Isolation, Characterization and Estimation of Ellagic Acid activity against *Porphyromonas gingivalis* Isolated from adult Periodontitis Patients in Kerbala City.

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ABSTRACT

Periodontitis is a chronic bacterial infection affects the gingiva, periodontium connective tissues and alveolar bone, results in alveolar bone resorption, ultimately, partially or completely tooth loss, indeed, it may causes various serious systemic complications like Diabetes Mellitus, Cardiovascular disorders, Rheumatoid Arthritis, Preeclampsia with low birth weight and Orodigestive cancer mortality. *Porphyromonas gingivalis* is considered the main, foremost and strongest periodontal pathogen. Isolation, characterization and monoplex PCR of *16S rRNA* and multiplex PCR of *fimA* geneotyping are the golden standard assays for detection of *P.gingivalis* in adult periodontitis patients. In the present study,Pomegranate peel Ethanol extract partially purified ellagic acid has an obvious effectiveness against oral pathogens (*P. gingivalis* and *S. mutans*) in both growth inhibition zone on solid medium and Percentage of growth reduction in a liquid medium in various concentrations with common MIC 16mg/ml.

عزل وتوصيف وتقييم فعالية حامض الايللاجيك ضد بكتريا *Porphyromonas gingivalis* من المرضى البالغين المصابين بالتهاب اللثة وما حول الاسنان في مدينة كربلاء

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الخلاصة

التهاب اللثة وماحول السن هو اصابة بكتيرية مزمنة تستهدف اللثة والانسجة الرابطة حول السن والعظم الحويصلي مسببة انهياره وبالتالي فقدان السن جزئيا اوكليا فضلا عن العديد من المضاعفات الجهازية الخطرة مثل داء السكري، الاختلالات القلبية الوعائية، التهاب المفاصل الروماتزمي، الولادة قبل الاوان مع نقصان وزن المولود والهلاك نتيجة سرطانات الفم والجهاز الهظمي. وتعتبر بكتريا *Porphyromonas gingivalis* اول واقوى المسببات الرئيسية للمرض. وان عزل البكتريا ودراسة خصائصها الكيميائية الحيوية وتشخيصها وراثيا عن طريق التفاعل المتسلسل لانزيم البلمرة لجيني (*16S rRNA , fimA*) هي الطرق الاساسية المعتمدة في التشخيص الدقيق للبكتريا الممرضة للثة وماحول الاسنان. ولقد اظهرت نتائج الدراسة الحالية ان مستخلص شحم الرمان بالايثانول والغني بحامض الايللاجيك (ellagic acid) المنقى جزئيا هو ذات فعالية واضحة في تثبيط نمو بكتريا (*P. gingivalis , Streptococcus mutans*) في

كل من تقنيتي تثبيط نموالبكتريا على الوسط الصلب وكذلك اختزال نمو البكتريا في الوسط السائل و بمختلف التراكيزللمستخلص، وان اوطا تركيز مثبط (MIC) موحد للبكتريا قيد التجربة هو 16ملغرام لكل ملليلتر .

1. INTRODUCTION

Periodontal diseases are the most common chronic bacterial infections that destroy connective tissue, periodontal ligaments and alveolar bone that surrounding the teeth, and ultimately leading to tooth loss [1]. They represent a serious oral health problem in adult populations in developing countries [2]. Worldwide, they affect about 750 million people or about 10.8% of the population as of 2010 [3], and associated with increased risk of various systemic complications including diabetes mellitus[4], cardiovascular diseases like myocardial infarction, atherosclerosis [5], rheumatoid arthritis [6], preeclampsia with low birth weight[7] and orodigestive cancer mortality [8].

Numerous bacteria are associated with the initiation and progression of periodontitis [9,] among which *Porphyromonas gingivalis* is considered the main, strongest, and foremost periodontal pathogen involved in onset of various form of periodontal diseases [10]. Bacterial culturing has been considered the classic diagnostic method widely used in the study of the composition of dental plaque and is still generally used as the gold or primary standard when determining the utility of a new microbial test in periodontal microbiology [11].

Different types of treatments have been accomplished to improve oral hygiene include mechanical removing plaques from all surfaces of the teeth, systemic or local use of antibiotics and antiseptics, laser removal of necrotic and granulation tissue and disinfection of periodontal pockets, use of water–powder system to mechanically remove bacteria or UV light systems for light-activated disinfection [12].

Over the years there have been many studies undertaken in different areas of the world on the bactericidal effects of pomegranates on a number of highly pathogenic and drug-resistant strains. These studies normally determine bactericidal potency of different extracts of the pomegranate plant against a range of different bacteria, utilizing disc diffusion assays or minimum inhibitory concentration (MIC). Methanol, ethanol extracts of the pomegranates fruit especially the peel exhibited the broadest antibacterial activity [13, 14]. This study was conducted to cultivation, phenotypic and molecular characterization of *P. gingivalis* isolated from adult periodontitis patients and *In vitro* estimation of inhibitory effect of pomegranate peel ethanolic extract partially purified (ellagic acid) against *P. gingivalis* isolate.

2. MATERIALS AND METHODS

Seventy two adult periodontitis patients aged between 2o and 60 years attending the Specialist Center of Dentistry division of periodontology and the clinics of dentistry in Kerbala governorate were included in this study during the period from September 2013 to July 2014. The permission for this study was obtained from the Ethical and Scientific Committee of the Medical Researches in Kerbala Health office.

Clinical examinations and Gingival Crevicular Fluid (GCF) sampling

Periodontitis Patients were clinically diagnosed by adentist, a full examination of the entire mouth of each patient was conducted and periodontal samples were collected. The

periodontal sites to be sampled were air-dried and isolated with cotton rolls, The supragingival plaque was first removed with a sterile Gracey curette, employed with care to avoid bleeding. A sterilized medium size (size 40, T.g., UK) two-four paper points were carefully inserted as deeply as possible into each gingival groove site (periodontal pocket) with a pocket depth (PD ≥ 5mm), and kept in place for (30-60) seconds. Then, the soaked paper points were rapidly transferred into 1.5 ml microcentrifuge Eppendorf tube contained 1 ml of sodium thioglycolate transport fluid (STTF) and subjected to the laboratory for bacterial cultivation, molecular detection and antibacterial estimation procedures.

Cultivation of *P. gingivalis*

The periodontal sample tubes were incubated vertically at 37°C for 48 hours, then, 100 µl aliquot from each periodontal sample was taken and streaked on *P. gingivalis* agar (P. GING) which is an enriched selective medium for isolation and presumptive identification of *P. gingivalis*[15]. The (P.GING) medium is locally prepared, consists of Columbia Agar Base, supplemented with Sheep blood, L-Cystein, Hemin, Vitamins K1, K3 and other requirements in table (1) which were selective agents for isolation of such fastidious, strictly anaerobic *P. gingivalis* from other periodontopathogenes.

Table1: cultural requirements of *P. gingivalis* in (P.GING) medium according to studies [15, 16].

Compositions	Dosage	Origin
Columbia Agar base	42.5 g /L.	(Oxoid)Basing stoke, U. K.
L-Cystein	1mg /ml (1g /L.)	(BD BBLTM).
Hemin	5µg /ml (5 mg /L.)	Sigma Chemical Co. USA
Vitamin K1, K3 (Konakion) inj.	1µg /ml (1mg /L.)	Hoffman-LaRocheLtd France
Agar Bacteriological powder	6.5 g./L.	(Oxoid)Basing stoke, U. K.
Bacitracin	10.0 mg/L.	Himedia Laboratories-India
Colistin methane sulfonate	15.37 mg/L	Himedia Laboratories-India
Nalidixic Acid	15.0 mg/L.	Himedia Laboratories-India
Sheep Blood	50.0 ml/L.	Local sheep
Distilled Water	1000.0 ml	Local product

Selective medium plates were incubated in a tightly packed anaerobic atmosphere jar using gas pack (OXOID England) at 37°C for(7- 14) days [15].

In Vitro* Identification of *P. gingivalis

Identification of *P. gingivalis* species was done on the basis of the ability on anaerobic growth, having the typical colony color and morphology, Grams staining, biochemical Indole reaction (Spot Indole Test) [17]. Hemagglutination with 3% sheep erythrocytes [18] and Vancomycin sensitivity[19] as well as molecular investigation.

Molecular Detection of *P. gingivalis* by essential genes

DNA Extraction

About (2-3) characteristic grown colonies on (P. GING) medium were suspended in 500 µl of 0.9% sterile normal saline solution, Genomic DNA Mini Kit (Geneaid, Korea) was

used for DNA isolation from bacteria in accordance with the manufacturer's instructions. Molecular detection of *P. gingivalis* was performed by monoplex PCR of *16S rRNA* gene amplification according to [20] and multiplex PCR of species specific *fimA* gene amplification according to [20, 21], Using the following amplification primers table 1 and cycling parameters (tables 2, 3).

Table 1: The Primers set was used in Molecular detection of *P.gingivalis*

Gene	Duplexing primers 5´- 3´	Product size (bp)	Reference
P. gingivalis 16S ribosomal RNA	AGG CAG CTT GCC ATA CTG CG ACT GTT AGC AAC TAC CGA TGT	404	[22]
Type I *fimA*	CTG TGT GTT TAT GGC AAA CTT C AAC CCC GCT CCC TGT ATT CCG A	392	[23]
Type Ib *fimA*	CAG CAG AGC CAA AAA CAA TCG TGT CAG ATA ATT AGC GTC TGC	271	[21]
Type II *fimA*	ACA ACT ATA CTT ATG ACA ATG G AAC CCC GCT CCC TGT ATT CCG A	257	[23]
Type III *fimA*	ATT ACA CCT ACA CAG GTG AGG C AAC CCC GCT CCC TGT ATT CCG A	247	[23]
Type IV *fimA*	CTA TTC AGG TGC TAT TAC CCA A AAC CCC GCT CCC TGT ATT CCG A	251	[23]
Type V *fimA*	AAC AAC AGT CTC CTT GAC AGT G TAT TGG GGG TCG AAC GTT CTG TC	462	[24]

Amplification Reaction programs

Table 2: Cycling parameters for monoplex PCR of *16S rRNA* gene amplification

No. of cycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
35	Denaturation	94	30 Sec.
	Annealing	60	30 Sec.
	Elongation	72	1 min.
1	Final extension	72	10 min.

Table 3: Cycling parameters for Multiplex PCR of species specific *fimA* gene amplification

No.ofcycles	Stage	Temperature °C	Time
1	Initial denaturation	95	5 min.
35	Denaturation	94	30 Sec.
	Annealing	58	30 Sec.
	Elongation	72	30 Sec.
1	Final extension	72	7 min.

Agarose Gel Electrophoresis

Agarose gel electrophoresis (1%) of PCR products was accomplished with the use of two types of DNA ladder (Accu Ladder 100 bp, Bioneer/Korea) and (50 bp DNA Step Ladder, Promega/ USA).

Antibacterial activity of Ellagic acid against *P. gingivalis*

Preparation of Ellagic acid powder from Pomegranate fruits

Pomegranate peel Ethanol extract partially purified (ellagic acid) was prepared according to ethanol extraction method [25, 26]. High performance liquid chromatography (HPLC) assay was used to determine the ellagic acid content in ethanol extracted powder and compared with the standard ellagic acid for Sigma chemical co. products of USA.

Preparation of Ellagic acid solution

Ellagic acid powder was dissolved in double distillated water to prepare the low concentrations of two fold serial dilutions of (32, 16, 8, 4, 2, 1, 0.5) mg/ml. These solutions were sterilized by filtration through a 0.45 μm sterilizing Millipore express filter (Biotech Germany) to be ready for antibacterial activity.

Test Microorganisms

The bacterial strains were used in the present experiments were cultivated, confirmatory diagnosed *P. gingivalis* as well as related *Streptococcus mutans* isolated from adult periodontitis patients indeed, *Escherichia coli* American type culture collection (ATCC®) 51813™, *Staphylococcus aureus* ATCC® 6538™ which maintained in central public health laboratory in Kerbala governorate microbank™vials at −70°C that used as additional test and control strains.

Antibacterial Activity of ellagic acid *in vitro* Experiments

The antibacterial activity of partially purified ellagic acid against *P. gingivalis* and test microorganisms was evaluated according to the following two methods:-

1- *In vitro* growth inhibition zone on solid (brain heart infusion agar) medium, according to agar well diffusion assay [27].
2- The percentage of growth reduction in a liquid (brain heart infusion broth) medium according to a comparative study [28].

Statistical Analysis:

Differences between means of data were compared by least significant difference (LSD) calculated using the Statistical Analysis System (S.A.S., Institute, Inc. Cary, NC, USA). Collected data were analyzed by using the available statistical system package of SPSS-18 (PASW statistical). Statistical analysis was done by using Chi-Square ($\chi 2$) test, Z test when applicable. P-value of ≤ 0.05.

3. Results and Discussion

Paper point sampling of (GCF) used for obtaining the predominant periodontal pathogen (*P. gingivalis*) because of its ability to persist and accumulate in the subgingival region, gingival pocket of chronic periodontitis patients [29] and the inflammatory exudate (GCF) is a rich source of essential nutrients for *P. gingivalis*, such as peptides and hemin-derived iron [30]. This technique was agreed with the most researches for *p. gingivalis* and other periodontal pathogenes sampling for molecular and microbiological detection [31].

Cultivation is the golden standard assay in microbiological detection of red complex periodontopathogens, although it possesses many drawbacks and difficulties, its time consuming, it has an expensive growth requirements, strict anaerobic growth conditions and

target microorganisms must survive during sampling, transportation and stay vital in order to be colonize, forming characteristic growth colony [11] as demonstrated in comparative study[32].

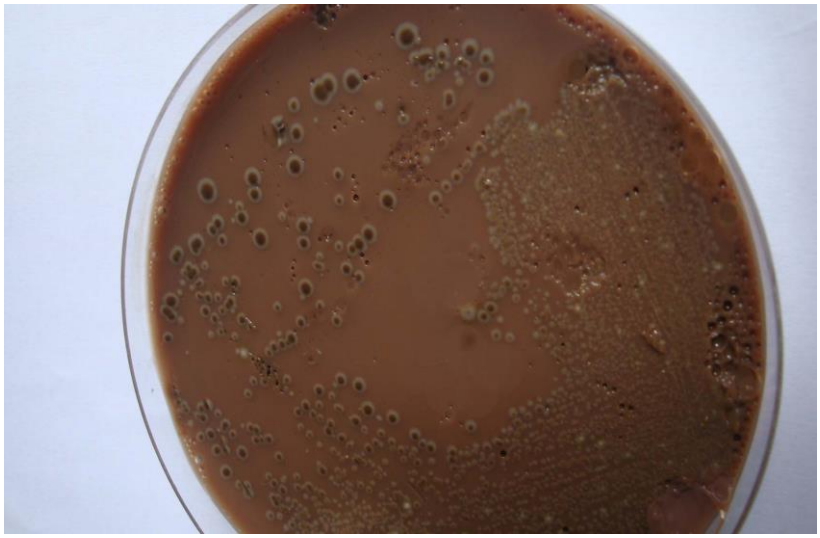

Figure 1: Colombia Blood Agar with characteristic black pigmented at the center of the colonies observed due to the aggregation of heme/iron on *P. gingivalis* cell walls.

Microscopic Examination and Biochemical Tests:-

The Gram's stain reaction of grown colonies revealed Gram negative coccobacilli, in some cases appeared as diplococci surrounded with a capsule or hallow as demonstrated in comparative study [33], as shown in figure (2).

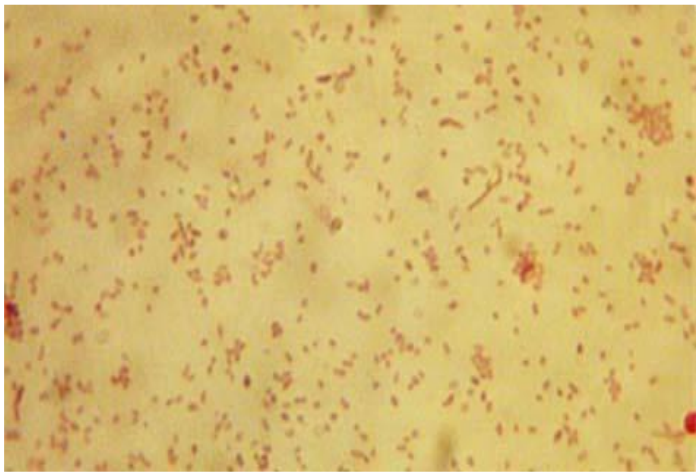

Figure 2: Gram negative coccobacilli, diplococci of *P. gingivalis* with a magnitude 100X.

Indole Reaction of *P.gingivalis* was Positive due to a blue to blue-green color appeared on a filter paper (within 30s) this is agree with another study [17]. Positive hemagglutination with 3% sheep RBCs indicates the presence of peritrichous fimbriae (fimbrillin) protein which is a main, strongest and formest virulence factor and characteristic property for fimbriated *P.gingivalis* isolate [10] as shown in figure (3).

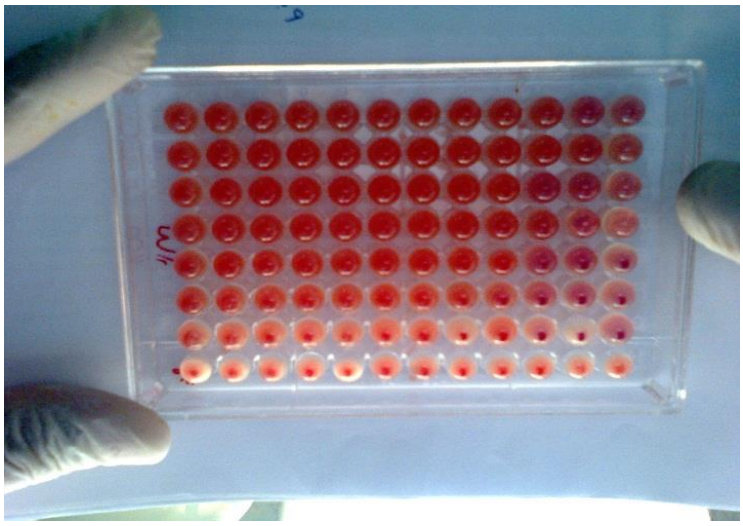

Figure 3: demonstrated positive haemagglutination reaction of fimbriated *P.gingivalis* (net shape), negative control appeared (dot shape).

Vancomycin sensitivity test revealed that vancomycin had 100% activity against *P. gingivalis*, and it's a characteristic feature for *in vivo* and *in vitro* characterization of this bacterium as demonstrated in figure (4), this has an agreement with other studies [16, 33].

Figure 4: positive result of vancomycin sensitivity test for *P.gingivalis* with inhibition zone 16mm according to two studies [16, 33].

Molecular Detection of *P. gingivalis* by essential genes

In the present study, molecular detection of bacterial colonies of *P. gingivalis* by essential genes revealed positive result with *16S rRNA* genotype with a single band of a lane 404 bp. as demonstrated in figure (5).

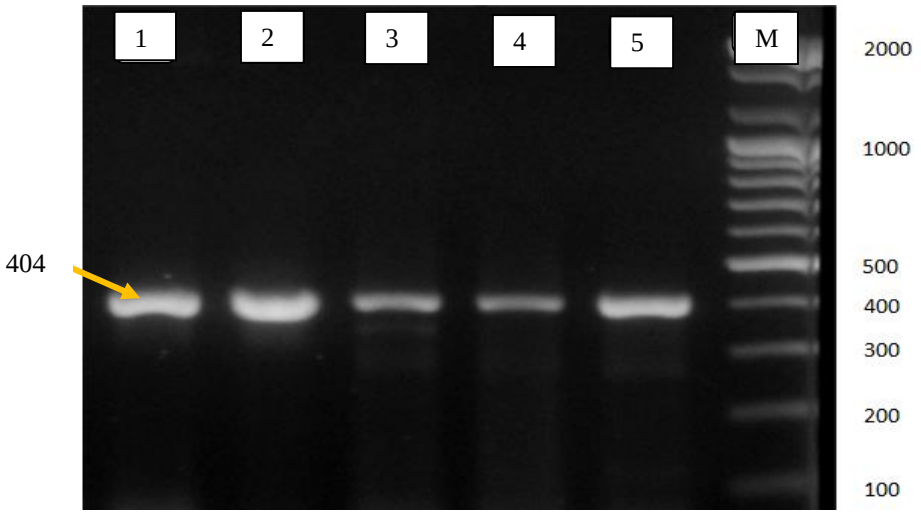

Figure 5: positive result of periodontal samples (1, 2, 3, 4, 5) with *16SrRNA* gene with a single band of a lane (404) bp.

As well as positive result with Species Specific *fimA* genotypes (I, II, III, IV, V) that demonstrated as a single band for each detected alleles (figure 6); these results have an agreement with worldwide various clinical, epidemiological and molecular studies for screening and detection of *P. gingivalis* in periodontal samples of adults chronic periodontitis patients [2, 34, 35, 36].

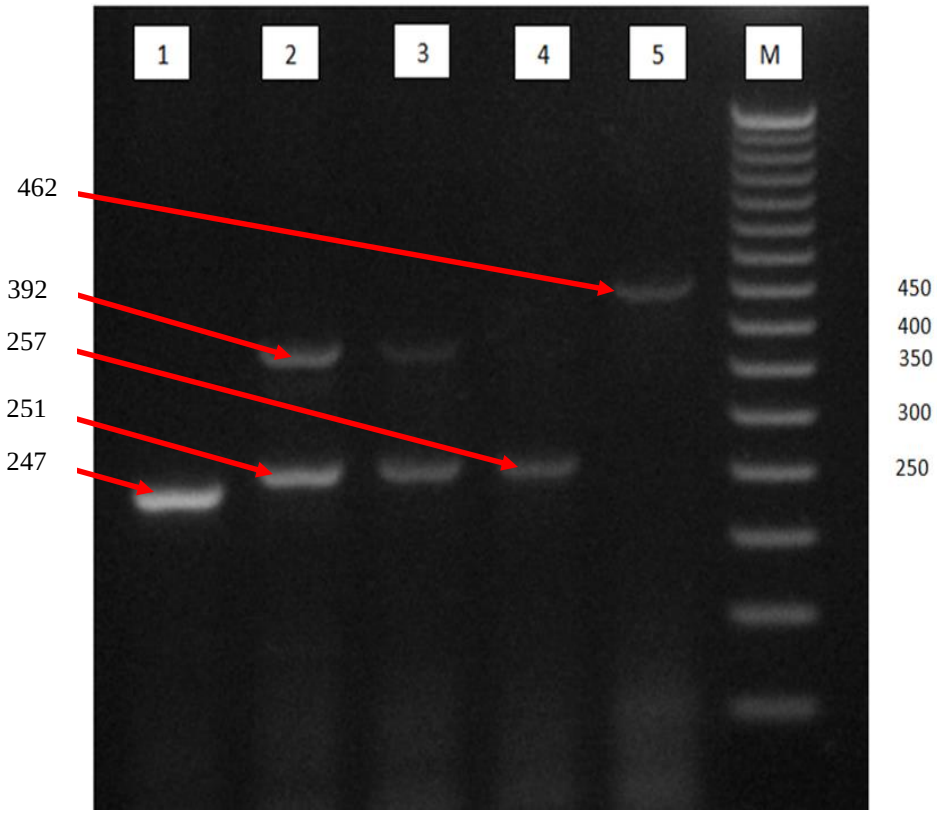

Figure 6: positive periodontal samples of *fimA* genotypes with lengths: sample (1) *fimA* genotype (III) 247 bp., sample (2, 3) *fimA* genotypes (IV) 251bp., and *fimA* genotypes (I) 392 bp., sample (4) *fimA* genotype (II) 257 bp.Sample (5) *fimA* genotype (V) 462 bp.

High performance liquid chromatography (HPLC) assay demonstrated an approximately analytical curves to both present ethanol extract and original ellagic acid of (Sigma chemical co./USA), this result has an agreement with previous studies for preparation and determination of ellagic acid in pomegranate peel ethanol extract powder [25, 37] as demonstrated in figure (7).

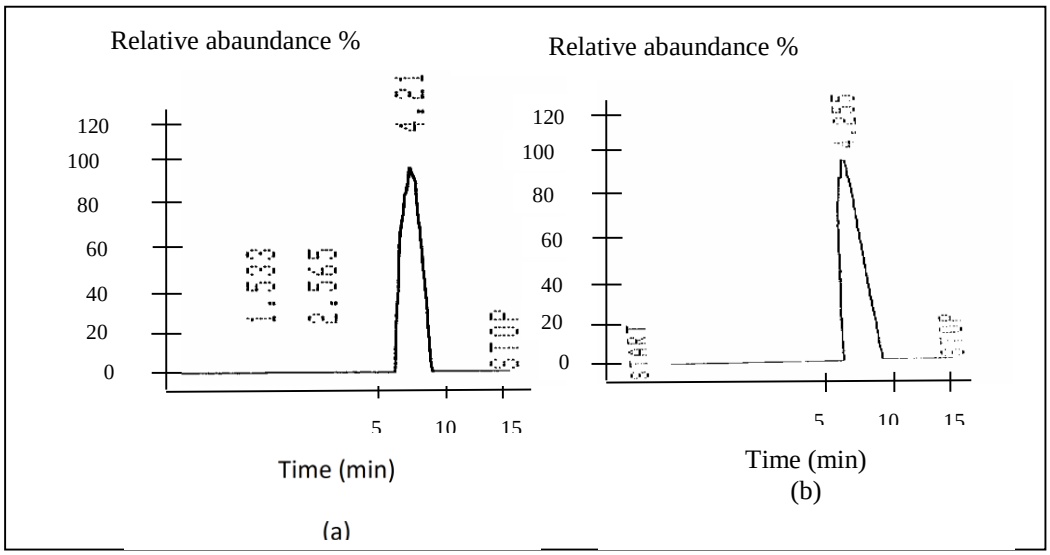

Figure 7: High performance liquid chromatography of (a) standard ellagic acid of Sigma chemical co. products USA, (b) prepared ellagic acid.

Antimicrobial Activity of ellagic acid *in vitro* Experiments

The use of plants with preventive and therapeutic effects contributes to health care needs [38]. There are three main reasons to be interested in the treating and healing power of plant extract. First, pharmacological studies have demonstrated that many of plants are known to possess antimicrobial agents; second, people are becoming aware of the side effects associated with the over prescription of traditional antibiotics; third, time to time resistant microorganisms against antibiotics are increasing [39, 40]. Among these plants, *Punica granatum* has an important role in folk medicine. Pomegranate is known as a rich source of pharmacological properties which have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic and anti-cancer effects as well as protection against herpes virus, inhibition of low density lipids (LDL) oxidation and decrease in atheromatous plaque formation and reduction of systolic blood pressure [40, 41].

In the present study, the ethanol extract of *Punica granatum* peel have an effect on tested microorganisms in many concentrations.

The first method an *in vitro* growth inhibition zone on brain heart infusion agar medium displayed gradually increasing of growth inhibition zone of *P. gingivalis* and other related test organisms with the elevation of ellagic acid concentrations as demonstrated in table (4).

Table 4: Pomegranate peel ethanol extract (ellagic acid) activity against *P. gingivalis* and other test organisms on brain heart infusion agar.

Growth inhibition zone on solid medium								
Ellagic acid concentrations (mg/ml)	32	16	8	4	2	1	0.5	control
Bacterial species	Zones of inhibition/mm							
P. gingivalis	33	28	23	17	8	6	0	0
Streptococcus mutans	32	24	20	17	8	6	0	0
Staphylococcus aureus	32	26	21	18	9	5	0	0
Escherichia coli	30	25	20	15	7	5	0	0

In the second method (growth reduction in a liquid medium) displayed an obvious effectiveness of pomegranate peel ethanol extract ellagic acid with all concentrations against *P. gingivalis*, conventionally related *Streptococcus mutans* as well as other test microorganisms with common MIC (Minimum Inhibitory Concentration) 16 mg/ml for all the tested bacteria table (5).

Table 5: Pomegranate peel ethanol extract (ellagic acid) activity against *P. gingivalis* and other test organisms in brain heart infusion broth.

Growth reduction in a liquid medium								
Ellagic acid concentrations (mg/ml)	32	16	8	4	2	1	0.5	Control
Bacterial species	Bacterial Growth Reduction (%)							
P. gingivalis	100	100	91.666	83.333	66.666	16.666	5.555	0
Streptococcus mutans	100	100	88.636	81.818	56.818	29.545	4.545	0
Staphylococcus aureus	100	100	90.909	78.787	45.454	24.242	2.272	0
Escherichia coli	100	100	92.307	73.076	42.307	15.384	7.692	0

These results were agreed with several clinical studies about (Reductions in Oral Bacteria) suggested a role for pomegranate extracts in reducing and preventing pathogenic dental bacteria and reducing the risk of plaque, gingivitis, and periodontal disease, all concentrations of the pomegranate extract had antibacterial activity against periodontal pathogens and the effects of three different concentrations of a methanolic pomegranate peel extract at 4mg/mL, 8mg/mL, and 12mg/mL on the growth of dental bacteria were highly effective using the disc diffusion method [44]. In other similar *in vitro* studies, ethanol and water extracts of pomegranate both had inhibitory effects against *S. mutans* and *P. gingivalis* [44, 45, 46, 47].

Various studies showed that pomegranate methanol, ethanol extracts were effective against many common oral pathogens such as *Streptococcus mutans* [37]. It is demonstrated that this antibacterial activity may be related to the presence of hydrolysable tannins and polyphenolics in the pomegranate extract specifically punicalagin, ellagic acid and gallagic acid, the real mechanism of the antimicrobial effect of tannins (the major components of *Punica granatum* extract) may be related to their toxicity, astringent, molecular structure or other ways. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins [40, 42, 43]. They may also suppress many enzymes such as glycosyltransferases [43]. Other studies [40, 41] demonstrated that ellagic acid (tannic acid) has the highest antibacterial effect against tested sensitive strains even at low concentrations. Hence, the antibacterial activity of *Punica granatum* may be related to polyphenol structures because polyphenols may affect the bacterial cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms [40, 43].

CONCLUSION

Cultivation, characterization and Molecular Detection of *P. gingivalis* using *16S rRNA*, species specific *fimA* genes are the golden standard assays in molecular and microbiological detection of periodontopathogens. Ethanol extract partially purified ellagic acid of pomegranate peel has an obvious effectiveness against oral pathogens (*P. gingivalis* and *S. mutans*) in both solid and liquid media in various concentrations with common MIC 16mg/ml, and might be used in the control (prevention and treatment) of common oral pathogens responsible for adult chronic periodontal diseases.

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REFERENCES

[1] Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K and Taylor R. (2012). Periodontitis and diabetes: a two-way relationship, **J**. **Periodontol**. 55:21–23.

[2] Krishnan Mahalakshmi, Padma Krishnan, S.C. Chandrasekaran, K.H. Panishankar, and Natarajan Subashini. (2012). Prevalence of Periodontopathic Bacteria in the Subgingival Plaque of a South Indian Population with Periodontitis. **Journal of Clinical and Diagnostic Research.** Vol-6(4): 747-752.

[3] Vos, T, Flaxman, D., Abraham, D; Naghavi, et al. (2012). Asystematic analysis for the Global Burden of Disease Study, **Lancet**, 380 (98): 2163–96.

[4]Pınar, G., Nurcan, B. (2013). Diabetes mellitus and periodontitis: signs of a bidirectional relationship, **EMJ Diabet**. 1:30-36.

[5] Zhang, B., Elmabsout A., Ateia H., Khalaf, V. T Basic, Kartheyaene, J., Robert, K., Torbjörn, B. and Allan, S. (2013). The periodontal pathogen *Porphyromonas gingivalis* changes the gene expression invascular smooth muscle cells involving the TGFbeta/Notch signalling pathway and increased cell proliferation, **BMC Genomics**, 14:770.

[6] Koziel, J. Mydel, P. and Potempa, J. (2014). The link between periodontal disease and rheumatoid arthritis: an updated review, **Current Rheumatology Report**, vol. 16, article 408.

[7] Perez-Chaparro, P., Gracieux P, Lafaurie G, Donnio P, Bonnaure Mallet M. (2008). Genotipic characterization of *Porphyromonas gingivalis* isolated from subgingival plaque and blood sample in positive subjects with periodontitis **J. Clin. Periodontol**. 35: 748-53.
[8] Jiyoung A., Stephanie S. and Richard H. (2012). Periodontal disease, *Porphyromonas gingivalis* serum antibody levels and orodigestive cancer mortality, **Carcinogenesis** 33 (5):1055–1058.
[9] Amano**, A.** (2003). Molecular interaction of *Porphyromonas gingivalis* with host cells implication for the microbial pathogenesis of periodontal disease, **J. Periodontol.** 74**:**90–96.
[10] Jaroslav M., Stepan P., Pavla S., Yelena Lyuya-Mi, Jirina B., Tatjana J., Jarmila P., and Jana D. (2014). *Porphyromonas gingivalis*: Major Periodontopathic Pathogen Overview, **J. Immunol. Res.,** Volume 2014. Review Article ID 476068, 8 pages.
[11] VERNER C., LEMAITRE P., DANIEL A., GIUMELLI B., LAKHSSASSI N., SIXOU M. (2006). Carpegen real-time polymerase chain reaction vs. culture for periodontal pathogen identification., **Oral Microbiology Immunology**. 21: 341-346.
[12] Khuller N, Sharma N. (2009). Recent trends in periodontal treatment (surgical and non-surgical): A review. **The Internet Journal of Dental Science**, 7(2).
[13] Fawole O. A., Makunga N. P., and Opara U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate Evidence-Based Complementary and Alternative Medicine fruit peel methanolic extract, **BMC Complementary and Alternative Medicine,** 12: 200–225.
[14] Ismail T., Sestili P., and Akhtar S. (2012). Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and antiinfective effects, **Journal of Ethnopharmacology** 143(2): 397–405.
[15] Jousimies-Somer, H. R., Summanen, P. C., Citron, D. M., Baron, E., Wexler, H. M. and Finegold, S. M. (2002). **Wadsworth – KTL Anaerobic Bacteriology Manual. Sixth Edition.** Star Publishing Co. Belmont, CA 94002.
[16] NCCLS. Quality Control for Commercially Prepared Microbiological Culture Media; Approved Standard- Third Edition. (2004). NCCLS document **M22-A3. NCCLS,** 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898.
[17] MacFaddin JF.(2000). Biochemical tests for identification of medical bacteria. **3rd ed. Philadelphia: Lippincott Williams & Wilkins.**
[18]Tomoko K., Atsuyo B., Naoko A., Ryosuke T., Munetaka H. Takayuki T., Shinji O, Yoshimitsu S., Tetsuji A., and Kenji Y. (2004). Suppression of Pathogenicity of *Porphyromonas gingivalis* by Newly Developed Gingipain Inhibitors, **Mol. Pharmacol**. 66:1599–1606.
[19] Forbes BA, Daniel FS, and Alice SW. (2007). Bailey and Scott's diagnostic microbiology. **12th. ed.; USA Mosby Elsevier Company**.
[20] Amano A, Kuboniwa M, Nakagawa I, Akiyama S, Morisaki I and Hamada S. (2000). Prevalence of specific genotypes of *Porphyromonas gingivalis* fimA and periodontal health status, **J. Dent. Res.** 79:1664-8.
[21] Nakagawa I, Amano A, Ohara-Nemoto Y, Endoh N, Morisaki I, Kimura S, Kawabata S and Hamada S. (2002). Identification of a new variant of *fimA* gene of *Porphyromonas gingivalis* and its distribution in adults and disabled populations with periodontitis, **J. Periodontal. Res.** 37: 425-432.
[22] Goncharoff P, Figurski DH, Stevens RH and Fine DH. (1993). Identification of *Actinobacillus actinomycetemcomitans*: polymerase chain reaction amplification of lktA-specific sequences, **Oral Microbiol Immunol.,** 8: 105-110.

[23] Amano A, Nakagawa I, Kataoka K, Morisaki I and Hamada S. (1999). Distribution of *Porphyromonas gingivalis* strains with *fimA* genotypes in periodontitis patients, **J. Clin. Microbiol**. 37:1426-1430.
[24] Nakagawa I, Amano A, Kimura RK, Nakamura T, Kawabata S and Hamada S. (2000). Distribution and molecular characterization of *Porphyromonas gingivalis* carrying a new type of *fimA* gene, **J. Clin. Microbiol**. 38: 1909-1914.
[25] FukumotoY, Asahi K, Yamazaki. (2002). Method for producing ellagic acid. **Jap. Patent**. 205993.
[26] Abdollahzadeh Sh., Mashouf RY., Mortazavi H., Moghaddam MH., Roozbahani N., and AVahedi M. (2011). Antibacterial and Antifungal Activities of *Punica granatum* Peel Extracts Against Oral Pathogens, **Journal of Dentistry, Tehran University of Medical Sciences,** Tehran, Iran (Vol. 8, No.1).
[27] NCCLS. (2002). National Committee for Clinical Laboratory Standards, Methods for dilution antimicrobial susceptibility tests of bacteria that grow aerobically. **NCCLS Approved Standard M100-S12.** Wayne PA.
[28] Paola D. S. and Marcello N. (2013). Antimicrobial Activity of a Neem Cake Extract in a Broth Model Meat System. **Int. J. Environ. Res. Public Health,** Aug. 10(8): 3282–3295.
[29] Clais S., Boulet G., Kerstens M., et al. (2014). Importance of biofilm formation and dipeptidyl peptidase IV for the pathogenicity of clinical *Porphyromonas gingivalis* isolates, **Pathogens and Disease.**
[30] Hajishengallis G. (2011). Immune evasion strategies of *Porphyromonas gingivalis*, **Journal of Oral Biosciences**. 53(3): 233–240.
[31] Azita H. , Hengameh K. , Abdollah B. , Molud A., and Amir E. (2013). Assessing the Antimicrobial Effect of the Essential Oil of *Myrtus communis* on the Clinical Isolates of *Porphyromonas gingivalis*: An *in vitro* Study, Jundishapur **J. Nat. Pharm. Prod.**, November; 8(4): 165-8.
[32] Smalley JW, Birss AJ, Szmigielski B and Potempa J. (2006). The HA2 haemagglutinin domain of the lysine-specific gingipain (Kgp) of *Porphyromonas gingivalis* promotes micro-oxo bishaem formation from monomeric iron (III) protoporphyrin IX., **Microbiology** 152: 1839–1845.
[33] Huda H. M. (2013). *In vitro* Antibacterial Activity of Propolis, Alum, Miswak, Green and Black Tea, Cloves Extracts Against *Porphyromonas gingivalis* Isolated from Periodontitis Patients in Hilla City, Iraq, **AJPCT**: 1(2): 140-148**.**
[34] Marieta D. Belcheva, Angelina K.-Y., Maya R., Tsonko U., Assya K., Stefan P., Victoria L., Sashka T., Milena G. and Nadia B. (2012). Transmission of *Porphyromonas gingivalis* from Carigivers to children, **Journal of IMAB** - Annual Proceeding 18, book 2.
[35] Fumiko H., Mitsugi O., Yuki O., Taro K. and Katsuyuki K. (2012)**.** Prevalence of *Porphyromonas gingivalis fimA* genotypes in Japanese children. **Journal of Oral Science**. 54(1): 77-83.
[36] Moreno, S.; Contreras, A. (2013). Functional differences of *Porphyromonas gingivalis* fimbriae in determining periodontal disease pathogenesis**:** a literature review **Colombia Medica**. 44(1**):** 51-60.
[37] Saadi J. M. and Mohammed AL Kasiey. (2009). The antibacterial effects of ellagic acid on *Mutans Streptococci* in comparison to Chlorhexidine**, J. Bagh. College Dentistry** 21(3): 2009.
[38] Holetz FB, Pessini GL, Sanches NR, Cortez DA, Nakamura CV, Filho BP. (2002). Screening of some plants used in the Brazilian folk medicine for the treatment of infectious diseases**. Mem. Inst. Oswaldo. Cruz.** Oct. 97(7): 1027-31.

[39] Meléndez PA, and Capriles VA. (2006). Antibacterial properties of tropical plants from Puerto Rico., **Phytomedicine** Mar.13(4): 272-6.
[40] Naz S, Siddiqi R, Ahmad S, Rasool SA, and Sayeed SA. (2007). Antibacterial activity directed isolation of compounds from *Punica granatum*. **J. Food Sci.** Nov; 72(9): 341-5.
[41] Reddy MK, Gupta SK, Jacob MR, Khan SI, and Ferreira D. (2007). Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* **Planta. Med.** May;73(5): 461-7.
[42] Vasconcelos LC, Sampaio FC, Sampaio MC, Pereira Mdo S, Higino JS, and Peixoto MH. (2006). Minimum inhibitory concentration of adherence of *Punica granatum* Linn (pomegranate) gel against *S. mutans, S. mitis and C. albicans*. **Braz. Dent. J.** 17(3): 223-7.
[43] Vasconcelos LC, Sampaio MC, Sampaio FC, and Higino JS. (2003). Use of *Punica granatum* as an antifungal agent against candidosis associated with denture stomatitis. **Mycoses**., Jun;46(5-6):192-6.
[44] Abdollahzadeh S., Mashouf R. Y., and H.Mortazavi et al. (2011). Antibacterial and antifungal activities of *Punica granatum* peel extracts against oral pathogens, **Journal of Dentistry** 8 (1): 1–6.
[45] Rosas-Pin´on Y., Mej´ıaa A., and D´ıaz G.-Ruiz et al. (2012). Ethnobotanical survey and antibacterial activity of plants used in the Altiplane region of Mexico for the treatment of oral cavity infections, **Journal of Ethnopharmacology** 141: 860–865.
[46] Bhadbhade S. J., Acharya A. B., Rodrigues S. V., and Thakur S. L.(2011). The antiplaque efficacy of pomegranate mouthrinse, **Quintessence International**, 42 (1): 29–36.
[47] Menezes S. M. S., Cordeiro L. N., and G. S. B. Viana. (2006). *Punica granatum* (pomegranate) extract is active against dental plaque**, Journal of Herbal Pharmacotherapy** 6 (2): 79–92.