



The antibacterial & antibiofilm activity of *Punica granatum* peel aqueous extract against some oral pathogen

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Abstract

This study was designed to evaluate the antibacterial & antibiofilm activity of *Punica granatum* peel aqueous extract against oral pathogens from patients suffering from gingivitis and dental carrier. These isolates were 58.8% gram positive (*Lactobacillus spp.*, *Streptococcus spp.* and *Staphylococcus aureus*) and 41.2% gram negative (*Klebseilla pneumonia* and *pseudomonas aeruginosa*). The susceptibility of these different bacterial species toward the aqueous extract of this plant was applied by using well diffusion agar method. The results were compared with each other and with selected antibiotic ciprofloxacin as positive control. Results showed that aqueous extract of *Punica granatum* had antibacterial activity against all isolates, on which the diameter of inhibition zone at concentration 200 mg/ml of this extract was 24mm for *Lactobacillus spp* and 23 mm for *Staph aureus* and *P.aeruginosa*, while the *Streptococcus spp.* was only 18 mm.

Also antibiotic sensitivity test was done for these isolates towards locally used drugs which ordinarily used to treat gingivitis and dental carrier. The results showed a resistance for most of these antibiotic and illustrated that most isolated bacteria undergo decreasing in biofilm formation activates after incubated with MIC of aqueous extract , this changing in biofilme formation activity for isolated bacteria was determined due to the average of optical density (O.D) at 540 nm.

Introduction

In the different sites of the mouth, the bacteria are able of cohabiting in saprophytism, depending directly on many factors such as pH, availability of nutrients and natural of mucous surface. The establishment and maintenance of oral microbiota is related to inter bacterial co aggregation and biofilm formation, which constitute the primary etiologic agents of oral diseases(1). The plaque (biofilm)related infection such as dental caries and Periodontal disease represent two most common types of dental disease. The dental plaque provides ground for the inhabitancy of pathogenic bacteria that lead to the tooth decay, where bacterial processes change sugar in food left on tooth to acid that demineralization hard tooth structure from calcium and progressively break down(2).

The demineralization which caused particularly by *Streptococcus* bacteria occurs within dental plaque that adheres to the tooth surfaces and become colonized by other bacteria such as *Lactobacillus sp*, some species of gram negative bacteria, yeas and that responsible to secondary infection in mouths (3). Antimicrobial agent against oral pathogen play an important roles in prevention of oral disease, but in same time the antibiotic use have been identified as major factors in the emergence of antibiotic resistance bacteria. Therefore the therapeutic effects of medical plants has increased dramatically and phytoplants have been shown to be good alternative to antibiotic in treatment of oral infection and against biofilm formation. The pomegranate is used in several systems of medicine because therapeutic properties for a variety of ailments, that may be used in treatment and prevention cancer,



diabetes and dental infection(4).

As far back as (5) demonstrated that pomegranate has many potential effects such as immune modulator stomachic, antifungal and antibacterial. Moreover it serve to decreases the effects of mouth lesion and periodontal disease, furthermore, pomegranate is an amazing source of Phenolic acid , Tannic acid and Flavones. The tannins can cross the cell wall composed of several polysaccharides and proteins, and bind to its surface, this can lead to effects on microbial metabolism(1).

So the purpose of this study was aimed to isolate and identification of oral pathogenic bacteria from patient suffering from gingivitis and dental carrier and make a comparison between the effect of antibacterial activity of aqueous extract of *Punica granatum* and antibiotic sensitivity test of ordinarily used antibiotic against oral pathogenic bacterial isolates beside studying of the effect of this extract on biofilm formation in mouth as attempt to find a safety method to prevent the biofilm formation and solve the problem of drug resistance.

Material and Methods

Specimens collection and bacterial identification. 1

Fifty six swabs were obtained from patients clinically diagnosed by dental physicians to have gingivitis and dental carrier. The swabs were streaked on general and selective media and incubated for 24h at 37C°. The growing bacteria were diagnosed depending on biochemical test (6) and according to methods described by (7).

2-Antibiotic Susceptibility tests

Antibiotics susceptibility test were carried out using disc diffusion methods on miller Hinton agar (oxid) accordance to (8) toward six antibiotic disc include used in susceptibility tests include (Ampicillin, Amoxycillin, Tetracycline, Kanamycin, Erythromycin and Trimetheprime). The diameters of inhibition zone (mm) were translated in term of sensitive or resistance by referring to interpretive chart (9).

3-Collection and processing of plant material

Fresh pomegranates were obtained from a public market of Karbala. The peels of *punica granatum* were sorted, cleaned and air dried at room temperature for 2 weeks and then crushed to get powder by a pestle and mortar (1).

4-Preparation of aqueous extract

About 20 gm. of the powdered sample was mixed with 400ml of distilled water in a conical flask. The mixture was stirred in a shaker incubator at 45C° for 24 h and then filtered using Whatman filter paper No. 1. The filtrate was then evaporated. The paper was collected and stored in a cup scroll bottle for next experiment(1).

5-Antibacterial activity of extract

The well diffusion agar method was adopted according to the method described by(7). In order to assess the antibacterial activity of the prepared extracts at several concentrations(100 mg/ml, 150 mg/ml and 200 mg/ml) an amount of 100 µl of tested bacteria were distributed into sterile Muller-Hinton agar Petri dishes. The agar was left to set in each of these plates one well by (10mm in diameter) were cut using a sterile cork borer No4. The agar well were filled with 0.1 ml of the extract and allowed to diffuse at room temperature for 15 min. The plates were then incubated in the upright position at 37c for 24h. After incubation, the diameters of growth inhibition zones were measured.

5-Determination of MIC concentration for aqueous peel extract

The MIC of the pomegranate peel aqueous extract was determine according to (10). The MIC recorded as the lowest concentration of extract that produce completely supuration of visible growth of bacterial suspension that added to the serial dilution of extract.

6-Determination of antibiofilm activity

The tissue culture plate method (TCP) were used as a qualitative assessment of biofilm formation these two methods was determined as previously described by (11).

Results and Discussion

1-Bacterial isolation from patient with gingivitis & dental carries

To detect the role of bacteria in role infection, 56 swabs were obtained from patient with gingivitis 22 and dental carrier 34. Table(1) illustrated that gram positive bacteria responsible for 58.8% from this infection, whereas gram negative bacteria were isolated from 41% of swabs ,this results may belong to that only a few specialized organism primarily *Streptococcus spp.* are able to adhere to oral surfaces and initiate plaque formation by their ability for using the glycosyl transferase enzyme to synthesize extra accumulation polysaccharide from sucrose, that regard critical to the development plaque(12).

The results also appeared that *K. pneumonia* (28%), *Lactobacillus spp.*(28%) and *Streptococcus spp.*(28%) were major pathogens associated with gingivitis oral infection whereas *S. aureus* (30%) and *K. pneumonia* (30%) were major pathogen associated with dental carries oral infection followed by *Streptococcus spp.*(20%) , *Lactobacillus spp.*(10%) and *P.aeruginosa*(10%). This finding is in agreement with studies carried out by (1 and 10) who found that *Streptococcus mutans* can creating favorable condition to adherence of opportunistic pathogens such as *S. aureus* to the surface of teeth. Moreover, primary colonizer such as *Streptococcus spp.* and *Lactobacillus spp.* able to prepare a favorable environment for secondary colonizer such as *K. pneumonia* which may responsible for primary infection in respiratory tract.

Table1: Bacterial isolated from patient with dental carries and gingivitis.

Type of infection	Number of swab	Number of isolates(%)	G+ve Isolates	Number(%)	G-ve Isolates	Number(%)
Gingivitis	22	7(31.8%)	<i>Lactobacillus spp.</i>	2(28%)	<i>k. pneumonia</i>	2(28%)
			<i>Streptococcus spp.</i>	2(28%)	<i>P.aeruginosa</i>	1(10%)
Dental carries	34	10(29.41)	<i>S.aureus</i>	3(30%)	<i>k.pneumonia</i>	3(30%)
			<i>Lactobacillus spp.</i>	1(10%)	<i>p.aeruginosa</i>	(10%) 1
			<i>Streptococcus spp.</i>	2(20%)		
Total	56	17		10(58.8%)		(41.1%) 7

Percentage from total isolates(N=17)



2- Antibiotic susceptibility assay

All strains were isolated from oral infections showed resistance towards most of used antibiotic . All *S. aureus* and *Streptococcus spp.* bacteria were resistance to Amp(100%)and kanamycin (100%),followed by Amoxicillin (66.6%,75%) and Trimethoprim (66.6%,50%), respectively. Whereas *K. pneumonia* and *p. aeruginosa* bacteria showed highly resistance to Trimethoprim (100%) followed by Ampicillin (60%, 100%) and Kanamycin (80%,50%), respectively, as shown in table (2). This result may belong to inappropriate or wide spread overuse of antibiotic to treatment oral infection and the oral pathogens may be exposure to repeat dose of antibiotic, when it was found in other parts of the body such as respiratory tract, that may be source for some oral pathogens such as *K. pneumonia* and *S. aureus* (13).

Table 2:Resistance of bacterial isolates to antibiotic.

Bacteria	No. isolate	Ampicillin	Amoxicillin	Tetracycline	Kanamycin	Erythromycin	Trimethoprim
<i>S.aureus</i>	3	3(100%)	2(66.6)	1(33.3)	3(100)	1(33.3)	2(66.6)
<i>Lactobacillus spp.</i>	3	1(33.3)	2(66.6)	2(66.6)	2(66.6)	1(33.3)	1(33.3)
<i>Streptococcus spp.</i>	4	4(100)	3(75)	1(25)	4(100)	2(50)	2(50)
<i>K.pneumonia</i>	5	3(60)	2(40)	3(60)	4(80)	3(60)	5(100)
<i>P.aeruginosa</i>	2	2(100)	2(100)	1(50)	1(50)	2(100)	2(100)

Percentage from total isolates (N=17)

3-Assesment of antibacterial efficacy of *P.granatum* peel aqueous extract

The result of antimicrobial properties of *Punica granatum* peel extract on the test organisms are shown in table (3), which shows the zones of inhibition of bacterial growth by aqueous extract in contrast to ciprofloxacin which is regarded as positive control. The results shows increasing of the inhibition zones with increasing in extract concentration to reach to maximum 23mm at 200 mg/ml for each one of *S.aureus* and *P.aeruginosa* while *Lactobacillus spp.* and *K. Pneumonia* reach to 24 mm and 21 mm respectively. Finally the *Streptococcus spp.* was gave narrow inhibition zone 18 mm at concentration 200 mg/ml in contrast to other isolated bacteria. This results are in agreement with (12)who demonstrated the specific antimicrobial actions of *Punica granatum* on dental biofilm bacteria and returned this action to the ability of this extract on disturbance of polyglycan synthesis, thus it reduces adherence mechanisms of these organism to dental surface. Many studies were shown highly sensitivity of oral pathogen to extracts of *Punica granatum* and contributed to, high tannin and polyphenols component in this fruit, which interfered with different mechanisms of toxicity and adherence of microorganism (14). The Tannins may act on the cell wall and cross the cell membrane of bacteria, they can precipitate protein and may suppress many enzyme such as glycosyl transferases (15).

Table 3: Sensitivity of bacterial strain to *Punica granatum* peel aqueous extract and ciprofloxacin

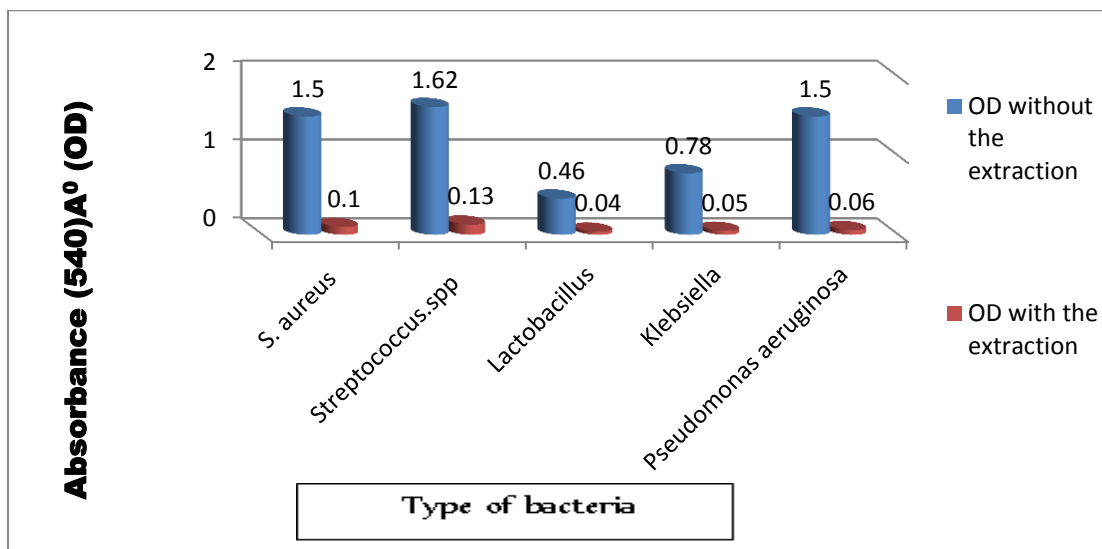
Bacterial strain	Isolates NO.	Diameter average of inhibition zones(mm) for extract and ciprofloxacin			
		extract			Ciprofloxacin
<i>G+ve</i>	10	100mg/ml	150mg/ml	200mg/ml	200mg/ml
<i>S. aureus</i>	3	18	19	23	29
<i>Streptococcus</i>	4	17	18	18	15
<i>Lactobacillus sp.</i>	3	26	21	24	18
<i>G-ve</i>	7				
<i>K. Pneumonia</i>	5	18	19	21	17
<i>P. aeruginosa</i>	2	17	25	23	9

4-Effect of extract on bacterial biofilm formation

The structure of biofilm after treatment with MIC concentration(140 mg/ml) of *Punica granatum* peel aqueous extract was assessed by using tissue culture plate method. The result obtained from this methods ,illustrated that most isolated bacteria undergo reduction in biofilm formation activates after incubated with MIC of aqueous extract , this transformation in biofilme formation activity for isolated bacteria could be seen through the average of optical density (O.D) at wave lengths 540nm for each bacterial genes after treated with extract , that decrease from 0.78 to 0.05 for *K. Pneumonia* and from 1.6 to 0.06 for *P. aeruginosa* bacteria , there for these tow genus become poor producer after treated with MIC of extract in contrast to control, in which these bacteria were high producer, while *S. aureus* and *Streptococcus* transformed from high producers in control,(O.D average 1.5 &1.6 respectively) to producer only through 0.1 O.D average for *S. aureus* and 0.13 *Streptococcus spp.* after treatment with extract, but the *Lactobacillus spp.* was produced only in control 0.46 then become poor producer 0.04 ,when treated with extract as show in fig (1).

This result approach with (16) who explain that only reduced antibiofilm activity and not eradication totally though used MIC for extract, may attributed to several factor that made cells in a biofilm are more resistant to antimicrobial agents compared to free floating cell, such as presence of an extrapolsaccharied (EPS) that surrounds a biofilm cells & slower growth rate in biofilms compared to plankton cells as a result of reducing nutrient and oxygen supply. In addition to solubility and diffusion of active compounds in agar media ,beside different condition the plant extract face the bacteria ,which can be influenced by the study design in vitro & in vivo, difference in extraction process and types of solvent used in these process ,these all factors affect directly on antibiofilm activity of extract (12).

Many searcher demonstrated the activity of *Punica granatum* peel aqueous extract on virulence factors of many oral pathogens. These finding can form the basis for further photochemical studies to obtain active compound from many plant such as *Punica granatum* and evaluated them against wide range of bacterial strain and dental plaque (13).



Fig(1): Antibiofilm activity of *Punica granatum* peel aqueous extract against some oral pathogens.

References

- 1-Vasconcelos** , L.C; Pereira ,M.V and Higino, J.S.(2006).Minimum inhibitory Concentration of adherence of *Punica granatum* Linn (pomegranate) get against *S.mutans* ,*S.mitis* and *C.albicans* Braz.Dent.J.17(3):223-227.
- 2-Gazzani,G.;** Daglia M. &Papetti. (2012).Food components with anticaries activity.Curr.Opin.Biote.23:153-159.
- 3-Devi** , A . ; Singh ,V. and Bhatt , A .B. (2011) Antibiotic Sensitivity pattern of *Streptococci* Agent commercially available drugs & Comparison with extract of *punica granatum* .Int J. of pharma &Bio sci 2(2).504-508.
- 4-Abdollah** Zadeh ,Sh.;Mashouf ,Ry.;Hortazavi ,MH.;Moghaddam ,N.; Roozbahari ,N. and Vahedi,M.(2011).Antibacterial and antifungal Activities of *punica granatum* peel extracts against oral pathogens J.of.Dentistry . 8(1):1-6.
- 5-Gould,W.J.;**Fielder,M.D.;Kelly,W.&Naughtion,D.P.(2009). Antimicro bial pomegranat rind extract enhancement by CuII and vitamin C combination against clinical isolation of *P. aerugenosa* Breti.J.Bio.Scie.66(3):129-131.
- 6-Holt, J.G. ;**Kriegean ,N.R .; Sneather ,P.H.; Staleyley ,J.T.and Williamsers ,S.T.(1994) Berges manules of determination bacteriablology 9th ed . , Williams & wilkins ,Baltimore ,U.S.A.
- 7-Macfaddin , J . F.**(2000) . Biochemical test for Identification of medical bacteria tested ;The Williams & wilkins ,Baltimore ,U.S.A.
- 8-Bauer** , A .w. ; Kirby ,WM . Sherris , JC. And Turck ,M.(1966). Antibiotic susceptibility Testing by a standardized single disk Method Am.J.clin .pathol. ,45:493-496.
- 9-NCCls –National committee for clinical laboratory Standards** (2004) performance standards For antimicrobial susceptibility testing Fourteenth informational supplemental M 100-s 14 ,wayne pA,USA.
- 10-Thenmzhi**, R.; Nithyanand, P.; Rathna, and Pandiun ,Sh.; K.(2009).Antibiofilm activity of cord associated bacteria against different clinical M serotypes of streptococcus pyogene .Immun.Med. Microbial.57:284-294.

- 11-Mathur, T.;**S.inghol,S.;K.hane,S.;Vpadhyay,D.;Fatma,T.& **Rattan, A.** (2006).Detection of biofilm formation among the clinical isolated of staphylococcus :an evaluation of three different screening methods Indian J. of Med .Micro.24(1):25-29.
- 12.Vahabi ,S.;**Najafi ,E.and Alizadeh,S.(2011) Invitro antimicrobial effects of some Herbed essences against oral pathogens .J.of med .plants. Res 5(19):4870-4878.
- 13-Yehia, H.M.;**Elkadragy,M.F. & Moneim, A.E.(2011).Antimicrobial activity of pomegranate rind peel extracts.Afri.J.Micro.Res.4(22):3664-3668.
- 14-Miguel, M.G.;**Neves,M.A. & Antunes,M.D.(2010).Pomegranate (punica grantum):A medical plant with myriad biological properties –A short review.J.Med.Plant.Res.4(25):2836-2847.
- 15-Jurenk ,J.MT .**(2008) .Therapeutic applications of pomegranate (punica granatum L.) A review . Alternative med .Rev .13(2):128-140.
- 16-Sandasi.M.,**Leonard,C.M. & Viljoen.(2010).The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against *Listeria monocytogenes*.Appl.Micro.50:30-35.

الفعالية التثبيطية والمضادة لتكوين الاغشية الحيوية للمستخلص المائي لقشور الرمان ضد بعض ممرضات الفم

صممت هذه الدراسة لتقييم الفعالية التثبيطية للمستخلص المائي لقشور الرمان ودراسة فعاليته المضادة لتكوين الاغشية الحيوية لبعض ممرضات الفم المعزولة من المرضى المصابين بالتهاب اللثة وتسوس الاسنان . حيث كانت 58.8% من العزلات المحصل عليها موجبه لصبغة كرام والمتمثلة بكل من بكتريا *Lactobacillus spp.*, *Staphylococcus aureus* , *Streptococcus spp.* و 41.2% منها عزلات سالبه لصبغة كرام والمتمثلة بكل من بكتريا *Klebseilla pneumonia* و *pseudomonas aeruginosa*.

استخدمت تقنية الحفر على الاكار لتحديد حساسية العزلات الجرثومية المعزولة للمستخلص المائي لقشور الرمان ومقارنة النتائج بتاثير المضاد الحيوي Ciprofloxacin كسيطره موجبه. حيث اظهرت النتائج فعاليته تثبيطية واضحة للمستخلص ضد العزلات الجرثومية حيث وصل قطر منطقة التثبيط عند التركيز 200ملغرام / مل من المستخلص 24 ملم لبكتريا *Lactobacillus spp.* و 23 ملم ضد كل من بكتريا *S. aureus* و *P.aeruginosa* تلتها بكتريا *K. pneumonia* 21 ملم بينما كان لبكتريا *Streptococcus spp.* 18 ملم فقط.

تم دراسة حساسية البكتريا المعزولة للمضادات الحيوية شائعة الاستعمال لعلاج التهاب اللثة وتسوس الاسنان . حيث اظهرت النتائج مقاومة العزلات الجرثومية لاغلب المضادات الحيوية المستخدمة. وعند بيان تاثير المستخلص على قابلية تلك الجراثيم على تكوين الاغشية الحيوية ظهر جليا ان اغلب العزلات عانت من انخفاض قابليتها على تكوين تلك الاغشية بعد حفظها مع التركيز المثبط الادنى للمستخلص ،ذلك الانخفاض الذي تم ملاحظته بقياس معدل الكثافة الضوئية عند طول موجي 540 نانومتر.