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EFFECT OF *ZIZIPHUS SPINA CHRISTY* EXTRACT IN BIOFILM FORMATION OF METHICILLIN RESISTANCE *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS HAEMOLYTICUS*

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ABSTRACT

This research aimed to investigate the antibacterial activity of hot an aqueous extract of leaves *Ziziphus spina Christy* (Sidr), against biofilm formation of clinical isolates *Staphylococcus aureus* and *Staphylococcus haemolyticus*. Ten isolates were diagnosed initially as Staphylococci then selected four isolates depending on virulence and resistance to different types of antibiotics. After that VITEK-2compact system (ID and AST) was used to confirm the species of Staphylococci. The results showed that three isolates reverting to *Staphylococcus aureus* and one to *Staphylococcus haemolyticus.*

Investigation of *S. aureus* and *S. haemolyticus* isolates ability to forming biofilm using of Microtiter plate (96 well) methods, the results indicated that all of the isolates were able to produce the biofilm.

The effect of Moxifloxacin and Penicillin G with (MIC), (Sub-MIC) and (Sub-Sub-MIC) were detected for preventing of *S.aureus* and *S.haemolyticus* biofilm production, as well as hot an aqueous extract of leaves *Ziziphus spina* Christy (Sidr) with50 mg/ml tested against the biofilm formation, the results showed ability of tow antibiotics and plant extract to prevent biofilm formation.

Key words: *Ziziphus spina Christy*; Biofilm; Methicillin; *Staphylococcus aureus*; *Staphylococcus haemolyticus*

INTRODUCTION

Medical plants have been very important in the last few decades, Despite significant development in manufacture of drugs and drugs prepared from pure chemicals In addition, the number of microorganisms resistant to traditional antibiotics are increasing (Al-Snafi, 2016, Hussein, 2017), So researchers looked to wider horizons through the introduction of chemicals taken from natural sources (plant) in the field of pharmaceutical industry and development especially for the control of multiresistant microorganisms of most traditional antibiotics, plant extracts are a rich source of secondary metabolites that have a lethal effect on microorganisms, among the most effective compounds in bactericidal agents (Alkaloids, Flavonoids, Terpenoids, Tannins, Saponins and Phenols) (Omojate *et al*., 2014).

Ziziphus spina-christi Sidr) is a traditional medicinal plant was used since ancient times, as a treatment in antiquity, In Iraq and the Middle East, it is called Sidr, while Christ's Thorn Jujube is called "Christ's Thorn" (Abdallah,2017).

Biofilm is known as a community of bacteria adhering to different Surfaces (live and non-living) and surround themselves with self-separating substances made up of extracellular polymers (Flemming *et al.*, 2016) The existence of the biofilm gives the pathogen a greater chance of antibiotic resistance. biofilm prevents antibiotic from reaching the bacterial cells, and bacteria inside of its undergo physiological changes and appearance patterns that enable it to resist antibiotic (Kırmusaoğlu, 2016, Abbas, *et al.,* 2017). Among the bacterial pathogens known to have high resistance to most antibiotics and great ability to form a biofilm, *Staphylococcus aureus* and *Staphylococcus haemolyticus*, Infections resulting from these two species enter into life-threatening diseases unless treated as quickly as possible, It causes a number of health problems ranging from mild, moderate and low-intensity infections to severe diseases requiring rapid medical treatment, including deep skin infections, endocarditis, chronic osteoarthritis, Pneumonia and other diseases that may lead to death (Greenwood et al., 2012).

MATERIALS AND METHODS

Collection of *Ziziphus spina- christi* leaves: Leaves of Ziziphus Spina- Christi were collected from the garden City of Najaf, leaves were washed with sterile water and then left to dry at room temperature, Grind using a blender Electric mill to get dry powder.

Preparation of aqueous extract of *Ziziphus spina-Christi*: Preparation of aqueous extract of *Ziziphius spina Christi* was prepared according to Adzu *et al.,* (2001) with some modifications, about 20 g of dry powder was mixed with 400 ml of hot distal Water, then it Placed in water bath at 45°C and 100° Cycle / minute for five hours. after this it removed from water bath and left at room temperature for 24 h. sterile medical gauze used for disposed of the plant residue, then it centrifuged at 3000RPM/min for 10 minutes, then it filtered using Millipore filter paper 0.22µg and dried the extract using the oven at 40° C it stored in refrigerator at 4°C.

Detection of biofilm formation: Biofilm production was detected using microtiter assay as described by Mathur *et al*., (2006) with modifications. In Briefly, *S. aureus* and *S. haemolyticus* isolates were inoculated overnight in Trypton soya broth (Himedia) with 0.1 % glucose (SIGMA), after comparing turbidity with McFarland tube Which, estimates the number of bacterial cells 1.5 × 10^8 cell/ml. Transfer 100µl from Bacterial culture to the Microtiter plates (96well flatbottom) and incubate 37 °C for 24 h, the supernatant was removed, and the wells were washed with phosphate buffer saline. Methanol was added, for fixation of the biofilm and the supernatant was removed again. Then, 0·1% crystal violet (CV) solution was added to wells, and after 20 minutes, the excess dye was removed by washing the plates under running tap water. Finally, bound crystal violet was released by adding 33% Glacial acetic acid. The absorbance was measured at 630 nm

Sensitive of bacterial biofilm for antibiotics: Minimum inhibitory concentrations were determined using Vitek (BioMérieux) (Koneman *et al*., 2006), Two antibiotics Moxifloxacin (Jamjoom) and Penicillin G (Drogsan) each of its dissolving in distilled water, three concentrations were used for each antibiotic based on the first inhibitory concentration (MIC), prepare Sub-MIC and Sub-Sub MIC, obtained from the vitek-2 compact system, starting from the minimum inhibitory concentration (MIC) of 0.5µg/ml and sub-MIC concentration of 0.25µg/ml and half the sub - sub -MIC concentration of 0.125µg/ml for Penicillin G, while the Moxifloxacin MIC concentration is 0.25g/ml and Sub-MIC concentration 0.12µg/ml and Sub-Sub-MIC concentration 0.0625µg/ml. The concentrates were placed in sterile tubes and kept in the refrigerator at 4°C.

Inhibition of bacterial biofilm formation by Moxifloxacin and Penicillin G detected by the crystal violet staining After 24 h, culturing isolates on Trypton soya broth then. The same steps were completed (Mathur *et al., 2006*).

Effect aqueous extract of *Ziziphus spina-Christi* against biofilm formation: Inhibition of bacterial biofilm formation by aqueous extract of *Ziziphius spina-christi* with 50 mg/ml concentration was detected by the crystal violet staining. After 24 h, culturing isolates on the nutrient agar. A bacterial colony was taken for culturing on Trypton soya broth then compare turbidity with McFarland tube Which estimates the number of bacterial cells 1.5 x 10^8 cell/ml, then the same steps were completed (Mathur *et al*., 2006).

Synergism effect of *Ziziphus spina-Christi* aqueous extract and two antibiotics against biofilm formation: Inhibition of bacterial biofilm formation by added 75µl with 50 mg/ml of Sider extract and 75µ for each concentration for two antibiotics, to 75µl bacterial culture in Microtiter plates (96 well flat bottom), then the same steps were completed (Mathur *et al*., 2006).

Statistical analysis: The results of the study were analyzed using the statistical program (SPSS) using a test ANOVA one way, Least Significant Difference (LSD) (Morgan *et al*., 2004).

RESULTS AND DISCUSSION

Ability of bacteria *Staphylococcus aureus* and *Staphylococcus haemolyticus* to biofilm formation: Biofilm is one of the important factors that contribute greatly to the ability of the microorganism to forming of the disease and its ability to resist various types of antibiotics, The Results showed the ability of bacteria (MRSA) and (MRSH) for biofilm formation with strong form, as show in Table**.**1. Variations in the process of forming the biofilm of studied isolates referred to effect of conditions were important in the formation of the biofilm, the type of medium used, growth conditions and PIA efficiency in adhesion contribute to the ability of bacteria to form the biofilm, Wang (2008) showed that the degree of adhesion of bacteria to surfaces depends largely on growth conditions and the type of medium used.

Table 1: biofilm formation Length of wave length (630nm).

Bacterial isolates	**Biofilm formation mean ± Standard deviation**
***S.aureus* (1)**	0.237±0.0404
***S.aureus* (2)**	0.267±0.0200
***S.aureus* (1)**	0.155±0.0121
S.haemolyticus	0.356 *± 0.0405

Detection of the effect of different concentrations of Penicillin G and Moxifloxacin in the biofilm formation of *Staphylococcus aureus* and *Staphylococcus haemolyticus* resistant to Methicillin: The effect of Penicillin G and Moxifloxacin was investigated as shown in Table .2. The results revealed effect of Penicillin G in preventing of the biofilm production for all isolates studied when compared with control, while when comparing the concentrations used the same and the best influence, all concentrations have shown the impact on the first isolation *S. aureus* (1), while the concentration of 0.125 µg/ml was the best in inhibiting the formation of the biofilm of the second isolates *S.*

aureus (2) and the fourth of *S. haemolyticus* bacteria, while the concentration 0.25µg/ml was the most efficient in inhibiting biofilm formation in the third isolation, Penicillin G inhibits bacterial cell wall synthesis by binding to Transpeptidase enzyme, which binds the peptidoglycan chains to the final stage of cell wall manufacturing. Penicillin G had lethal effects on biofilm viability, the remaining viable cells were indicated to combat this antibiotic by reinforcement peptidoglycan, increasing adaptation and virulence (Savijoki *et al*., 2016), As for the effect of Moxifloxacin with its three concentrations (MIC, Sub-MIC, Sub-Sub-MIC) respectively, the results reflected the extent of impact all its concentrations on biofilm formation when compared with the control Which was a bacterial culture. The concentration 0.0625µg/ml showed an effect in preventing biofilm formation of *S. aureus* (1) and *S. aureus* (2) more than other two isolates, while other concentrations showed a similar effect in preventing biofilm formation. Moxifloxacin belongs to fluoroquinolone, a fluoroquinolone group that inhibits the action of the enzyme Gyrase, which is involved in the repair of DNA, any defect in its work makes the bacteria unable to repair damage to the genetic material as well as loss of the ability to divide (Jaiswal and Khan, 2017).

Table 2: Effect of inhibitors different concentrations of Penicillin G and Moxifloxacin against biofilm formation of *S. aureus* and *S. haemolyticus* isolates

Bacterial isolates	**antibiotics**	**concentration (µg/ml)**		**Biofilm formation mean ± Standard deviation**
***S.aureus*(1)**	Penicillin G	0. 5	MIC	0.095±0.001 a
		0. 25	Sub MIC	0.172±0.001 b
		0.1 25	Sub- Sub MIC	0.208±0.002 c
Control 0.647 ± 0.017				
	Moxifloxacin	0.25	MIC	0.183±0.016 b
		0.125	Sub- MIC	0.205 ±0.00 b
		0.0625	Sub -Sub MIC	0.001 ± 0.143 c
Control 0.647 ± 0.017				
***S.aureus* (2)**	Penicillin G	0.5	MIC	0.224 ± 0.003 b
		0.25	Sub -MIC	0.248 ± 0.014 b
		0.125	Sub-Sub- MIC	0.193± 0.003 c
Control 0.463 ± 0.023				
	Moxifloxacin	0.5	MIC	0.313±0.012 b
		0.125	Sub -MIC	0.237±0.013 b
		0.0625	Sub-Sub- MIC	0.468±0.021 c
Control 0.463 ± 0.023				
***S.aureus* (3)**	Penicillin G	0.5	MIC	0.139±0.005 a
		0.25	Sub -MIC	0.114±0.005 b
		0.125	Sub-Sub- MIC	0.144±0.008 a
Control 0.265 ± 0.004				
	Moxifloxacin	0.25	MIC	0.166±0.020 b
		0.125	Sub- MIC	0.164±0.010 b
		0.0625	Sub-Sub- MIC	0.224±0.010 b
Control 0.265 ± 0.004				
S.haemolyticus	Penicillin G	0.5	MIC	0.113±0.003 b
		0.25	Sub -MIC	0.113±0.001 b
		0.125	Sub-Sub MIC	0.0740±0.010 c
Control 0.262 ± 0.010				
	Moxifloxacin	1	MIC	0.135±0.0128 b
		0.5	Sub- MIC	0.178±0.025 b
		0.25	Sub-Sub MIC	0.234±0.022 b
Control 0.262 ± 0.010				

Similar English letters indicate no significant differences between different treatment (P≤0.05).

Effect of hot an aqueous extract of *Ziziphus spina- christi* leaves on biofilm formation: The results of the plant extract revealed the effect preventing biofilm formation for all clinical isolated which tested as shown in Table.3. The ability of an aqueous extract is due to the presence of a high percentage of flavonoids in the leaves, in addition, the presence of alkaloids and tannins, which were known to include secondary compounds inhibiting the process of quorum sensing which plays important role in biofilm formation (Chieu and John, 2016) so, bacteria in the biofilm high resistance to most antibiotics, we conclude that plant extracts, which have a largely variety of phytochemicals, will provide a biodegradable effect to eliminate microorganisms.

Table 3: Effect of *Ziziphus spina- Christi* leaves extract on the biofilm formation

Bacterial isolates	**Biofilm formation mean ± Standard deviation**	**Biofilm formation mean ± Standard deviation control**
***S.aureus*.(1)**	0.0390 ± 0.024 a	0.017 ± 0.647
***S.aureus*(2)**	0.022 ± 0.001 b	0.023 ± 0.463
***S.aureus*(3)**	0.025 ± 0.013 c	0.004 ± 0.265
S.haemolyticus	0.027 ± 0.005 d	0.010 ± 0.262

Similar English letters indicate no significant differences between different treatment (P≤0.05).

Investigation of the possibility of synergistic action between plant extract, Penicillin G and Moxifloxacin against biofilm formation: The results are shown in Table 4, display the synergistic effect of *Ziziphus spina christi* leaves extract with 50mg/ml concentration, penicillin G and Moxifloxacin with three concentrations (Sub-MIC, MIC, Sub-MIC). When compared with the positive control group of the bacterial culture without any addition, the two isolates *S. aureus* (2) and *S. haemolyticus* (4) were more affected when compared with other isolates with P≤0.05. Sidr leaves are a rich source of active substances that have the potential to penetrate the cellular wall of bacteria therefore possible to counteract the effect on the bacteria and stop their growth as well Provide inadequate conditions to inhibit biofilm formation thus enabling the antibiotic to effect on bacteria and stop its growth, as well as to provide inappropriate conditions that prevent biofilm formation.

Tabe 4: synergistic action between plant extract ,Penicillin G and Moxifloxacin against biofilm formation.

Bacterial isolates	**Antibiotics**	**concentration (μg/ml)**		**Biofilm formation Rate ± mean**
***S.aureus*(1)**	Penicillin G + sider	0. 5	MIC	0.028 ± 0.138 a
		0. 25	Sub MIC	0.003 ± 0.092 a
		0.1 25	Sub- Sub MIC	0.002± 0.122 a
Control				0.017 ± 0.647
	Moxifloxacin + sider	0.25	MIC	0.002±0.212 a
		0.125	Sub- MIC	0.009± 0.221 b
		0.0625	Sub -Sub MIC	0.005± 0.190 a
Control				0.017 ± 0.647
***S.aureus* (2)**	Penicillin G + sider	0.5	MIC	0.025± 0.147 a
		0.25	Sub -MIC	0.011±0.133 a
		0.125	Sub-Sub- MIC	0.009±0.178 a
Control				0.023 ± 0.463
	Moxifloxacin + sider	0.5	MIC	0.014± 0.258 a
		0.125	Sub -MIC	0.017± 0.241 a
		0.0625	Sub-Sub- MIC	0.019± 0.347 b
Control				0.023 ± 0.463
***S.aureus* (3)**	Penicillin G + sider	0.5	MIC	0.010±0.180 a
		0.25	Sub -MIC	0.024± 0.352 b
		0.125	Sub-Sub- MIC	0.027± 0.626 c
Control				0.004 ± 0.265
	Moxifloxacin + sider	0.25	MIC	0.008± 0.142 a

		0.125	Sub- MIC	0.005± 0.175	a
		0.0625	Sub-Sub- MIC	0.030± 0.153	a
Control				0.004 ± 0.265	
S.haemolyticus	Penicillin G + sider	0.5	MIC	0.002± 0.102	a
		0.25	Sub -MIC	0.006± 0.076	b
		0.125	Sub-Sub MIC	0.001±0.141	c
Control				0.010 ± 0.262	
	Moxifloxacin+ sider	1	MIC	0.005± 0.303	a
		0.5	Sub- MIC	0.009±0.076	b
		0.25	Sub-Sub MIC	0.009± 0.076	b
Control				0.010 ± 0.262	

Similar English letters refer to no significant differences (P≤0.05).

Conclusion

We can conclude that, the synergism effect of penicillin G, Moxifloxacin with (MIC), (Sub-MIC) and (Sub-Sub-MIC) and hot an aqueous extract of leaves *Ziziphus spina christi* (Sidr) with 50 mg/ml investigated, the results revealed that high synergism effect between two antibiotics and plant extract.

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