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Antibacterial Effects of Pomegranate Extract (Ellagic Acid) on Some Clinically Isolated Periodontal Pathogens in Vitro Study

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Abstract: ***Background: Periodontal disease is considered as one of the most worldwide spread infectious diseases, that if it's not treated it may lead to functionally, aesthetically and systemically problems, hence the importance of treatment of this disease came from. And because the side effects of chemical antiseptic products that used with mechanical treatment of periodontal disease, the interesting with plant antimicrobial substances began. Pomegranate (Punica granatum Linne) is one of the oldest fruits that is used in all medical intervention because it has many biochemical constituents, from these, ellagic acid that has antioxidant and antibacterial effects. Aims of the study: To test the antibacterial effect of pomegranate extract on Aggregatibacter actinomycetemcomitans (A.a) and Porphyromonas gingivalis (P.g) (as alone and in combination) in comparison with 0.2% chlorhexidine gluconate (CHX) and 10% Dimethyl Sulfoxide (DMSO) in vitro. And laboratory analysis of the active ingredients of pomegranate extract using high performance liquid chromatography (HPLC). Materials and Methods: subgingival plaque samples were collected from 56 patients with no signs of any systemic disease, suffering from chronic periodontitis with probing pocket range from 5-6 mm in depth, A.a and P.g were isolated and diagnosed according to morphological characteristics and biochemical tests. The pomegranate white flesh was extracted by using a mixture 0f 200 ml triethylamine and 20% aqueous ethanol in a ratio 1: 9 (v/v). This study involved two experiments in vitro, the first experiments involved testing the sensitivity of A.a and P.g as alone and in combination to (0.0625mg/ml, 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml, 16mg/ml and 32mg/ml) of the pomegranate extracts in addition to 0.2% CHX and 10% DMSO use agar well diffusion method, the second experiment involved determination of the minimum inhibitory concentration (MIC) of the extracts that inhibits the bacterial growth and then the determination of the minimum bactericidal concentration (MBC) of the extract that was required to kill the bacteria. The present study also involved laboratory analysis of pomegranate extract HPLC. Results: pomegranate extract was effective in inhibition of A.a and P.g as alone and in combination using the agar well diffusion method, CHX showed higher inhibition zones than all pomegranate extract concentrations. The MIC of pomegranate extract that inhibit A.a growth was 8mg/ml and it was the same for P.g, also it was the same for A.a and P.g in combination. The MBC of pomegranate extract that kills A.a was 32mg/ml, the MBC of pomegranate extract that kills P.g was 16mg/ml, the MBC of pomegranate extract that kills A.a and P.g in combination was 32mg/ml. The HPLC analysis of pomegranate extract that was used in this study revealed that the extract contained higher concentrations of ellagic acid about 97%. Conclusion: Pomegranate extract was effective against Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis as alone and in combination, it showed bactericidal activity at 32mg/ml and 16mg/ml concentrations respectively.***

Keywords: Pomegranate, ellagic acid, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, chlorhexidine gluconate, Dimethyl Sulfoxide, high performance liquid chromatography

1. Introduction

Importance of periodontal diseases are coming from its consideration a disease among the most prevalent oral infection affecting mankind worldwide. Periodontitis is the inflammatory diseases caused by a certain bacterial complex in dental plaque biofilm. The disease could result in loss of periodontal ligament and alveolar bone. Clinically, this situation is determined by the formation of plaque and/or gingival recession. Mild to moderate forms of chronic periodontitis are mostly seen and the rate of prevalence, is estimated to be 13-57%. Severe periodontitis involved 5-15% of the population and considered as one of the most important oral health issues.[1] Chronic periodontitis if left untreated, it may progress to more advance face, characterized by tooth mobility, pain with and without eating and unaesthetic appearance of the gingiva due to loss of scalloped outline of the gingivae, this features are easily seen by the patient.[2] The main cause of periodontal disease is the multiplication of opportunistic pathogens within the gingival crevice.[3] Although the oral cavity is contained a wide range of microorganisms, approximately 630 different taxa are presented, just little number have been implicated with active periodontal disease. In 1996 officially consider the *A.a* and *P.g* are a causative agent of periodontitis,[4] and their metabolites play major roles in the initiation and progression of these infections.[5]

A.a is highly correlated with periodontitis and also colonizes the oral cavity in normal condition, it's characterized by a nonmotile, Gram-negative, facultative anaerobic and coccobacillus bacteria.[6, 7]

P.g, it consider the major bacteria that is responsible for development of periodontal infection especially chronic periodontitis with other oral bacteria and also found in normal gingival tissue; it is described as black-pigmented, Gram-negative and anaerobic rod found in subgingival biofilms.[8]

In the last years, attention in the effect of plants and its medicament product development has increase significantly,[9, 10] as 80% of the people depend on substitutional medication for their health care needs.[11, 12] And because of the most manufacturing antimicrobial agent have many side effects and also probability of development of antimicrobial resistance, therefor the plants and there natural products consider a good alternate to these agents.[13, 14]

Pomegranate (Punica granatum Linne), a common fruit in the Mediterranean area, is widely used for therapeutic formulae, cosmetics, and food seasoning,[15] in the last decade, many studies have shown that pomegranate has many therapeutic effects like: bactericidal, antiviral, antifungal, vermifuge, immune modulation, stimulant, refrigerant, astringent, stomachic, styptic, laxative, diuretic and anthelmintic.[16,17] Furthermore, pomegranate is an amazing source of anthocyanidins, phenolic acids, gallic acid, ellagic acid (tannic acids), luteolin, quercetin (flavones), kaempferol (a flavonol), naringenin (a flavanone) as well as 17-alpha estradiol, estrone, estriol, testosterone, betasistosterol, coumesterol, gamma-tocopherol, punicie acid, campesterol and stigmasterol in its juice, peels and seed oil that are chemopreventive and therapeutic potentials of this plant.[18] Among the great variety of bioactives present in the pomegranate fruit, ellagic acid compound is considered one of the most important component have antibacterial effect.[19, 20] Therefore this study was carried out in order to test the antibacterial effect of pomegranate extract on *Aggregatibacter actinomycetemcomitans* (*A.a*) and *Porphyromonas gingivalis* (*P.g*) (as alone and in combination) in comparison with 0.2% chlorhexidine gluconate (CHX) and 10% Dimethyl Sulfoxide (DMSO) in vitro.

2. Materials and Methods

The present study involved three experiments in vitro, the first experiment concerning the effects of pomegranate extract on the sensitivity of *A.a* and *P.g* as alone and in combination; the second experiment involved determination of MIC and MBC of pomegranate extract against *A.a* and *P.g* as alone and in combination; It was conducted at the unit of Bacterial Laboratory in the General Health Laboratory, during the period from February 2015 to October 2015. While the third one involved laboratory analysis of pomegranate extracts using HPLC technique. It was conducted by the Ministry of Science and Technology, Department of material research.

Human sampling: 56 participants, the age range was (35-55) years old have chronic periodontitis with no systemic disease. The subgingival dental plaque samples were collected from the deepest part of periodontal pockets with probing pocket depth (PPD) range from (5-6) mm in depth, using a sterilized curette.

The Exclusion criteria included: any chronic systemic disease, pregnancy, and smoking. Also the patients should, not do professional scaling for at least 6 months prior to the study or use of antibiotics and/or anti-inflammatory medicament within at least 3 months before the study.

The patients were informed about the study and patient consents and approvals were obtained prior to collecting the samples.

The excavation of plaque was done by periodontal curette then transferred the sample to the swab that is inserted immediately into a transfer media, the sample was spread on selective media, then incubated in an anaerobic jar with anaerobic gas bags for 72 hours anaerobically, all this procedure should be done within a 30 minutes from taking the sample from the patient to the incubation.[21]

Culture media used in this study:
1) Blood agar.[22]
2) *Aggregatibacter actinomycetemcomitans* agars.[23]
3) *Porphyromonas gingivalis* agars.[22]
4) Thioglycolate broth liquid media suitable for anaerobic micro-organisms.[24]

Identification of micro-organisms: The procedures used to identify the micro-organisms were the same for both *A.a* and *P.g* and were performed separately, according to:
1) Morphological characteristic.[25]
2) Gram stain.[26]
3) Biochemical tests.[27, 28, 29]
4) Hemolytic ability.[30]
5) Antibiotic sensitivity tests.[31]

Extraction procedure of ellagic acid from pomegranate: 100g fresh weight of the white flesh pomegranate was ground and mixed using mechanical mixer to obtain a soft slurry. A mixture 0f 200 ml triethylamine and 20% aqueous ethanol in a ratio 1: 9 (v/v), was added to the formed slurry and mixed for 1 hour at 25 ^{0}C in soxhlet extractor, the resulting mixture was allowed to stand for 24 hours and then filtered by vacuum. The suspension was dried with a rotary evaporator at 50 ^{0}C for 1h and speed 300rpm, then with a lyophilizer to obtain 50 g. of dry powder. Then the extracts were collected and stored at -20 0C. The filtrate was dried at room temperature to evaluate the extract by HPLC method and antimicrobial activity test. This procedure is done according to[32, 18] in The Ministry of Science and Technology, Department of material research.

Solutions of ellagic acid with different concentrations were prepared for this study, the concentrations prepared were (0.0625mg/ml, 0.125mg/ml, 0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml, 16mg/ml and 32mg/ml) by using two fold dilution and 10% DMSO was used in order to prepare the solutions.

Experiments performed in this study:

Experiment no. 1:
Sensitivity of *A.a* and *P.g* as alone and in combination to different concentrations of ellagic acid in vitro: In this experiment agar well diffusion method was used to study the antimicrobial effects of ellagic acid on ten isolates of *A.a* and *P.g*.

Procedure: Using a sterile loop, three colonies were picked up and emulsified in the saline with a turbidity equivalent to a 0.5 McFarland standard and then spread 0.1 ml on blood agar plate in a mattress fashion by using cotton swap, after that four wells of equal size and depth (6 mm diameter) was prepared in each agar plate using Pasteur pipette under aseptic conditions, afterwards each well was filled with the selected agent (100 microliter) then the plates were incubated anaerobically for 48 hours.

Inhibition zone represents the clear zone across the diameter of each well where no bacterial growth is present. No zone

of inhibition indicates resistance of the bacteria to the agent. The inhibition zones were measured in millimeters using a ruler.
The same procedure was performed for *P.g* and *A.a* separately and *A.a* and *P.g* together.

Experiment no. 2:
Determination of MIC and MBC of ellagic acid against *A.a* and *P.g* as alone and in combination:

Procedure:
A-Serial microdilution: A sterile microtiter (96 wells plate) plate near an alcohol burner in aseptic atmosphere is prepared for a serial dilution method for standardization.

Using a micropipette, 150 µl of thioglycolate broth is dispersed into one of the Microtiter plate wells. Using a sterile loop, a colony of the micro-organism was picked up and mixed with the thioglycolate broth in the well, one for each organism. Then from the first well the micropipette was used to take 15µl and place it into the next well, and then we complete the other 135µl with thioglycolate broth in order to have a total volume of 150µl in each well.

Then we repeat the procedure for 5 times in 5 sequential wells to reach 5 serial dilutions of 1:10 ratio each time. Each well carries the number of its dilution (e.g. 1 for the first well, 5 for the 5th dilution well and so on). Same procedure of the serial dilution is repeated for the both *P.g* and *A.a* as alone and in combination.[33]

B- Determination of MIC: Eppendorf tubes were labeled and arranged in a rack, 100 µl of bacterial suspension (10^3 concentration) were added to each tube, then 50 µl of the tested agent were added to its designated tube. Then the tubes were incubated anaerobically for 72 hours.

C-Determination of MBC: The tubes that were identified as the MIC were then subcultures in order to determine the MBC, 150 µl were taken from each tube using a micropipette and then spread on a blood agar plate using a sterile spreader and incubated anaerobically for 48 hours.

Experiment no. 3:
Determination of active constituent of pomegranate extract by using HPLC:
0.1g of the extract was dissolved in 5 ml of 20% aqueous ethanol and triethylamine mixture with a ratio of (9:1). The solution completed to10 ml with same solvent mixture in a volumetric flask. The resulted solution was diluted twice more. The analysis conditions were carried on reverse phase OSD-C18 (250 * 4.6mm. I.D) column, mobile phase 0.03M phosphate buffer PH (7): methanol 70:30 (v/v), flow rate 1ml / min; temperature 35 0C and UV-VIS. detector at 256 nm. (Fukumoto *et al*., 2002). This study was done in the Ministry of Science and Technology, Department of material research.

Statistical analysis: Data processing and analysis were carried out by using SPSS program, which provide the following:-
1) Calculation and presentation of statistical parameters: mean and SD of the variables in the study.
2) Analysis of variance (ANOVA) for testing the significant differences among means of different groups.
3) For all the above mentioned tests, the analysis was accepted at $p<0.05$, as the limit of significance, when $p<0.001$ were regarded as highly significant.

3. Results

Production of pomegranate extraction
In this study, the prepared ellagic acid was purified by dissolving with 20% aqueous ethanol and triethylamine mixture, filtered, acidified to PH-2, filtered, washed and dried. The final weight of ellagic acid is about 33mg and its color is creamy white to yellow.

Experiment No.1: A- Sensitivity of *A.a* to different concentrations of ellagic acid (Agar well diffusion method): Pomegranate extract (ellagic acid) showed increase in the diameter of inhibition zone as the concentration increase from 0.0625mg/ml to 32 mg/ml, mean of inhibition for 32mg/ml was 19.7mm, chlorhexidine (positive control) showed the largest diameter of inhibition zone which was 21.3mm while 10%DMSO (negative control) showed no inhibition zone. Table (1).

Table 1: Descriptive statistics of inhibition zones (mm.) of *A.a* bacteria using different concentration of pomegranate extract (ellagic acid) and 0.2%CHX and 10% DMSO

Conc.	Mean	SD
0.0625	0	0
0.125	7.10	0.32
0.25	7.9	0.74
0.5	9.8	1.23
1	11.7	1.49
2	13.2	1.48
4	14.6	1.65
8	16.3	1.49
16	17.9	1.52
32	19.7	1.42
CHX	21.3	1.83
DMSO	0	0

Statistical analysis using **ANOVA** test (analysis of variance test) was shows a highly significant difference among different concentrations of ellagic acid and the positive and negative control agents when tested against *A.a*, F-test value was306. 622 and p-value was 0.000.

Least significant difference (LSD) Each concentration of pomegranate extract (ellagic acid) is compared with other conc. of pomegranate extract and with positive and negative controls.

0.0625mg/ml. showed non-significant difference with 10% DMSO; while it showed highly significant differences with all the other concentrations and with the +ve controls.

0.125mg/ml. showed non-significant difference with 0.25mg/ml. ; while it showed highly significant differences with all the other concentrations and with the +ve and –ve controls.

0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml, 16mg/ml and 32mg/ml concentrations showed highly

significant differences when comparing each of them to the other concentrations and to the control agents.

B- Sensitivity of *P.g* to different concentrations of pomegranate extract (Agar well diffusion method):

Ellagic acid showed increase in the diameter of the inhibition zone as the concentration increased, chlorhexidine (positive control) showed the largest mean of inhibition zones (mean of inhibition zones for CHX was 22.3); while 10% DMSO (negative control) showed no inhibition zone. Table (2).

Table 2: Descriptive statistics of inhibition zones (mm.) of *P.g* bacteria using different concentration of pomegranate extract (ellagic acid) and 0.2%CHX and 10% DMSO

Conc.	Mean	SD
0.0625	0	0
0.125	7.5	0.70711
0.25	8.7	1.059
0.5	10.9	1.59
1	12.9	2.46
2	14.1	1.72
4	15.4	2.01
8	17.3	1.49
16	19.0	1.05
32	20.6	1.42
CHX	22.3	1.88
DMSO	0	0

Statistical analysis using **ANOVA** test (analysis of variance test) was shows highly significant difference among different concentrations of ellagic acid and the positive and negative control agents when tested against *P.g*, F-test value was 282.965 and p-value was 0.000.

Least significant difference (LSD) test: Each concentration of pomegranate extract (ellagic acid) is compared with other conc. of pomegranate extract and with positive and negative controls.

0.0625mg/ml. showed non-significant difference with 10%DMSO; while it showed highly significant differences with all the other concentrations and with the +ve.

0.125mg/ml. showed non-significant difference with 0.25mg/ml. ; while it showed highly significant differences with all the other concentrations and with the +ve and –ve controls.

0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 4mg/ml. 8mg/ml 16mg/ml and 32mg/ml concentrations showed highly significant differences when comparing each of them to the other concentrations and to the control agents.

C- Sensitivity of *A.a* and *P.g* in combination to different concentrations of pomegranate extract (Agar well diffusion method):

Ellagic acid showed increase in the diameter of the inhibition zone as the concentration increased, chlorhexidine (positive control) showed the largest mean of inhibition zones (mean of inhibition zones for CHX was 21.0); while 10% DMSO (negative control) showed no inhibition zone. Table (3).

Table 3: Descriptive statistics of inhibition zones (mm.) of *A.a* and *P.g* in combination using different concentration of pomegranate extract (ellagic acid) and 0.2%CHX and 10% DMSO

Conc.	Mean	SD
0.0625	0	0
0.125	7.1	0.31623
0.25	7.8	0.78881
0.5	9.5	0.97183
1	11.5	1.35401
2	12.8	1.54919
4	14.5	1.43372
8	15.9	1.152388
16	17.4	1.71270
32	19.3	1.33749
0.2% CHX	21.0	2.05480
10% DMSO	0	0

Statistical analysis using **ANOVA** test (analysis of variance test) was shows highly significant difference among different concentrations of ellagic and the positive and negative control groups when tested against *A.a* and *P.g* in combination, F-test value was 293.626 and p-value was 0.000.

Least significant difference (LSD) Each concentration of pomegranate extract (ellagic acid) is compared with other conc. pomegranate extract and with positive and negative controls.

0.0625mg/ml. showed non-significant difference with 10%DMSO; while it showed highly significant differences with all the other concentrations and with the +ve controls.

0.125mg/ml. showed non-significant difference with 0.25mg/ml. ; while it showed highly significant differences with all the other concentrations and with the +ve and –ve controls.

0.25mg/ml, 0.5mg/ml, 1mg/ml, 2mg/ml, 8mg/ml, 16mg/ml and 32mg/ml concentrations showed highly significant difference when comparing each of them to the other concentrations and to the control agents.

Experiment No.2: A- Determination of minimum inhibitory of pomegranate extracts (ellagic acid) against *A.a* and *P.g* as alone and in combination:

The concentration 8mg/ml of pomegranate extract (ellagic acid) is considered as the MIC against *A.a* and *P.g* as alone and in combination. Table (4).

Table 4: MIC of pomegranate extract against *A.a* and *P.g* as alone and in combination

Conc.	*A.a*	*P.g*	*A.a & P.g*
4mg/ml	+	+	+
8mg/ml	-	-	-
16mg/ml	-	-	-
32mg/ml	-	-	-
CHX	-	-	-
Bacteria alone	+	+	+

B- Determination of Minimum bactericidal concentration (MBC) of pomegranate extracts (ellagic acid) against *A.a* and *P.g* as alone and in combination:

The MBC for pomegranate extract (ellagic acid) that kills *A.a* was 32mg/ml, *P.g* was 16mg/ml and *A.a* and *P.g* in combination was 32mg/ml. Table (5).

Table 5: MBC of pomegranate extract against *A.a* and *P.g* as alone and in combination

Conc.	*A.a*	*P.g*	*A.a & P.g*
8mg/ml	+	+	+
16mg/ml	+	-	+
32mg/ml	-	-	-
CHX	+	+	+
Bacteria alone	+	+	+

Experiment No.3: determination of pomegranate extracts active constituents using HPLC:

High-performance liquid chromatography (HPLC), is used here to separate active compounds of pomegranate peel extract.

Using column (250x 4.6 mm i. d) for separation ellagic acid from pomegranate extract, the eluted peak were monitored by UV set at 264 nm, the chromatograms of the studied samples were recorded using HPLC, Shimadzu 10A *VP* , Japan.

The analysis results were appeared that the retention time for sample is 4.25min fig. (1a) and 4.21min fig. (1b) for the standard. The purity of the prepared ellagic acid in term of concentration was calculated related to standard compound and it is found to be 97%. Fig.1

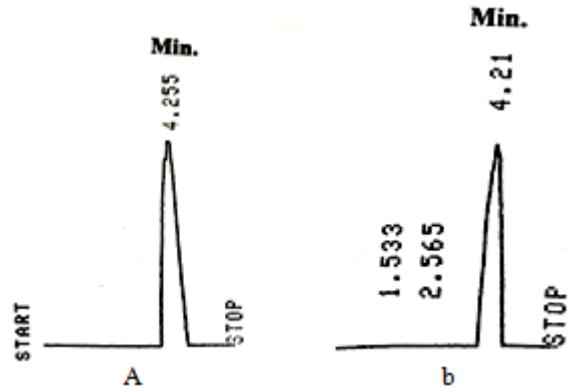

Figure 1: HPLC Chromatogram of (a) prepared ellagic acid (b) standard ellagic acid

4. Discussion

It is the first Iraqi study that researched the antibacterial effect of pomegranate extract (ellagic acid) and sensitivity of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* as alone and in combination with different concentration of the extract.

Sensitivity of *A.a* to different concentrations of pomegranate extracts (ellagic acid) in vitro (agar well diffusion):

Results showed that pomegranate extracts (ellagic acid) were able to inhibit the growth of *A.a* this finding was in agreement with Bhadbhade *et.al* in 2011.[34]

This could be related to the fact that when pomegranate extract used in this study were analyzed using HPLC technique, it contained higher concentration of ellagic acid (about 97%) and studies revealed that ellagic acid exhibited strong antimicrobial abilities.[19, 20]

10%DMSO used as –ve control, had no effect on the bacteria appearing by the absence of inhibition zone, this was in agreement with Fani and Kohanteb in 2012.[5]

Sensitivity of *P.g* to different concentrations of pomegranate extract (ellagic acid) in vitro (agar well diffusion):

Results showed that pomegranate extract (ellagic acid) were able to inhibit the growth of *P.g*, this finding was in coincidence with other studies like Cai and Wu in 1996 and Rosas-Pin´on *et al* in 2012.[35, 36]

The diameter of inhibition zones was increased as the concentration of pomegranate extract (ellagic acid) increased from 0.125mg/ml to 32mg/ml, this was in agreement with (Adel *et al*, 2015) who found that the increase in the consternation of pomegranate extract from 0.5mg/ml to 32mg/ml lead to increase in the diameter of inhibition zone.

Loo *et al*. in 2010 evaluated the effect of Ellagic acid on the growth of oral bacteria (*Porphyromonas gingivals* other type of oral bacteria) as well as their generation of water insoluble glucan and adhesion to saliva-coated hydroxyapatite (S-HA) beads. Antibacterial activity of Ellagic acid was determined by using adenosine triphosphate (ATP) bioluminescence assay at various concentrations from 0.125 to 8 mg/ml.[37]

Sensitivity of *A.a* and *P.g* in combination to different concentrations of pomegranate extract (ellagic acid) in vitro (agar well diffusion):

Results showed that pomegranate extract (ellagic acid) were able to inhibit the growth of both A.a and *P.g*. There is no study before to compare with this study.

Determination of Minimum inhibitory concentration:

The MIC of pomegranate extract (ellagic acid) that inhibit *A.a* and *P.g* as alone and in combination was 8mg/ml. Which means that 8mg/ml of pomegranate extract was able to inhibit the bacterial growth in broth media but when bacteria samples taken from this broth media were subcultured on blood agar bacterial growth was observed which indicates that the pomegranate extract (ellagic acid) at 8mg/ml conc. exhibited bacteriostatic effect.

Determination of Minimum bactericidal concentration:

The MBC of pomegranate extract (ellagic acid) that kills *A.a* was 32mg/ml, for *Porphyromonas gingivalis* was 16mg/ml; that's mean *A.a* is more resistant than *P.g* to ellagic acid.

The MBC of pomegranate extract (ellagic acid) that kills *A.a* and *P.g* as in combination was 32mg/ml.

The HPLC analysis of pomegranate extracts:

The HPLC analysis of pomegranate extracts that were used in this study revealed that the extract contained highest concentration of ellagic acid, this result is agree with the published literature.[38, 39] The purity of the prepared ellagic acid in term of concentration was calculated related to standard compound and it is found to be 97%.

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