ellagic acid to decrease the metabolic activity of the MRSA biofilmseparately or in combined with ciprofloxacin or gentamicin.

References

Abuelsaad, Abdelaziz S A, Imad Mohamed, Gamal Allam, and Adnan A Al-Solumani. 2013. 'Antimicrobial and Immunomodulating Activities of Hesperidin and Ellagic Acid against Diarrheic Aeromonas Hydrophila in a Murine Model'. *Life sciences* 93(20): 714–22.

Akanbi, Olufemi Emmanuel et al. 2017. 'Antimicrobial Susceptibility of Staphylococcus Aureus Isolated from Recreational Waters and Beach Sand in Eastern Cape Province of South Africa'. *International journal of environmental research and public health* 14(9): 1001.

Aldulaimi, Omar A. 2017. 'General Overview of Phenolics from Plant to Laboratory, Good Antibacterials or Not'. *Pharmacognosy reviews* 11(22): 123.

Araújo Silva, Viviane et al. 2016. 'Ocimum Basilicum: Antibacterial Activity and Association Study with Antibiotics against Bacteria of Clinical Importance'. *Pharmaceutical biology* 54(5): 863–67.

Bakkiyaraj, Dhamodharan, Janarthanam Rathna Nandhini, Balakumar Malathy, and Shunmugiah Karutha Pandian. 2013. 'The Anti-Biofilm Potential of Pomegranate (Punica Granatum L.) Extract against Human Bacterial and Fungal Pathogens'. *Biofouling* 29(8): 929–37.

Chusri, Sasitorn, Ivan Villanueva, Supayang Piyawan Voravuthikunchai, and Julian Davies. 2009. 'Enhancing Antibiotic Activity: A Strategy to Control Acinetobacter Infections'. *Journal of antimicrobial chemotherapy* 64(6): 1203–11.

Coenye, Tom, and Hans J Nelis. 2010. 'In Vitro and in Vivo Model Systems to Study Microbial Biofilm Formation'. *Journal of microbiological methods* 83(2): 89–105.

Daglia, Maria. 2012. 'Polyphenols as Antimicrobial Agents'. *Current opinion in biotechnology* 23(2): 174–81.

Djordjevic, D, M Wiedmann, and L A McLandsborough. 2002. 'Microtiter Plate Assay for Assessment of Listeria Monocytogenes Biofilm Formation'. *Applied and environmental microbiology* 68(6): 2950–58.

García-Niño, Wylly Ramsés, and Cecilia Zazueta. 2015. 'Ellagic Acid: Pharmacological Activities and Molecular Mechanisms Involved in Liver Protection'. *Pharmacological Research* 97: 84–103.

Gnanamani, Arumugam, Periasamy Hariharan, and Maneesh Paul-Satyaseela. 2017. 'Staphylococcus Aureus: Overview of Bacteriology, Clinical Diseases, Epidemiology, Antibiotic Resistance and Therapeutic Approach'. *Frontiers in Staphylococcus aureus*: 4–28.

Gurung, Raja Ram, Prashanna Maharjan, and Ganga Gharti Chhetri. 2020. 'Antibiotic Resistance Pattern of Staphylococcus Aureus with Reference to MRSA Isolates from Pediatric Patients'. *Future Science OA* 6(4): FSO464.

Gyawali, Rabin, and Salam A Ibrahim. 2014. 'Natural Products as Antimicrobial Agents'. *Food control* 46: 412–29.

Jafari-Sales, Abolfazl, Parisa Hossein-Nezhad, and Parisa Bolouri. 2019. 'Identification of Chemical Composition of Essential Oil and Evaluation of Antimicrobial Effects of Ethanolic Extract of Mentha Pulegium on Staphylococcus Aureus and Escherichia Coli'.

Health Biotechnology and Biopharma 3: 29–38.

Kawamura, Hideki et al. 2011. 'Quantitative Analysis of Biofilm Formation of Methicillin-Resistant Staphylococcus Aureus (MRSA) Strains from Patients with Orthopaedic Device-Related Infections'. *FEMS Immunology & Medical Microbiology* 63(1): 10–15.

Langeveld, Wendy T, Edwin J A Veldhuizen, and Sara A Burt. 2014. 'Synergy between Essential Oil Components and Antibiotics: A Review'. *Critical reviews in microbiology* 40(1): 76–94.

Lin, Mei-Hui et al. 2011. 'Inhibitory Effects of 1, 2, 3, 4, 6-Penta-O-Galloyl-β-D-Glucopyranose on Biofilm Formation by Staphylococcus Aureus'. *Antimicrobial Agents and Chemotherapy* 55(3): 1021–27.

Matuschek, Erika, Derek F J Brown, and Gunnar Kahlmeter. 2014. 'Development of the EUCAST Disk Diffusion Antimicrobial Susceptibility Testing Method and Its Implementation in Routine Microbiology Laboratories'. *Clinical Microbiology and Infection* 20(4): O255–66.

Mekkawy, Mai Hamdy Ahmed. 2013. 'Synergistic Effect of Ellagic Acid and Certain Trace Element on Some Biochemical Disorders Induced by Gamma-Irradiation in Male Albino Rats'.

Metsämuuronen, Sari, and Heli Sirén. 2019. 'Bioactive Phenolic Compounds, Metabolism and Properties: A Review on Valuable Chemical Compounds in Scots Pine and Norway Spruce'. *Phytochemistry Reviews* 18(3): 623–64.

Miragaia, Maria. 2018. 'Factors Contributing to the Evolution of Meca-Mediated β-Lactam Resistance in Staphylococci: Update and New Insights from Whole Genome Sequencing (WGS)'. *Frontiers in microbiology* 9: 2723.

Mutambuze, Jean Wilson. 2014. 'In Vitro Antimicrobial Activity of BTZ043 and PNU-100480 Against Mycobacterium Ulcerans'.

Nowicka, Danuta, and Ewelina Grywalska. 2019. 'Staphylococcus Aureus and Host Immunity in Recurrent Furunculosis'. *Dermatology* 235(4): 295–305.

Park, Sang Wook et al. 2014. 'Antiviral Activity and Possible Mode of Action of Ellagic Acid Identified in Lagerstroemia Speciosa Leaves toward Human Rhinoviruses'. *BMC complementary and alternative medicine* 14(1): 171.

Periasamy, Saravanan et al. 2012. 'How Staphylococcus Aureus Biofilms Develop Their Characteristic Structure'. *Proceedings of the National Academy of Sciences* 109(4): 1281–86.

Rendeková, Katarína et al. 2016. 'The Activity of Cotinus Coggygria Scop. Leaves on Staphylococcus Aureus Strains in Planktonic and Biofilm Growth Forms'. *Molecules* 21(1): 50.

Sandasi, M1, C M Leonard, and A M Viljoen. 2010. 'The in Vitro Antibiofilm Activity of Selected Culinary Herbs and Medicinal Plants against Listeria Monocytogenes'. *Letters in applied microbiology* 50(1): 30–35.

dos Santos Rodrigues, Jessica Bezerra et al. 2017. 'Effects of Oregano Essential Oil and Carvacrol on Biofilms of Staphylococcus Aureus from Food-Contact Surfaces'. *Food Control* 73: 1237–46.

Sarker, Satyajit D, Lutfun Nahar, and Yashodharan Kumarasamy. 2007. 'Microtitre Plate-Based Antibacterial Assay Incorporating Resazurin as an Indicator of Cell Growth, and Its Application in the in Vitro Antibacterial Screening of Phytochemicals'. *Methods* 42(4): 321–24.

Schillaci, D et al. 2008. 'In Vitro Anti-biofilm Activity of Boswellia Spp. Oleogum Resin Essential Oils'. *Letters in Applied Microbiology* 47(5): 433–38.

Sultan, Fairoz Bahar, and Safaa Abed Latef Al Meani. 2019. 'Prevalence of Staphylococcus Aureus Toxins Genes in Clinical and Food Isolates in Iraq'. *Journal of Pharmaceutical Sciences and Research* 11(2): 636–42.

Takó, Miklós et al. 2020. 'Plant Phenolics and Phenolic-Enriched Extracts as Antimicrobial Agents against Food-Contaminating Microorganisms'. *Antioxidants* 9(2): 165.