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RESEARCH ARTICLE

THE ANTIBACTERIAL EFFECT OF ETHANOLIC EXTRACT OF *Allium sativum* ON BIOFILM FORMING *Staphylococcus aureus* WHICH CAUSE FOLLICULITIS.

Rawaa AlChalabi, Ruqia Mohammed, Rana Adnan and Shatha AlBayati.

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Abstract

This study included isolation and identification of *Staphylococcus aureus* from folliculitis patients then detection the bacterial ability for biofilm formation, because biofilm play very important role in pathogenesis and antibiotics resistance of bacteria. Fifty specimens have been collected, 47(94%) specimens collected by swabbing gave positive culture and 3 (6%) specimens gave negative culture. After Gram 44(93.6%) isolates were Gram positive bacteria and 3(6.4%) isolates were Gram negative bacteria when identification of positive and negative bacterial isolates has been completed the results showed that 2 (4.3%) isolates were *Pseudomonas sp.*, 1(2.1%) isolates was *Klebsiella sp.* and 44 (93.6%) isolates were *S. aureus*. Thirty nine (88.6%) isolates of *S. aureus* produced biofilm and only 5 (11.4%) isolates not produced biofilm although isolates obtained from folliculitis patients and reflected resistance to antibiotics. Ciprofloxacin reflect high efficiency against *S. aureus* with inhibition zone (26mm) while ethanolic extract of locally garlic which showed high inhibitive ability against *S. aureus* with inhibition zone (27mm).

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Introduction:-

Medicinal plants are used in various tribal medicine due to minimal side effect and cost effectiveness. Garlic one of the oldest plants used in medicine ranks the highest of all the herbal remedies consumed for its health benefits. Scientific and clinical studies have shown that garlic can enhance immunity, protect against infection and inflammation and help lower the risk of cancer (Karwan, 2013 and Chekkiet *al.*, 2014). In addition, garlic may be effective against drug-resistant bacteria, and research has revealed that as allicin digests in your body, it produces sulfonic acid, a compound that reacts with dangerous free radicals faster than any other known compound. In order to get the health benefits, the fresh clove must be crushed or chopped in order to stimulate the release of an enzyme called alliinase, which in turn catalyzes the formation of allicin (Tessemæet *al.*, 2006 and Taylor, 2013).

Folliculitis is defined histologically as the presence of inflammatory cells within the wall and ostia of the hair follicle, creating a follicular-based pustule. The type of inflammatory cells varies depending on the etiology of the folliculitis and/or the stage at which the biopsy specimen was obtained (Saegemanet *al.*, 2017). When hair follicles are damaged, they may be invaded by viruses, bacteria and fungi, leading to infections such as folliculitis. Superficial folliculitis affects the upper part of the hair follicle and the skin directly next to the follicle while deep folliculitis affects the deeper portion of the follicle and can involve the entire hair follicle (Andre and Soares, 2015). Folliculitis is often caused by *Staphylococcus aureus* and may also be caused by viruses, fungi. Follicles are densest on your

scalp, and they occur everywhere on your body except your palms, soles, lips and mucous membranes and the treatments receive for folliculitis depend on the type and severity of your condition (Otberget *et al.*, 2008 and Sillaniet *et al.*, 2010).

Folliculitis occurs in both sexes and in all ethnicities and age groups. The most superficial form of skin infection is staphylococcal folliculitis, manifested by minute erythematous follicular pustules without involvement of the surrounding skin. Gram-negative organisms that commonly cause folliculitis in this setting are *Klebsiella* sp., *Pseudomonas* sp. (Sharquieet *et al.*, 2012). Regardless of the causative agent, individual lesions of folliculitis may be asymptomatic, painful, or pruritic. Lesions often heal spontaneously without scarring, although deep infection and excoriation may lead to scars. (Degitzet *et al.*, 2007 and Tchernev, 2011). Also, *Candida* may cause folliculitis in diabetics, the immunocompromised, and patients with chronic antibiotic therapy (eg, taking a tetracycline for acne vulgaris).

Staphylococcus aureus are Gram-positive, catalase positive cocci belonging to the *Staphylococcaceae* family. They are approximately 0.5-1.5 μm in diameter, non-motile, non-spore-forming, facultative anaerobes that usually form in clusters. Many strains produce staphylococcal enterotoxins, the super antigen toxic shock syndrome toxin (TSST-1), and exfoliative toxins. *Staphylococcus aureus* are part of human flora, and are primarily found in the nose and skin (Chambers and Deleo, 2009). *Staphylococcus aureus* is a virulent pathogen that is currently the most common cause of infections in hospitalized patients. *Staphylococcus aureus* infection can involve any organ system. The success of *Staphylococcus aureus* as a pathogen and its ability to cause such a wide range of infections are the result of its extensive virulence factors. The increase in the resistance of this virulent pathogen to antibacterial agents, coupled with its increasing prevalence as a nosocomial pathogen, is of major concern (Liu, 2010).

Biofilm is thin usually resistant layer of microorganisms (as bacteria) that form on and coat various surfaces (Bendouahet *et al.*, 2006). *Staphylococcus aureus* can produce a multilayered biofilm embedded within a glycocalyx or slime layer with heterogeneous protein expression. *Staphylococcus aureus* biofilm mode of growth is tightly regulated by complex genetic factors. Host immune responses against persistent biofilm infections are largely ineffective and lead to chronic disease (Corriganet *et al.*, 2009).

Materials and Methods:-

Specimens Collection:-

Specimens are collected by swabbing the involved areas of the skin. When pustules or vesicles are present, the roof or crust is removed with a sterile surgical blade. The pus or exudate is spread as thinly as possible on a clear glass slide for Gram staining. Specimens collected from patients of (Al- Yarmouk hospital and Al-Jabchi private Hospital during the period from Dec.-1-2016 to Mar.-1-2017).

Isolation of bacteria:-

The swabs were spread on nutrient agar, mannitol salt agar and chromo agar plates. Plates were incubated overnight at 37 °C. This process was repeated several times for purity before use for further diagnosis steps.

Detection the ability of bacteria for biofilm formation (Test tube Method):-

By using Christensen method for detection the ability of bacterial isolates for biofilm formation, this method included inoculation 5 ml of (Tryptcase soya broth) with particular isolates and incubated for 48 hours at 37 °C, after that, the contents of the tubes were removed carefully and added the crystal violet stain (1%) to each tube for 15 minutes then rinsed the tubes and let tubes to dry at room temperature (20-25)°C. The result was read by notice the formation of biofilm as a layer at the internal wall of tubes by naked eye and comprise with the negative control (tube contains Tsb medium without inoculation), thickness and color of layer consider a parameter of bacterial ability for biofilm formation (Christensen *et al.*, 1982).

Antibiotic sensitivity test:-

Ten ml of nutrient broth medium was inoculated with bacterial isolate, and incubated at 37 °C for 18 hours, transfer 0.1 ml (1.5×10^8 cell/ ml) of freshly broth (Growth of bacteria was monitored by McFarland tube No. 5 turbidity standard, which is equivalent to bacterial concentration for inoculum 1.5×10^8 organism / ml) to Muller-Hinton agar plate and streaked by sterile cotton swab three times by rotating the plate approximately 60 mm between streaking to ensure even distribution of the inoculum, the inoculated plates were placed at room temperature for 10 minutes to allow absorption of excess moisture, then antibiotic disks were applied by sterile forceps on the surface

of plates and incubated at 37 °C for 18 hours in an inverted position .After incubation, measured the diameter of inhibition zone (clear area around disks) by ruler which indicate the sensitivity of bacteria to that antibiotic and the result were compared with NCCLS (Atlas *et al.*, 1995) .

Plant Extraction:-

Garlic (*Allium sativum*) used in the present study was purchased from the local market of Baghdad, Iraq. The fresh garlic cloves were washed, peeled, sliced and sun dried for seven days. After drying, garlic was ground to fine powder by using an electric blender. 60 gm powder of garlic was soaked in 300 ml of 90% ethanol , The flask was incubated at room temperature for 5 days with shaking at 140 rpm. The crude extract was filtered by using 0.22 filter unit then concentrated in a rotary evaporator. Dried crude extract was dissolved in DMSO separately to the final concentration of 300 mg/ml (Gull *et al.*, 2012) .

Antibacterial activity by agar well diffusion Method:-

Agar well diffusion method is widely used to evaluate the antimicrobial activity of plants extracts . The agar plate surface is inoculated by spreading 100 µl of the bacterial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip and (100 µl) of the extract solution at desired concentration is introduced into the well. Then, agar plates are incubated overnight at 37°C. The antibacterial activity determined by measurement of inhibition zone (Balouiri *et al.*, 2015) .

Results and Discussion:-

Fifty specimens were collected from patients suffering from folliculitis by swabbing from two different hospitals in Baghdad. All the isolates were identified by using cultural, morphological and biochemical tests (Cruckshank *et al.*, 1975). Results showed that 47 out of 50 gave positive culture as shown in table (1) .

Table 1:- percentage of positive and negative culture of specimens.

Culture		No. of Isolates	Percentage(%)
1-	Positive	47	96
2-	Negative	3	6

At the beginning, microscopically examination showed that 3 (6.4 %) isolates out of 47 samples were classified as Gram negative bacteria and 44 (93.6 %) isolates as Gram positive bacteria . This result agreed with result reported by Suzanne and Maximo ,(2009) who recorded bacterial skin infections are a common problem encountered in clinical practice range from superficial epidermal infections to life-threatening necrotizing fasciitis. Although most infections can be managed on an outpatient basis, physicians must remain alert for signs and symptoms indicative of a more serious infection requiring rapid evaluation and hospital admission and most bacterial infections are caused by gram-positive organisms, including *Staphylococcus aureus*, group and, *Streptococcus viridans*, and *Enterococcus faecalis*. Less common causes of infection include gram negative organisms such as, *Pseudomonas* sp., *Aeromonas* sp. and *Klebsiella* sp.

After performing biochemical tests and api 20 for bacterial isolates , results showed that 2(4.3%) isolates was identified as *Pseudomonas* sp., 1 (2.1. %) *Klebsiella* sp. and 44(93.6%) isolates identified as *Staphylococcus aureus* as shown in table(2) .

Table (2):- percentage of bacterial isolates.

Staining pattern		No. of Isolates	Percentage %	Bacterial isolates	No. of Isolates	Percentage %
1.	G- VE	3	6.4	<i>Pseudomonas sp.</i>	2	4.3
				<i>Klebsiellasp.</i>	1	2.1
2.	G+ VE	44	93.6	<i>Staphylococcus aureus</i>	44	93.6

This result is compatible with result recorded by Raza *et al.*, (1996) they found that skin diseases can be caused by viruses, bacteria, fungi, or parasites and the most common bacterial skin pathogens is *Staphylococcus aureus*. Also, Findley and Grice, (2014) reported that specific microbes in skin disease, but whose pathogenesis may be complicated by microbial community interactions and/or host-microbe interactions. The specific microbes strongly associated include *Staphylococcus aureus*, *Propionibacterium acnes*, and *Malassezia* spp., all of which are known skin commensals but also exhibit pathogenic potential under certain conditions. There are well-characterized skin pathogens that have been definitively linked to dermatological disorders(Wang *et al.*, 2013),.

Biofilm Formation:-

Test tube method used to detect ability of pathogenic *Staphylococcus aureus* isolates which isolated from skin of folliculitis patients for biofilm formation. The result illustrated in table (3) showed high percent of *Staphylococcus aureus* isolates were able to form biofilm. 39 (88.6%) *Staphylococcus aureus* isolates formed biofilm with different degree of thickness and only 5 (11.4%) isolates unable to form biofilm .

Table 3:- percentage of biofilm formation by *S. aureus* isolates.

<i>S. aureus</i> Isolates		No.	Percentage(%)
1.	Biofilm Former	39	88.6
2.	Biofilm Non-former	5	11.4

Nitsche-Schmitz *et al.*, (2007)notice that microorganisms differ in it's ability to produce biofilm, thickness of biofilm differ according to the genus and species of producing bacteria, conditions like temperature, pH and type of folliculitis .The *Staphylococcus aureus* isolates formed biofilm with different thickness .Bacterial adhesion and subsequent colonization of surfaces are the first step toward biofilm formation . Biofilm consisted of microcolonies encased in extracellular polysaccharide material which formed under selected conditions (Olson *et al.*, 2002;Anderson *et al.*, 2007),.

The biofilm develops on both living surfaces and artificial implants which allow the bacteria to persist for long period by establishment of dormant reservoir of pathogens , re-emergence of bacteria from this reservoir might be the source of recurrent infection(Deleo *et al.*, 2010). *Staphylococcus aureus* represents the most common bacteria caused skin diseases with possess high ability to produce biofilm which enable them to find safe haven and subvert clearance by innate host response. Because of the matrix of biofilm modify the environment of adherent cells by concentrating nutrients and protecting the cells from surfactant, biocides , phagocytic cells and antibiotic are generally not very effective against organism embedded in biofilm. Cells form biofilm express properties distinct from plank tonic cells which increased resistance to antimicrobial agents. Biofilm formed by *Staphylococcus aureus* significantly enhances antibiotic resistance by inhibiting the penetration of antibiotics, resulting in an increasingly serious situation(Yan *et al.*, 2016 and Suviet *et al.*, 2017)

Antibiotics Sensitivity:-

Antibiotics are biochemical compounds naturally produced by certain types of microorganism (bacteria and fungi) that inhibit the growth or kill other microorganism(David, 2013). The emergence of resistance of bacteria to antibiotics is a common phenomenon which considered major problem reflects evolutionary processes that take place during antibiotic therapy. The antibiotic treatment may select for bacterial strains with physiologically or genetically enhanced capacity to survive high doses of antibiotics. Under certain conditions, it may result in preferential growth of resistant bacteria, while growth of susceptible bacteria is inhibited by the drug(Rhee and Gardiner , 2004 and Ocampo *et al.*, 2014). In this study the effect of antibiotics on *Staphylococcus aureus* isolates

were tested by using standard disk diffusion method and results were obtained compared with the NCCLs .Results illustrated in tables (4) and figure (1) .

Table 4:-The percentage of antibiotics resistance of *S. aureus* isolates

Antibiotics		Resistance	
		No.	Percentage (%)
Penicillin	P	44	100%
Cephalexin	CL	44	100%
Cefotaxime	CTX	44	100%
Vancomycin	VA	41	93.2 %
Clindamycin	CD	5	11.4%
Tetracycline	TE	44	100%
Amoxicillin +Clavunic acid	AMC	44	100%
Nalidexic acid	NA	44	100 %
Ciprofloxacin	CIP	0	0%
Rifampin	RA	13	29.7%

Moreover the table (4),indicate rang of resistance of *Staphylococcus aureus* isolates which gave very high resistance percentage to penicillin (100%), (cephalexin 100%, Cefotaxime 100 % ,Tetracycline 100% and Nalidexic acid 100%) and gave varied resistance percentage to Amoxicillin +Clavunic acid (100%),Vancomycin (93.2 %), Rifampin (29.7%) and Clindamycin (11.4%). while gave no resistance to ciprofloxacin . The prevalence of antibiotic resistance bacteria therapeutic problems that could be explained by several hypothesis such as influence of excessive in appropriate antibiotic used, antibiotic resistance among pathogenic bacteria that cause infections . Different types of antibiotics are discovered, some of them classified as broad spectrum antibiotic which effect on a wide range of bacteria (Gram positive and Gram negative), while others classified as narrow spectrum antibiotics effected on a limited type of microorganism (Tanet *et al.*, 2015).Certain types of bacteria are inherently resistant to the effect of particular antibiotic, this is called innate or intrinsic resistance, while resistance of other bacteria to antibiotic types considered as acquired resistance which may result through spontaneous mutation or the acquisition of new genetic information (Gill *et al.* , 2014).

The resistance of bacteria to particular antibiotic may result from mutation which change the components of bacterial cell or the bacteria may have the plasmid carrying genes encoded for these resistance or by transposons that encoded for resistance and have the ability to transfer to another plasmid which lack to the resistance property, the acquired of resistance between the bacterial cells may result from conjugation , or transformation or transduction . The determination of the resistance or sensitivity of bacterial isolates depends on the measurement of the diameter of inhibition zone. The results show that *Staphylococcus aureus* isolates gave very high resistance to β -lactam antibiotic because it produce β -lactamase. Also ,*S. aureus* isolates reflect high resistance to cephalexin (100%) which represent the first generation of cephalosporine and resist to cefotaxime with percentage (100 %),this resistance belong to the production of cephalosporinase .

Biofilm has an active role in bacterial pathogenicity because bacteria embedded in a matrix of host proteins and microbial slime, which provided a home for organism and promote increased drug resistance thus antibiotic less effective in biofilm cells than in planktonic cells.

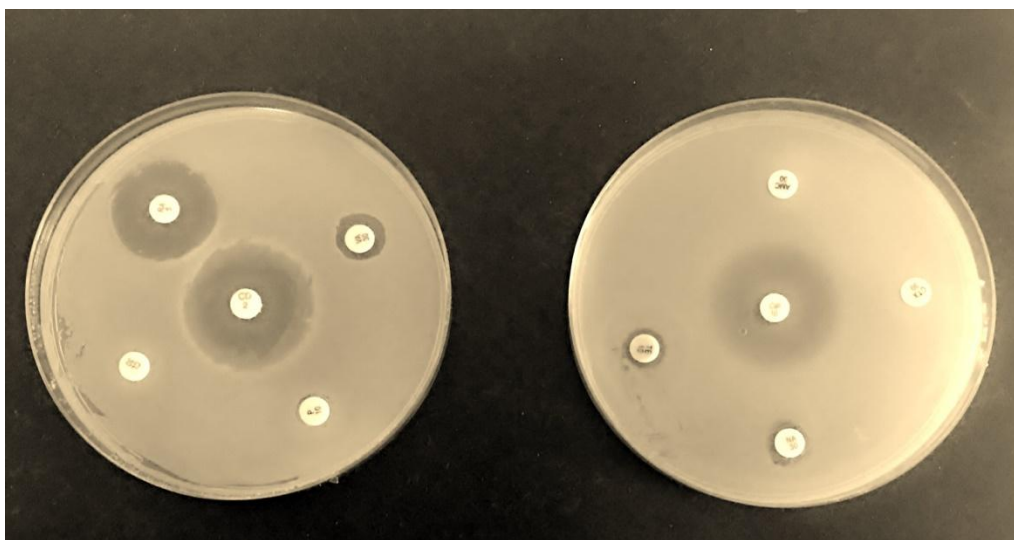


Figure 1:- Antibiotic sensitivity test by disk diffusion method.

Determination of antibacterial activity of *Allium sativum* methanolic extract against *S. aureus* isolated from Folliculitis infection:-

Antibacterial activity of *Allium sativum* methanolic extract against *Staphylococcus aureus* isolated from folliculitis infections were diagnosed according to laboratory culture was determined by agar well diffusion method with different concentrations of *Allium sativum* methanolic extract. Results showed that ethanolic extract at concentration (300 mg/ml) have strong antibacterial activity against more efficient biofilm forming and multidrug-resistant *Staphylococcus aureus* isolates and the diameter of inhibition zone (23-28)mm as shown in figure (2) a while other concentration of extracts (150, 75 mg/ml) have no antibacterial activity as shown in table (5). The inhibition activity depends on concentrations (increased the concentrations of *Allium sativum* methanolic extract lead to increase the diameter of the inhibition zone).

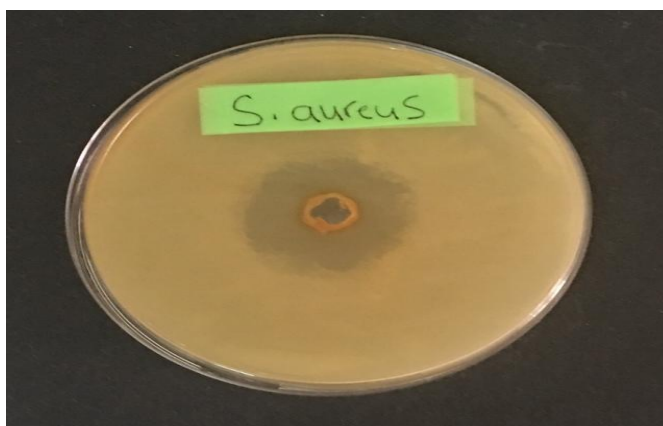


Figure 2:- Antibacterial activity of the *Allium sativum* methanolic extract

Table 5:- Antibacterial activity of the *Allium sativum* methanolic extract on more efficient biofilm forming *Staphylococcus aureus* isolates.

Bacterial Isolates	Concentrations of <i>Allium sativum</i> methanolic extract (mg/ml)		
	Zone of inhibition (mm)		
	75 mg/ml	150 mg/ml	300 mg/ml
S1	---	----	28
S2	----	----	25
S3	----	----	23
S4	----	----	23

S: *Staphylococcus aureus*

Garlic is a plant with various biological properties like antimicrobial, anti-cancer and antioxidant. Garlic extracts demonstrated activity against Gram negative and Gram-positive bacteria including species of *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus*, *Clostridium*, *Helicobacter pylori*. Allicin is thiosulfate compound of garlic reported for its antibacterial activity. Allicin is proved to be anti-bacterial as it inhibits RNA synthesis (Akintobiet al., 2013 and Desalegn, 2014). The composition of *Allium* bulbs is complex. Garlic contains volatile oil (0.1-0.36%), the major components are Sulphur compounds like alliin. It contains also proteins (amino acids, glutamyl peptides), , enzymes (alliinase, peroxidase, myronidase). Allicin is formed from alliin by the alliinase. It is considered that 1 mg of alliin is equivalent to 0.45 mg of allicin. Organo-sulfur compounds, flavonoids, saponins and saponins, selenium compounds and fructosamines have been recognized as the main bioactive principles in raw garlic and different garlic supplements (Fleischauer et al., 2000 and Viswanathan et al., 2014). Evidence from several investigations suggests that the biological and medical functions of garlic are mainly due to their high organo-sulphur compounds content. Drugs derived from plants are effective, easily available, and less expensive and rarely have side effects. The practitioners of traditional and indigenous medicine rely mainly on medicinal plants and herbs for preparation of therapeutic substance. Initial screening for the potential antibacterial and antifungal compounds from plants may be performed by using the crude extracts (Duraijet al., 2010).

Furthermore, comparison between the inhibitory activity of the garlic extracts and antibiotics on Gram-positive *Staphylococcus aureus* revealed that ciprofloxacin had the high inhibitive activity with inhibition zone smaller than zone of extract against the susceptible *S. aureus* isolates. The variation in the size of the inhibition zone among the different isolates may be due to the content of the membranes, the permeability of allicin and other garlic constituents. There are a greater number of studies showing antimicrobial activity of garlic against bacteria, fungi, virus and human intestinal protozoan parasites.

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