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Anti-Virulence Effects of Pomegranate Peel Extracts on Most Common Urinary Tract Infection Pathogens in Pregnant Women

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Abstract
A total of 100 morning mid-stream urine samples from pregnant women were streaked on general and selective media, the growing bacteria were diagnosed biochemically and by using Api system.The results revealed the occurrence of 52 isolates of pathogenic bacteria which were dividedin to Gram– positive bacteria 43 (83%) and Gram – negative bacteria9 (17%).Then the ability of *S. aureus* and *E. coli* bacteria which were responsible for the highest percentage of urinary tract infection to form biofilm were tested by using tube methods (TM), in order to select one isolate for each bacteria that having the highest ability to form the biofilm to continue the other steps of the study. Then the ability of these two isolates to form some virulence factors such as hemolysin, protease, β – lactamase , biofilm and adherenceare tested.Pomegranate peel had been extracted by three solvents (alcoholic 96%, aqueous and the acetone 70%). The inhibition zones diameters of the pomegranate peel extracts against studied bacteria increase significantly with concentrations increase. The MICof the extracts was determined against studied bacteria which reached to 0.04 g/ml when used both the ethanol and the acetone extracts, while reached to 0.06 g/ml in the aqueous extract. The ability of two studied bacteria to produce virulence factors were compared before and after treated with MIC of extracts which revealed there is not effect of the extracts on the ability of studied bacteria to produce hemolysin and protease enzymes, while both *S. aureus* and *E. coli* bacteria loss its ability to produce β – lactamaseenzyme after treated with MIC of the extracts. In addition extracts were affected largely on adherence activity & biofilm forming ability of tested bacteria and there affect difference with the different kinds of the extracts.

Introduction
Urinary tract infection (UTIs) is an infection caused by the presence and the growth of microorganism anywhere in the urinary tract, so it could be defined as the colonization of and invasion of the structures in the urinary tract by micro-organisms(1). In human the urinary tract was one of the most common sites of bacterial infection and most case of urinary tract infection was caused by bacteria which ascend from the perineum, and the reason of the ascent of bacteria was raised by conditions like pregnancy (2&3). Urinary tract infections are relatively common problems during pregnancybecause the Physiologic changes which are related to pregnancy that make otherwise healthy women susceptible to serious infectious complications (4).Urinary tract infections are caused by gram-negative&positive bacteria like *Escherichia coli, Klebsiella* species, *Proteus mirabilis*, *Pseudomonas aeruginosa, Enterococcus, Staphylococcus, and Streptococcus agalactiae*(5).The isolate bacteria produce deferent virulence factors which play essential roles in pathogenesity of these bacteria such as hemolysin(is an important virulence factor which attached with especial receptors on the erythrocytes wall then making pours in the cell wall so the erythrocytes will lyses), β -lactamase (is enzyme that cleaves the ß-lactam ring and inactivates the antibiotic) contain serine amino acid in their hydroxlic group which represented the active site of this enzyme(6).

Pathogenic bacteria adhesion to the host tissue was an important initiating step in many types of infection by helping the bacteria to resist the defense mechanism in the body, enable colonization and growth, and might be the first phase in bacterial invasion into mammalian cells (7). Bacteria living in the outer environment as well as the pathogens bacteria special the human pathogens, which have the ability to form biofilm, a slimy layer with embedded micro colonies and this is the most important and most widespread mode for increasing pathogen of the microorganism and the biofilm formation helps bacteria to resist the surrounding environment condition(8).

Some resistant pathogens which responsible for the infection of urinary tract are routinely complicated to be dealt with due to their virulence factors and because of a relatively limited choice of antimicrobial agents. Thus, it is extremely important to find novel antimicrobials or new techniques that are effective for the treatment of infectious diseases caused by drug-resistant microorganisms. Many studies have demonstrated that plants either contain antimicrobials that can operate in synergy with antibiotics or possess compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to a previously ineffective antibiotic (9).Many of plants are used in medicine for treatment of disease such as pomegranate which was one of the oldest fruits that have no changed much through the history of man. The pomegranate (*Punica granatum*) has been used to treat several diseases and consumed mainly fresh or in beverages and is a rich source of phenolic compounds, including hydrolyzable tannins, which possess high antioxidant activity. (10 &11). So the purpose of this study was aimed to find a safety methods to reduce pathogenicity of high virulence pathogenic bacteria

responsible for UTI by use natural material & attempt to find a safety method to solve the problem of multi drug resistance of pathogenic bacteria.

Materials and methods

1.Isolation and diagnosis bacteria

One hundred morning midstream urine were collected from pregnant women attending to Maternity and Women's Hospital in Karbala Province during December 2011 to March 2012 samples. The isolated bacteria were diagnosed biochemically according to methods described by (12, 13).Then the diagnosed bacteria were confirmed by APi20E and Api Staph system accomplished according to manufacturer's instructions. The isolated bacteria used in the study were chosen according to their ability to gave deep violet color on the wall and bottom of the test tubes, which was used for determination ofthe biofilm forming as described by(14).

2. Ability of isolated bacteria to produce virulence factors

Blood agar plates and skim milk agar plates were used to determined theability of isolated bacteria to producehemolysinand protease enzyme respectively as described by (12&13). The β – lactamase production was prepared according to(15).The adherence activity for studied bacteria was carried out according to(16) . In addition we screwed isolates for their ability to form biofilm by tube &tissue culture plat methods as described by (14 &17).

3.Plants extracts

Plant extracts(aqueous,alcohol & acetone) were prepared according to (18& 19).Then stock solution was prepared for each extract by dissolving 1g of dry extract with 10 ml of distilled water, so the final concentration of extract would be 0.1 g/ml, from this solution other concentration were prepared (0.01-0.1)g/ml, which was used to determine antibacterial activity of peel extracts against *S.aureus* and *E .coli* bacteria byagar well diffusion method as mentioned in (21) but agar dilution method was used to detect MIC of the plant extracts according to (22). The extracts were subjected to phytochemical screening according to(20).

4. Effect of pomegranate peels extracts on the bacterial virulence factors

Minimim inhibition concentration (MIC) of each extract was added to the bacterial suspension, andalltests were made as mentioned in steps2

Result and discussion

1.Frequency of urinary tract pathogens in pregnant women

Fifty two bacterial isolates were isolated in this study ,gram– positive bacteria43(83%) occurred more frequently than gram- negative bacteria 9(18%) where *S. aureus* 20(39%) and *E. coli* 6(11%) were the commonest offending isolated as shown in figure (1). This might be duefirstly to environment , the socioeconomic conditions of the pregnant and the reinfection, when infection was happened in the first trimester (some time it is possible in the third trimester) of pregnancywhich was ensured by the patients' information.

One isolate of both *S. aureus* and *E. coli* had been chosen to continue and complete other steps of the study. these two isolates were subjected to standard tests for determination their ability to produce different virulence factors such as hemolysin ,protease , β –lactamase,adherence and biofilm formation ability. From the result ,it appeared that these two isolates gave positive results for the previous testtests.Many studies indecatethe relationship between the bacterial virulence factors and their pathogenicity such as (23) who found that the bacteria which had the ability to produce the hemolysin and protease enzymes in some way they were showed increase in its invasion activity and ability to resist host immune system.While(24) reported that several virulence factors such as hemolysin, cytotoxic necrotizing factor, aerobactin, biofilm and different types of adhesion have been responsible for *E. coli* pathogenesis, because of the relationship between the bacterial ability to produce virulence factors and their infectivity or pathogenicity.

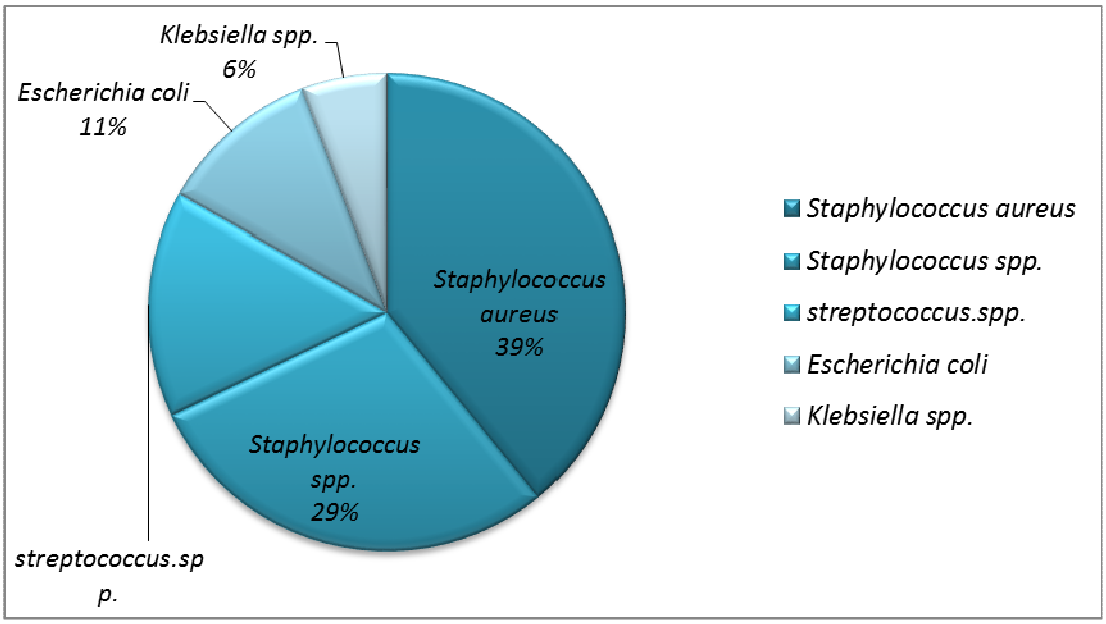

Figure (1): Frequency of urinary tract pathogens in pregnant women.

2- Plant extracts

Pomegranate peel extracts screening alkaloid, saponins, flavonoids, tannins, phenolic, glycosides and resinsas a phytochemical. The flavonoids and saponins were absent in aqueous extract, but during ethanol extraction, only saponins were not extracted the. While in the acetone extraction this solvent succeeded to extract nearly all the studied active material. The active material in the pomegranate peel needed the polar solvent to be extracted in a good way and that mean that most of these materials were extracted in good amount by the ethanol .The mode of action of these compounds such as polyphenols is generally attributed to polyphenol-protein interactions, though different mechanisms have been suggested including inhibition of microbial enzymes and action on membranes or deprivation of substrates were required for microbial growth (25).The antibacterial activity of plant extracts depends on the extraction conditions such as type and concentration of the solvent, time and temperature for the extraction process, all these factors effect on the type and the amount of the active material that extracted (26).

3. The antibacterial activity of pomegranate peel extracts

The resultsshowed clearly that pomegranate peel extracts were active against *S.aureus* and *E .coli* bacteria in comparison to ciprofloxacin as a positive control and the distilled water as a negative control. The alcohol solvent could be considered as the best one among the three solvents which were used in this study. Acetone follow alcohol and distilled water might be good solvent with respect to their activity against the chosen isolates as shown in tables (1& 2). The results showed a relationship between the value of inhibition zone diameter for each one of studied bacteria and type of solvents used in the extraction process. When the three solvents were used at different concentrations against *S. aureus* bacteria, alcohol solvent gave the widest inhibition zone, when used at (0.01, 0.05, 0.075 and 0.1) g/ml and it was followed by the value of inhibition zone diameter for the acetone extract, but the aqueous extract had the lowest effect when it used at the concentrations above and when 0,025 g/ml concentration of these three types of extracts were used against *S. aureus*, the acetone extract was the best one as the highest effect then followed by the alcohol and the aqueous extracts.Among the solvent and according to their effects on *E .coli*isolate, acetone extract was considered as the best one when it used at 0.01 g/ml and 0.075 g/ml, but the alcohol extract had the highest effect on *E.coli*bacteria at 0.025 g/ml, 0.05 g/ml and 0.1 g/ml concentration in contrast with the other extracts.

When the pomegranate peel extracts were used as an antibacterial the best solvent chosen as extractor could be the polar solvents especially the ethanol solvent due to the best effect on both selected isolates. The results agree with (27) who attributed to the antibacterial activity of pomegranate peel extracts to the presenceof the broad spectrum antimicrobial compounds that act against both selected isolates.

Table (1): Antibacterial activity of extracts against *S. aureus*bacteria.

Concentration g/ml	Inhibition zone rate (cm)			Concentration Range
	Aqueouspomegranate peel extract	Alcohol pomegranate peel extract	Acetone pomegranate peel extract	
0.01	1.25*	1.65*	1.45*	1.45*
0.025	1.6*	1.73*	1.9*	1.74*
0.05	1.61	1.95*	1.91	1.82
0.075	1.81*	2.21*	2.00	2.01*
0.1	1.88	2.26	2.5*	1.96*
Extraction Range	1.63*	1.96*	2.03*	
Control (Ciprofloxacin) Antibiotic 5µg/disc	1.2			

LSD $_{0.05}$(concentration =0.064, extraction solvent =0.58,interaction = 1.296)
P≤ 0.0001* = Significant different

Table (2): Antibacterial activity of extracts against*E. coli*bacteria.

Concentration g/ml	Inhibition zone rate (cm)			Concentration Range
	Aqueouspomegranate peel extract	Alcohol pomegranate peel extract	Acetone pomegranate peel extract	
0.01	1.38*	1.50*	1.68*	1.52*
0.025	1.51*	1.90*	1.76*	1.72*
0.05	1.63*	1.93	1.91*	1.82*
0.075	1.73*	2.08*	2.21*	2.01*
0.1	1.78	2.36*	2.33	2.15*
Extraction Range	1.64*	1.97*	1.98*	
Control (Ciprofloxacin) Antibiotic 5µg/disc	2.5			

LSD $_{0.05}$(concentration =0.086, extraction solvent =0.077,interaction = 1.737)
P≤ 0.0001* = Significant different

3. Ant-virulence activity of pomegranate peel extracts

The studied bacteria which had the ability to produce number of the virulence factors (hemolysin, Protease, β – lactamase) were treated with the (MIC)of each one of the plant extracts (which reached to 0.006 g/ml in aqueous extract ,0.004 in both alcohol and acetone extracts).The results explained that capacity of *S .aureus* and *E.coli* bacteria to produce hemolysin toxin and protease enzyme had not been affected and remained without any alteration after incubation period with these extracts in compare with the control. While these bacteria were completely lost their ability to produce β – lactamase enzymes after their treatment with the MIC of each extract, although these bacteria were active producer for this enzyme before they have been treated with the extracts, as shown in table (3).

Forty cells of *S.aureus* bacteria adhered on the assayed epithelial cell, while only 20 cells of *E. coli* adhered to the epithelial cells. These data were regarded as control for detection the effects of the MIC of pomegranate peel extracts on adhesion ability of studied bacteria.

As show in figure (2) the number of adhered *S. aureus* bacterial cells on the epithelial cell which was clearly declined when the bacteria treated with MIC of extracts. The aqueous extract reduced the number of the adherence bacterial to only 10 bacteria /cell, but the number of the adherence cells reach to 3 and 1 bacteria /cell in the presence of the acetone and the alcohol extracts respectively.

The adherence of *E. coli* cell reached to one bacteria /cell when the acetone extract was added to bacterial suspension, but only three bacterial cell seen to be attached to epithelial cells after the bacterial suspension was incubated with alcohol extract, while only 5 bacteria/ cell were attached after treatment with the aqueous extract, in contrast with control (*E. coli* without the extracts), as shown in figure (3)

As a comparison ethanol extract was the best anti adhesive factor, and followed by the acetone, while the aqueous extract had the least effect. This may return to the weak ability of distilled water to extract the active materials from plants peel in affected amounts in compare with acetone and alcohol solvents.

The pomegranate peel extracts have been worked as anti –adhesive, because of the large amounts of the saponins, flavonoids, alkaloids, tannins, phenolic, glycosides and resinsin, which were directly responsible for the anti-adhesive activity against pathogen (28). The effect of the plant extracts is return to its ability to inhibiting cell attachment,therefore that pretreatment of the body surface with plant extracts produced an unfavorable film that preventing and reduce the surface adhesion of pathogenic bacteria (29).

Table (3): Ability of isolated bacteria to produce virulence factors with and without extracts

Virulence factors	*S .aureus*	*S .aureus* with Pomegranate peel extracts by			*E .coli*	*E .coli* with Pomegranate peel extracts by		
		Distell water	96% ethanol	70% acetone		Distell water	96% ethanol	70% acetone
Hemolysin	+	+	+	+	+	+	+	+
Protease	+	+	+	+	+	+	+	+
β –lactamase production	+	-	-	-	+	-	-	-

Key: (+) indicates present and (-) indicates absent

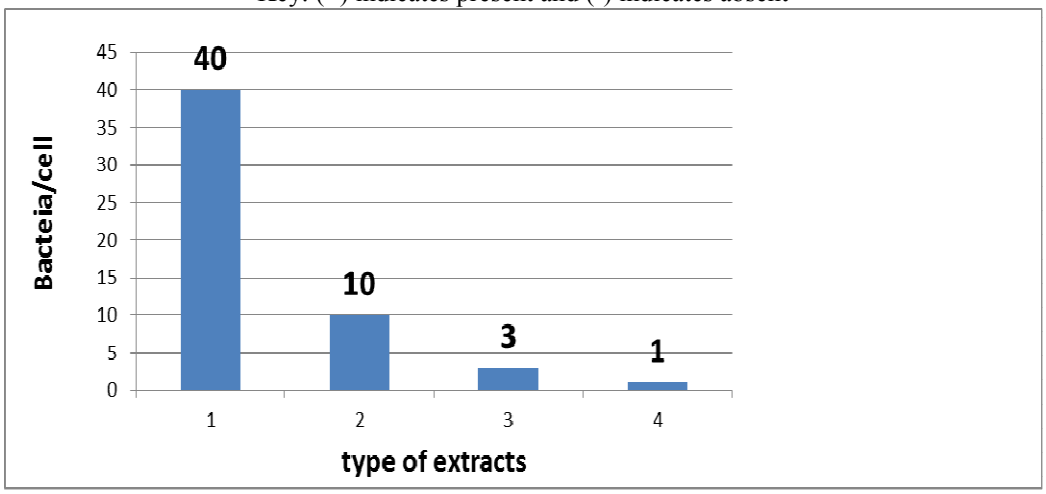

Figure (2): Effects of extracts on adherence activity of *S. aureus*bacteria.

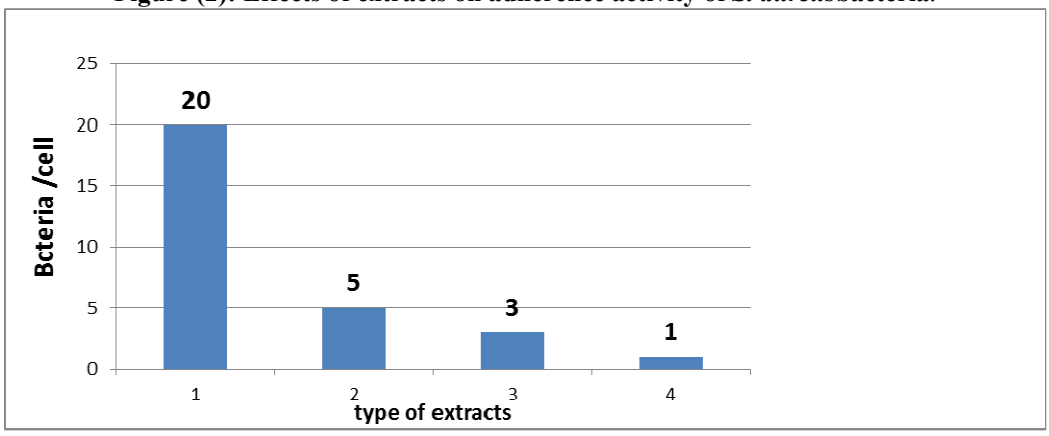

Figure (3):Effects of extracts on adherence activity of *E. coli*bacteria

Figure (4) illustrated that *S .aureus* was high producer for biofilm formation; this was shown through its optical density that reaches to 1.7 when it was measured for bacterial suspension without any types of the extracts (regarded as control). But, the optical density came to be reduced largely when the MIC of each one of the extracts was added to *S .aureus* suspension before reading the optical density. The optical density(OD) of *S .aureus* suspension with the MIC of the aqueous and the acetone extracts almost the same, and this results was different from the results of the other studies which found that the acetone extract had more effect than the aqueous extract. Whereas the alcohol extracts still the best solvent in its effects on studied bacteria.*E. coli* was regarded as high producer biofilm because the OD of its suspension without extracts reached to 1.5. But when

the suspension of *E.coli* bacteria treated with the MIC of the aqueous and acetone extracts the OD of it reduced. that mean the biofilm formation activity were declined clearly in compared with the control, as shown in figure (5)

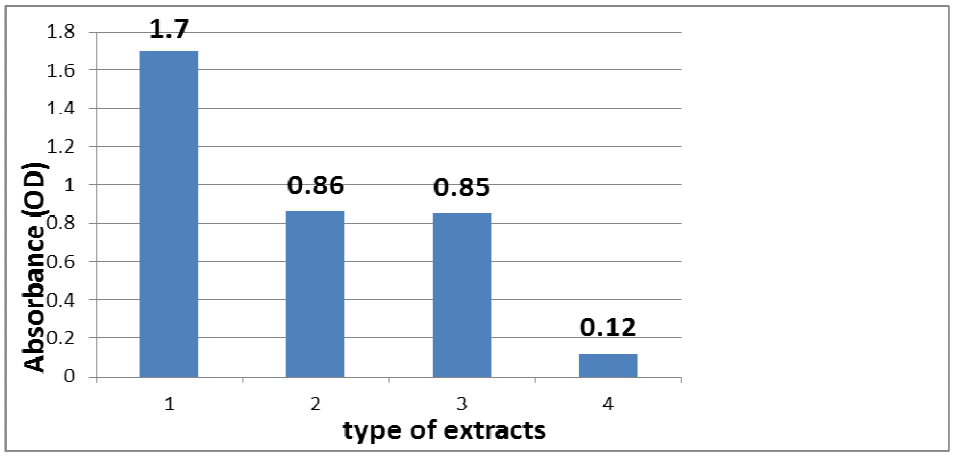

Figure (4):Effects of extracts on biofilm formation activity of *S. aureus* bacteria

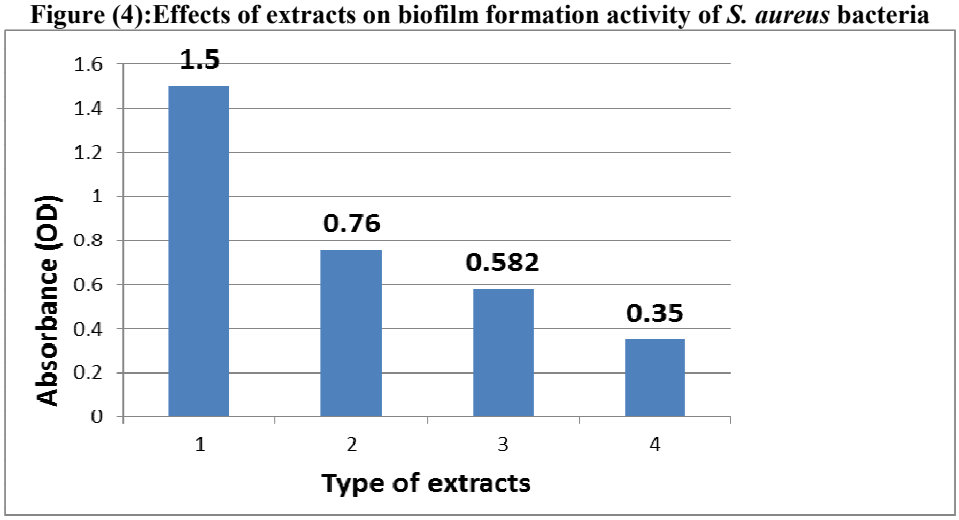

Figure (5):Effects of extracts on biofilm formation activity of *E. coli* bacteria

The biofilm formation activity of bacteria were declined after treatment with extracts but this decline do not transfer bacteria from high producer (The control) to the poor producer (OD lower than 0.1) after treatment with extracts. The *S .aureus* and *E.coli* bacteria were treated with the aqueous andthe acetone extracts remained as a high producer (OD high than 0.5), but when the bacteria treated with alcohol extract it came to be transferred from high producer to producer only (OD between 0.5 – 0.1). This proved that alcohol solvent represented as the best one in extraction and preserve the activity of active compounds in pomegranate peel extract in compared with the acetone and aqueous extracts and these results may be due to form the variety of biological properties and the activity of the extracts chemical composition when different solvents were used in preparing these extracts.

Many searches search for any material could be inhibitthe cell attachment which is the initial stage in biofilm formation following surface conditioning (which is achieved by the adsorption of substances that include nutrients, organic and inorganic molecules) that creates a favorable environment for bacterial attachment, growth and aggregation(29& 30).

Bacterial exopolysaccharides have always been suggested to play crucial roles in the bacterial initial adhesion and development of complex architecture in the later stages of bacterial biofilm formation. Therefore; many searchers were emphasizing by many trials to find some material that inhibit the activity of these bacterial materials such as chemical material that extract from plant(30& 31).

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