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Antibiofilm impact of *ziziphus spina christi* on methicillin resistance of *Staphylococcus warneri*

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***Abstract*---**This research aimed to study the antibacterial activity and Antibiotic effect of hot aqueous extract of leaves of Ziziphus spina Christy (Sidr), against clinical isolate of Staphylococcus wernari. Four isolates were initially diagnosed as Staphylococci bacteria, then one isolation was chosen depending on its sensitivity and resistance to several different types of antibiotics. The VITEK-2 compact system (ID and AST) and PCR were used to confirm the type of staphylococcal isolates. The results showed that one isolate was due to Staphylococcus wernari. The ability of isolate to form biofilm was tested using the Microtiter plate method (96 wells). The results indicated that the extracted isolate was able to produce a biofilm. Where there was a clear effect of moxifloxacin and penicillin G with

(MIC), (Sub-MIC) and (Sub-MIC) in inhibiting the growth of bacteria, A significant (P < 0.001).Synergistic effect between Penicillin G, Moxifloxacin with (MIC), (Sub-MIC) and (Sub-MIC) were tested with hot aqueous extract of Ziziphus spina christi (Sidr) leaves with 50 mg/mL. The results showed a high synergy between two types of antibiotics and plant extracts.

***Keywords*---**Antibiofilm, ziziphus spina Christi, methicillin resistance, Staphylococcus warneri.

Introduction

Ziziphus species *(Rhamnaceae)* are generally spread from Africa to India and are one of the most significant trees that raise in the dry parts of tropical Asia, Africa and also South of Iran (Godini *et al.*,2009) This genus approximately consists of 100 species locally known as sidr in Iran (Waggas ,2007). *Staphylococcus warneri* is a type of coagulase negative staphylococcus (CNS), oxidase-negative, catalase-positive and blood-coagulant negative, a communal organism present as part skin humans and animals. Like other thrombotic-negative staphylococcus, *S. warneri* infrequently causes disease, but rarely it may cause infection in patients with declining immune systems as a result of any factor that weakens it. *S. warneri* has been recommended as a cause of spontaneous abortion in cattle and humans and has been associated with disc inflammation (Kamath *et al.*,1992) this type of bacteria cause urinary tract infection, osteomyelitis, meningitis, and infection. ventricular and Endocarditis thrust infections. Similar to other anticoagulant negative staphylococci, the presence of *S. warneri* in CSF cultures and blood could also represent an infection rather than a true infection. Biofilm development is a process of biofilm formation that includes a set of complex stages starting with the adhesion phase, then the assembly phase, and then the phase dispersion phase, which is the phase in which bacteria spread outside the biofilm, causing infection (Otto *et al.*, 2013). Subsequently, the bacteria multiply to form a multilayer biofilm, associated with PIA production that mediates cell-to-cell adhesion (Becker *et al.*, 2014). The biofilm causes bacteria to survive in the stress conditions such as UV damage, metal toxicity, anaerobic conditions, bacteriophages, metal toxicity, acid exposure, salinity, desiccation, pH gradients and amoebae and to resist antibiotics, antimicrobials, and host immune defense (Archer *et al.*,2011).

Materials and methods

Several leaves of *Ziziphus spina- christi* were collected from the garden, then washed with sterile water and left to dry for an appropriate period of time at room temperature, and ground using an electric mill in order to obtain a dry powder. Prepare an aqueous extract of *Ziziphus spina christi* according to (Adzu, *et al.*, 2001). Twenty grams of dry powder was taken and mixed with 400 ml of remote hot water, and placed in a water bath at 45°C and 100°C/min for approximately five hours. Then remove from the water bath and leave at room temperature for 24 hours. A sterile medical gauze was used to get rid of plant residues and then expelled at 3000 rpm for 10 minutes, after which the extract is filtered using

Millipore 0.22 micrograms filter paper, the extract was dried using an electric oven at 40 ° C and then stored in the refrigerator at 4 °C.

Method of wells diffusion

Muller-Hinton agar, where the bacteria were grown by homogeneous diffusion over all parts of the culture medium than medium was left at room temperature to allow the bacterial culture to fully penetrate the medium, wells with a diameter of 8 mm were made on the surface of the medium with two concentrations (50 and 25) mg / ml were added to wells . The plates were left at room temperature for 10 minutes to allow the extract to diffuse through the medium then incubated at 37 ° C for 24 hours, after which the diameters of the inhibition zones around the well were measured (Zinedine and Faid., 2007).

Measurement of plant extracts on bacteria by using a spectrophotometer

The procedure was carried out in accordance with (Alan,A. and Hennessy, (2009) some of the changes we made to accommodate the research conditions. The colonies were collected from nutrient agar plates and cultivated in tubes with 1 mL of tryptone soy broth medium, which promotes bacterial growth, The turbidity was measured and compared to the McFarland standard solution No. 1.5 x 10^8 cells / ml. Bacteria were put to tubes and incubated for various periods of time, including (0m, 30m, 60m, 90m, 120m, 24h). A spectrophotometer was used to measure the optical density of the tubes using a 630 nm wavelength, and a tryptone soy broth medium was used to zero the device before each reading.

Detection of biofilm production of MR-CoNS by the microtiter plate (MTP) method

The biofilm formation was testing by quantitative microtiter plate (MTP). investigation was done Bekir *et al* (2011). The MR-CoNS isolate was grown in trypticase soy broth supplemented with 0.25 percent glucose in 96-well polystyrene tissue culture microtiter plates (Nunc, Denmark) overnight at 37°C. The culture media was removed after incubation, and adhering cells were fixed in 95 percent ethanol and stained with 1% crystal violet. At 630 nm, the absorbance was measured.

Detection of biofilm production of MR-CoNS by the Congo red agar (CRA) method

As described by Freeman and coworkers (1989), biofilm creation was carried out on Congo red agar (CRA) plates. The isolate was streaked on the CRA plate and incubated for 24 to 48 hours at 35°C under aerobic conditions. The non-biofilm producer bacteria created red colonies, while the staphylococci biofilm producer strains formed black and very black colonies. A darkening of the colony was indicated by the absence of dry crystalline colonial morphology, whereas very black isolates created a darkening of the colony with the presence of dry crystalline colonial morphology.

Sensitive the bacterial biofilm for antibiotics

Low inhibitory concentrations for antibiotics were determined by using by Vitek (BioMérieux). Two from antibiotics Moxifloxacin (Jamjoom) and Penicillin G (Drogsan) each dissolved 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 compac system, starting from the minimum inhibitory concentration (MIC) of 0.5 mg/ml and sub-MIC concentration of 0.25 mg/ml and half the sub - sub -MIC concentration of 0.125 mg/ml for Penicillin G, while the Moxifloxacin(MIC)concentration is 0.25 g/ml and Sub-MIC concentration 0.12 mg/ml and Sub-Sub-MIC concentration 0.0625 mg/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 examined by the crystal violet staine After 24 hours, culturing isolates on Trypton soya broth then. The same steps were completed by (Mathur *et al.*, 2006).

Effect aqueous extract of Ziziphus spina-Christi against of biofilm formation

The effect of aqueous extract of Ziziphus spina-Christi against inhibition of biofilm of bacterial biofilm formation by the aqueous extract of Ziziphius spina-christi with 50 mg/ml concentration was examined by the crystal violet staining. After 24 h, culture are isolates on the nutrient agar medium. A bacterial colony was taken for cultivation in Trypton soya broth then compare turbidity with McFarland tube, which estimating the number of bacterial cells 1.5×10^{-8} cell/ml, then The same steps were completed (Mathur *et al.*, 2006).

Statistical analysis

The data obtained in this research study was analyzed using SPSS statistical program. As the one-way variance was analyzed (ANOVA) and less significant Fisher difference (LSD) The tests were used to determine the source of variation and p Valuable in all experiences. P-value <0.05 was considered prominent (Morgan *et al* .,2004).

Results

Table (1)
Effect of antibiotics on biofilm

Sample	Antibiotics	μg/ml		(standard deviation ±average)
Staphylococcus warneri	Mixofloxacin only	0.25	MIC	0.011±0.164
		0.125	Sub- MIC	0.228± 0.228
		0.0625	Sub -Sub MIC	0.000[a] ± 0.260
0.020±0.425 control				
Staphylococcus warneri	Penicillin G only	0. 5	MIC	0.0138 ± 0.120
		0. 25	Sub MIC	0.008± 0.089

		0.1 25	Sub- Sub MIC	0.005 ± 0.151
0.017±0.647 control				
Staphylococcus warneri	sider	50		0.002±0 .272
0.0205± control				0.425

Table (2)
Synergic between antibiotic and extract effect on biofilm

Sample	Antibiotics	µg/ml		(standard deviation ±average)
Staphylococcus warneri	Mixofloxacin + sider	0.25	MIC	0.011±0.164
		0.125	Sub- MIC	0.009± 0.228
		0.0625	Sub -Sub MIC	0.000[a] ± 0.260
0.020±0.425 control				
Staphylococcus warneri	Penicillin G + sider	0. 5	MIC	0.013 ± 0.120
		0. 25	Sub MIC	0.008± 0.089
		0.1 25	Sub- Sub MIC	0.005± 0.151
0.020± control				.0425
Staphylococcus warneri	sider	50		0.002±0 .272
0.0205± control				0.425

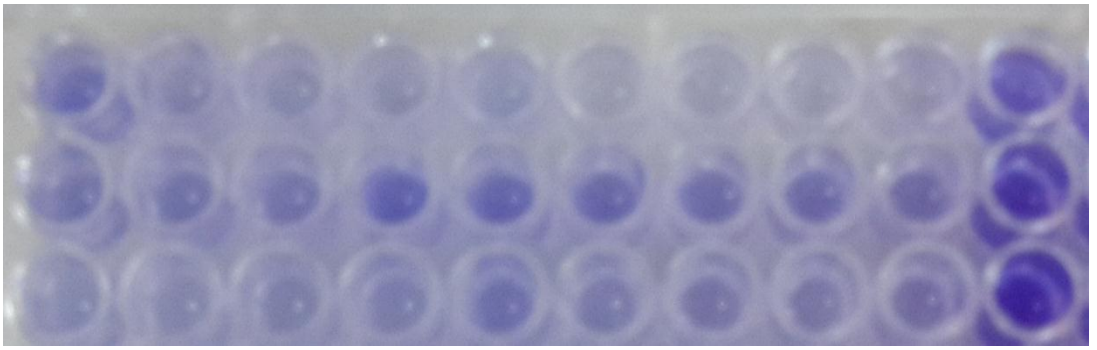

Figure (1) effect of extract and antibiotics on biofilm formation

Discussion

The inhibitory efficacy was tested by using microtiter plate method 96 well. The hot aqueous extract of sidr leaves showed an effect on the bacteria with concentrations (50) mg / ml this result was consistent with the results of both studies (Al-Mutairi *et al.*, 2016) when testing Sidr extract on a group of microorganisms, including bacteria. The inhibitory activity of the leaves is due to the active ingredients that contain, most notably alkaloids and saponins, which are known to be effective against microorganisms, Where it can be very effective in killing bacteria (Shahat, 2001), as well as being rich in flavonoids and furocoumarin, which inhibit the growth of bacteria through their attachment to DNA, and thus prevent their reproduction. The cells within the biofilms of bacteria, which are part of the biomass, have a high resistance to most antibiotics; from this it can be concluded that plant extracts containing a huge range of phytochemicals will provide biological efficacy against biofilms (Al Mussawi *et al*,2017).the results show the effect of two types of antibiotics with different concentration on biofilm formation of bacteria ,the concentration of 0.25 of Penicillin G was more efficiency than other concentration , this result appearance in table (1),(2),(3)

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