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Evaluated bioactive component extracted from *Punica granatum* peel and its Ag NPs forms as mouthwash against dental plaque

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ABSTRACT

The bioactive components of the hydroalcoholic (MeOH 70%) *Punica granatum* peel crude extract (PGPC) and its methanol fraction (PGPM) were extracted and tentatively identified by LCMS/MS as a number of phenolic compounds which are used as capping agents fasting the reduction of Ag^{+} to $Ag_{(0)}$. The prepared silver nanoparticles Ag NPs PGPC and Ag NPs PGPM were characterized by the transmission electron microscopy (TEM) and UV–vis spectra. Four mouthwashes were designed using PGPC, PGPM, Ag NPs PGPC and Ag NPs PGPM. The prepared mouthwashes exhibited a significant antimicrobial activity against the *Streptococcus mutans* and the three dental plaque isolates called *Lysinibacillus cresolivorans, Lysinibacillus cresolivorans* and *Lysinibacillus boronitolerans.* As well as, the prepared Ag NPs mouthwashes led to reduction in the cytotoxicity against BJ-1 cell line equal 35 and 23.66% with LC_{50} = 185.16 and 134.8 µg/mL. Finally, phenolics promote Ag NPs PGPM mouthwash to be potent anti-calculus and anti-hemorrhagic agents.

1. Introduction

Dental plaque considered the most common disease worldwide. It is caused by a mixture of pathogenic microorganisms and food remains. Acid-producing bacteria, specifically *Streptococcus mutans*, inhabit the dental surface and cause destruction to the hard tooth in the occurrence of fermentable sucrose and fructose (Forssten et al., 2010). In order to reduce the prevalence of dental plaque, the role of the microorganisms in dental diseases needs more understanding and clarification. The tooth surface is enclosed by a biofilm containing a paste layer including millions of bacterial cells, salivary polymers, and food remains. This biofilm can easily reach a thickness of hundreds of cells on the surfaces of the teeth and be a good environment for colonization and growth of many bacterial species also, the plaque provides an excellent adhesion site for the colonization. So far, there is no sufficient information explains the mechanism which led to this conversion. Most strategies usually depended on decreasing the growth or activity of *Streptococcus mutans* (Marsh, 2003). For controlling the dental plaque formation and accordingly, their pathogenic effects the mechanical oral hygiene processes were used, but in most cases, this is not enough. In order to overcome the shortcomings of mechanical plaque control methods, various chemotherapeutic agents have been employed and developed to improve the efficacy of daily oral hygiene (Somu et al., 2012).

Punica granatum L. (pomegranate) belongs to the family Punicaceae is a deciduous shrub in Iran, Spain, Italy, Afghanistan, America, India, Turkey, China, Russia, Uzbekistan, Morocco, Chile and Greece (Rahimi et al., 2012; Shaygannia et al., 2016). Therefore, the demand gradually grown is due to the increasing consumer awareness of the potential health benefits of pomegranates. Its traditional uses, medicinal properties and its constituents have attracted the interest of researchers and mentioned as fruits, seeds, peel and leaves of pomegranate contain various types of valuable ingredients and such ingredients show therapeutics role in disease cure such as treatment and prevention of cancer, cardiovascular disease, diabetes, bacterial infections and antibiotic resistance, dental conditions, erectile dysfunction, and ultraviolet radiation-induced skin damage. Other important applications include infant brain ischemia, male infertility, Alzheimer's disease, arthritis, and obesity. (Ambigaipalan et al., 2016; Rahmani et al., 2017). Recent researches reveal that pomegranates could be implemented in oral health care process. Also, it was used as a successful therapy for strengthening gums and fastening loose teeth. Previously, Abdollahzadeh et al. (2011) reported in *Punica Granatum* Peel Extracts as strong antibacterial against the oral pathogens like *Streptococcus mutans and Staphylococcus aureus*. Al-Obaidi et al. (2017) mentioned

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that until this moment there are limited studies concerning the using of pomegranate extracts as an anti-bacterial mouthwash and suggested the possibility of preparing a mouthwash from pomegranate extract. Pomegranates have polyphenols, tannins, ellagic acid and anthocyanins which are powerful antioxidants (Ashoush et al., 2013). Flavonoids, ellagitannins and proanthocyanidin compounds and minerals such as calcium, magnesium, phosphorus, potassium and sodium are the most ingredients of the peels of the pomegranate that covers around 60% of the fruit (Mirdehghan and Rahemi, 2007; Rahmani et al., 2017).

In previous study the treatment with *Punica granatum* extract significantly improved the chronic periodontal in a number of clinical trials (Sastravaha et al., 2005). Later, the extraction of 13 Brazilian medicinal agents was done from different plants. They were selected for their antimicrobial activity against bacteria and yeasts. The results showed that pomegranate has a promising activity on *Staphylococcus aureus* bacteria also, anti-candidal activity was identified (Kote et al., 2011).

The present study aimed to design mouthwash had antimicrobial activity against the *Streptococcus mutans* and the three isolates from dental plaque patient. Within this context, the *P. granatum* peel extract (PGPC) and its methanol fraction (PGPM) were identified. Then, they converted to their nano-forms which were characterized by various techniques. The antimicrobial activity for PGPC, PGPM, Ag NPs PGPC and Ag NPs PGPM were evaluated. Finally, the mouthwash containing silver nitrate nano-particles (Ag NPs) using PGP extract and its methanol fraction were designed, also, their cytotoxicity was evaluated for the nano-forms and their mouth washes. I*n vitro* anti-calculus and anti-bleeding activities were studied.

2. Material and methods

2.1. Materials

Silver nitrate ($AgNO_3$, Merck), pluronic® F-68, peppermint oil, saccharin, ethanol was purchased from Sigma Chemical Company, USA. thymol 99% and glycerol were obtained from ACROS Organics™.

2.2. Isolation and identification of bacteria

A swab was taken from dental plaque patient. The sample was inoculated separately into 25 mL of broth nutrient agar. The culture flasks were incubated at 37 °C for 48 h. The freshly, grown culture (1 mL) from each dental plaque was diluted serially up to 10 − 5 using the distilled water. Then, one hundred μL was serially dilutedand was spread over freshly prepared nutrient agar (NB) plates. The culture plates were incubated and the culture conditions were 37 °C and 24 h under aerobic conditions. The isolated colonies were picked up and subsequently streaked on slant of NBI for preservation. The purity of each culture was examined by microscope. All the isolates were identified according to 16s rRNA.

2.3. Amplification and sequencing of 16S rRNAgene

The 16s rRNA gene was amplified using the universal primers and the conditions illustrated by Weisburg et al. (1991). The PCR was carried out in a thermal cycler system Mj Mini (Bio Rad, Hercules, CA, USA) using 2X PCR Master Mix (Ferment as Life Sciences, Vilnius, Lithuania). The used primers were purchased from Sigma Scientific Services, Cairo, Egypt. The PCR products were purified by using a QIA quick PCR purification kit (Qiagen, Hilden, Germany) in according to the manufacturer's instructions. The isolates were identified through sequencing the amplified 16S rRNA fragment. The DNA sequences were evaluated by the dideoxy chain termination method (Sanger et al., 1977). DNA similarity was determined by the use of BLAST search tool within the National Centre of Biotechnology Information, Gene Bank (Altschul et al., 1997). All the isolates were culture on medium consists of g/L: wheat flour, 20; sucrose, 100; K_2HPO_4, 5; $MgSO_4.7H_2O$, 0.2, and yeast extract, 2.5. The initial pH was also adjusted to 7.0 prior to sterilization.

2.4. Glucan detection

The hydrolysis products were separated chromatographically by using the Whatman No. 1 filter paper and the solvent system *n*-butanol-acetone-water (4:5:1 v/v) according to (Jayme and knolle, 1956).

2.5. Plant collection and preparation

The fresh *Punica granatum* peel (PGP) from Egyptian market was collected. The peels of PGP were washed several times with domestic water then distilled water after that dried in shade for 8 days at room temperature. The dried PGP (500 g) was milled until reaching the powder form (50–100 mesh) then macerated with 70% (*v/v*) aqueous methanol (MeOH) for several times. The obtained PGP filtrate was filtered and dried on arotary evaporator (Rotavapor® R-300, BÜCHI, Switzerland). The PGPC extract was fractionated using MeOH and then the PGPM fraction was dried under reduced pressure using rotavapor®. All samples were freeze dried till using.

2.6. Total carbohydrate and phenolic content

The total carbohydrate content (TC) for each of the analyzing materials was evaluated, after hydrolysis quantitatively as described in details (Ragab et al., 2014). The total phenolic content was determined according to the method mentioned by Makkar et al. (1997). One mL of the extract was taken in a test tube, and then 0.5 mL of Folin Ciocalteu reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added sequentially in each tube. After 1 h of incubation at room temperature, the absorbance was measured at 725 nm using the spectrophotometer and compared to a gallic acid calibration curve. Total phenols were determined as gallic acid equivalents (mg gallic acid equivalent/g extract) and the values are presented as means of triplicat

2.7. High performance liquid chromatography (HPLC-(−) ESI-MS/MS)

The phytochemical analysis was done by the high-performance liquid chromatography-mass spectrometry. The LC system called Thermofinnigan (Thermo electron Corporation, USA) coupled with an LCQ-Duo ion trap mass spectrometer with an ESI source (ThermoQuest). The separation process was done through a C18 reversed-phase column (Zorbax Eclipse XDB-C18, Rapid resolution, 4.6 × 150 mm, 3.5 μm, Agilent, USA). A gradient of water and acetonitrile (ACN) (0.1% formic acid each) was done from 5% to 30% ACN in 60 min in flow rate of 1 mL/min with a 1:1 split before the ESI source. The MS was operated in the following conditions: capillary voltage (− 10 V), the source temperature was set at 200 °C. In addition, the nitrogen was used as a sheath and auxiliary gas at a flow rate of 80 and 40 (arbitrary units), respectively in the negative mode. The MS/MS fragmentation was registered with collision energy of 35%. The ions were detected in a full scan mode and mass range of 50–2000 m/z and finally the machine was controlled using the Xcalibur software (XcaliburTM 2.0.7, Thermo Scientific) (Sobeh et al., 2017).

2.8. Bio-synthesis of Ag NPs

Ag NPs PGPC and Ag NPs PGPM were done by reducing of 10 mL of aqueous $AgNO_3$ solution (1 mM) with different concentrations (100–500 μL) of PGPC and PGPM at room temperature for examination their effects upon the reduction and the size of the biosynthesized nanoparticles (Emam et al., 2017). Then, the mixture was shaken slowly and left for standingat dark room temperature.

2.9. Characterization of the bio-synthesized Ag NPs

The UV–vis spectra measurements were registered by using UV-2401 (PC)S, UV–Vis recording spectrophotometer (Shimadzu, Japan). The shapes and sizes of the as-prepared samples were done byusing the transmission electron microscope (TEM) (JEOL-JEM-1011, Japan).

2.10. Antimicrobial assay

The different samples including the PGPC, PGPM, and their modified nanoparticles and prepared mouthwashes were examined for their antimicrobial activity against *Streptococcus mutans* and the three dental plaques by using the well diffusion method (Shalaby et al., 2018). The experiment conditions were fixed at 37 °C for 24 h. Twenty mL of nutrient agar were poured into sterile petri-dishes and left to solidify. The wells were done in agar plates by using the sterile cork pore which had 4 mm diameter. The cultures were fixed to approximately 10^6 CFU/mL with sterile saline solution. Then, 150 µL of the suspensions were spreading over the agar plate's surface by a sterile glass spreader. Each tested sample was dissolved in 1 mL of DMSO and sterilized by filtration through a 0.22 µm membrane filter (by using Milliopore membrane filter apparatus). Each sample (150 µg/mL) was added separately to the convenient wells in the Petri dishes.

2.11. MIC assay

The minimum inhibitory concentration assay values (MIC) was done according to Andrews (2001). Then, the incubation was occurred at 37 °C for 24h for bacteria. The well-cut diffusion technique was done for determination of the minimal inhibitory effect. The final concentrations of the antibacterial agents adjusted to be 25, 50, 100, 200 µg/mL DMSO against all studied pathogens.

2.12. Cell viability (cytotoxicity) assay

The skin normal human cell line BJ-1 (A telomerase immortalized normal for skin fibroblast cell line) was kept in DMEM medium. The medium was supplied with 10% fetal bovine serum and incubated at 37 °C in 5% CO_2 and 95% humidity. The cells were sub-cultured using trypsin 0.15%. The cell line was provided from Karolinska Centre, Department of Oncology and Pathology, Karolinska Institute and Hospital, Stockholm, Sweden. After 24 h of seeding 50,000 cells per well in 96 well plates, the medium was converted to a complete medium contained a sample with final concentration equal 100 µg/mL in triplicates. The cells were treated for 48 h. 100 µg/mL doxorubicin was considered as the positive control and 0.5% DMSO was considered as the negative control. Cell viability was shown by using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as mentioned by (Mosmann, 1983). The equation which used for percentage cytotoxicity calculation: (1- (av(x)/(a (NC)))* 100.

Where Av: average, X: the absorbance of sample and negative control (NC) will measure at 595 nm with reference 690 nm.

2.13. Mouthwash preparation

The mouthwash was done by using the following materials: 20% w/v glycerol, 0.1% w/v thymol, 0.2% w/v saccharin, peppermint oil (q.s.), 1% w/v pluronic® F-68, 10% w/v ethanol and distilled water. 100%. The PGPC extract, PGPM fraction and their Ag NPs were dissolved in glycerol and incorporated into the prepared solution of mouthwash. The concentration of the extract done in the prepared mouthwash was determined according to MIC results; also, the concentration of the two native extracts was 2% w/v while that of the two nano-forms was 1% w/v.

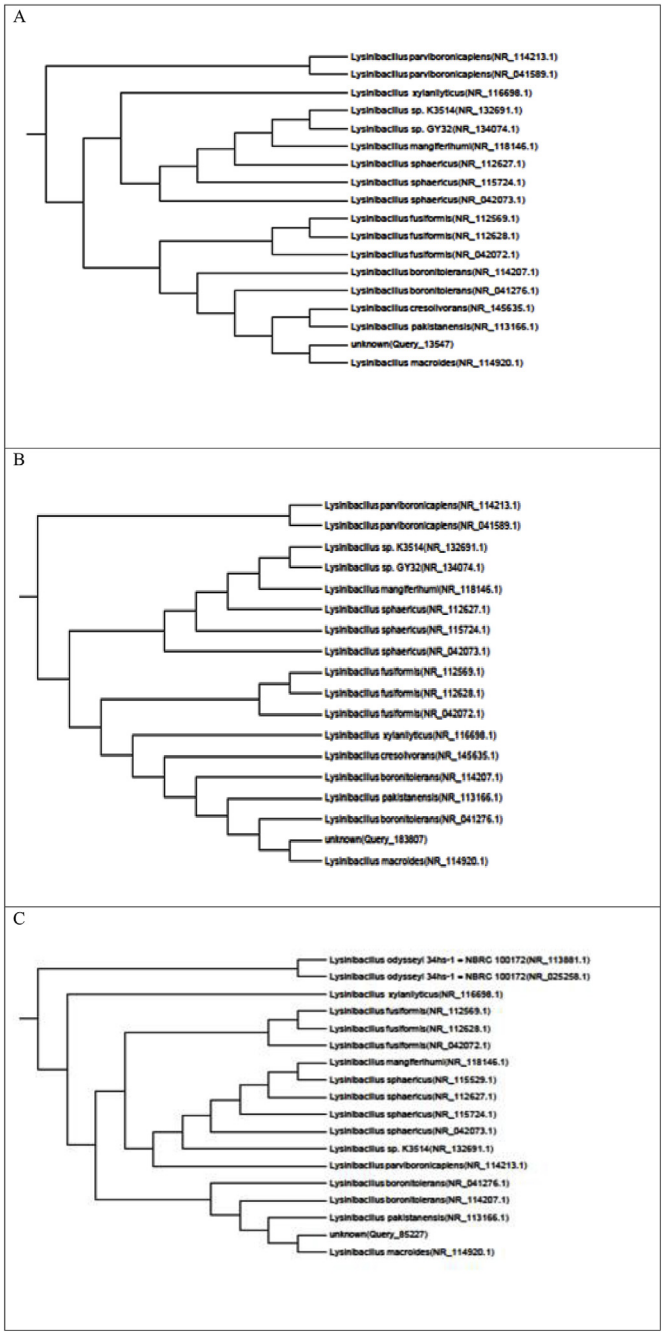

Fig. 1. 16S rRNA and identification of dental caries bacterial isolates
(A). Lysinibacillus cresolivorans
(B). Lysinibacillus cresolivorans
(C). Lysinibacillus boronitolerans.

2.14. In vitro anti-calculus

In privet Dental clinic the calculus stones were obtained. The four prepared mouthwashes 15 mL was added to 0.015 g calculus at different time intervals (0.5, 1, 1.5 and 2 min) with gently stirring at 37 °C, separately. The reaction mixture was filtrated at the definite time and the remained calculus was dried and weighted.

2.15. Anti-hemorrhagic activity

The assay was evaluated by the examination of the effect of the four prepared mouthwash on human plasma. This was achieved through sets

Table 1
LCMS/MS profiles of PGPC extract.

Id.	Metabolites	$[M-H]^-$ (*m/z*)	MS^2 ion fragments (*m/z*)	t_R (min)	References
1	5-O-caffeoylquinic acid	353	173, 111, 191	0.99	Ambigaipalan et al. (2016)
2	Citric acid derivative	391	217, 191, 173, 155, 111	1.03	Mena et al. (2012)
3	Citric acid	191	111, 173	1.54	Mena et al. (2012)
4	HHDP-hexoside	481	301, 275, 257	1.97	Mena et al. (2012)
5	Citric acid derivative	205	111, 173, 143	2.31	
6	Citric acid derivative	219	173, 111,143, 101, 87	2.62	
7	Di(HHDP-galloylglucose)-pentose	1415	1397, 783, 933, 1113, 633	3.04	Mena et al. (2012)
8	Punicalin α/A	781	601, 721	3.65	Mena et al. (2012)
9	Pedunculagin I isomer	783	481, 602, 721, 722, 765, 301	3.81	Mena et al. (2012)
10	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907	3.86	Fischer et al. (2011)
11	HHDP-gallagyl-hexoside (punicalagin)	1083	302, 575, 601, 603, 781, 807	4.01	(Ambigaipalan et al., 2016; Fischer et al., 2011)
12	Galloyl-HHDP-hexoside (corilagin)	633	275, 301, 302, 463, 615	4.51	Mena et al. (2012)
13	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907	4.66	Fischer et al. (2011)
14	HHDP-gallagyl-hexoside (punicalagin)	1083	302, 575, 601, 603, 781, 807	4.01	(Ambigaipalan et al., 2016; Fischer et al., 2011)
15	Castalagin, 2-O-galloyl-4,6(S,S) gallagoyl-D-glucose	933	915, 781, 763, 721, 601, 575	4.75	
16	Digalloyl-gallagyl-hexoside	1085	765, 783, 633, 602, 451	4.80	Mena et al. (2012)
17	Digalloyl-HHDP-glucoside (punigluconin)	801	301, 347, 649	4.99	Ambigaipalan et al. (2016)
18	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907	5.08	Fischer et al. (2011)
19	HHDP-gallagyl-hexoside (punicalagin)	1083	302, 575, 601, 603, 781, 807	5.30	(Ambigaipalan et al., 2016; Fischer et al., 2011)
20	Pedunculagin I isomer	783	481, 602, 721, 722, 765, 301	5.67	Mena et al. (2012)
21	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907	5.95/6.34	Fischer et al. (2011)
22	Galloyl-HHDP-DHHDP-hexoside(granatin B)	951	933, 907	7.06/7.42	Fischer et al. (2011)
23	Di(HHDP-galloylglucose)-pentose	1415	1397, 783, 933, 1113, 633	8.14	Mena et al. (2012)
24	Digalloyl-gallagyl-hexoside	1085	765, 783, 633, 602, 451	8.39	Mena et al. (2012)
25	Punicalagin isomers	541	301, 532, 275	8.54	Mena et al. (2012)
26	HHDP-gallagyl-hexoside (punicalagin)	1083	302, 575, 601, 603, 781, 807	8.70	(Ambigaipalan et al., 2016; Fischer et al., 2011)
27	Pedunculagin I isomer	783	481, 602, 721, 722, 765, 301	9.03	Mena et al. (2012)
28	HHDP-gallagyl-hexoside (punicalagin)	1083	302, 575, 601, 603, 781, 807	13.10	(Ambigaipalan et al., 2016; Fischer et al., 2011)
29	Di(HHDP-galloylglucose)-pentose	1415	1397, 783, 933, 1113, 633	13.74	Mena et al. (2012)
30	Valoneic acid dilactone	469	425	18.03	(Ambigaipalan et al., 2016; Fischer et al., 2011)
31	galloyl-bis-HHDP-hexoside (casuarinin)	935	481, 571, 615, 633, 639, 659	19.59	Ambigaipalan et al. (2016)
32	DigalloyltriHHDP-diglucose (sanguiin H10) isomer	1567	765, 935, 783, 915, 1209, 1265	19.84	Mena et al. (2012)
33	Ellagic acid hexoside	463	300, 301	20.33	Ambigaipalan et al. (2016)
34	digalloyl-HHDP-gluc (pedunculagin II)	785	301, 483, 613, 633, 765	20.42	Ambigaipalan et al. (2016)
35	Dihydrokaempferol-hexoside	449	287, 269, 259, 431	20.46	Mena et al. (2012)
36	Galloyl-HHDP-hexoside (corilagin)	633	275, 301, 302, 463, 615	20.88	Mena et al. (2012)
37	Ellagic acid hexoside	463	300, 301	21.43	Ambigaipalan et al. (2016)
38	trigalloylglucopyranose I	635	301, 465, 483	21.78	Ambigaipalan et al. (2016)
39	Ellagic acid hexoside	463	300, 301	22.53	Ambigaipalan et al. (2016)
40	Caffeic acid hex der	497	451	24.91	Fischer et al. (2011)
41	Ellagic acid-pentoside	433	300, 301	30.31	Mena et al. (2012)
42	Nt	491	328, 313, 284	31.31	
43	Ellagic acid deoxyhexoside	447	300, 301, 302	31.70	Ambigaipalan et al. (2016)
44	Ellagic acid	301	185, 229, 257, 283, 301	33.97	Ambigaipalan et al. (2016)
45	Kaempferolrutinoside	593	285, 547	34.99	Mena et al. (2012)
46	Kaempferolhexoside	447	285, 284, 327, 255	36.40	Mena et al. (2012)
47	Ellagic acid	301	301, 257, 229, 185	37.03/38.31	Fischer et al. (2011)
48	Kaempferolhexoside	447	285	45.73	Mena et al. (2012)
49	Phloretin-hexoside (Phlorizin)	435	273, 297, 167	47.23	Mena et al. (2012)
50	Kaempferol-3-O-pentose	417	285	50.45	

of three-hard glass test tubes (31 × 100mm) were cleaned by immersing overnight in chromic acid. After that, the mouthwash tube was prepared by mixing 0.8 mL mouthwash (PGPC, PGPM fraction and their Ag NPs, 1 mL plasma and 0.2 mL calcium chloride solution (1% w/v). After that, the tubes were placed in a water bath at 37 °C. The blank was prepared with same method by adding 0.8 mL saline solution (0.89% w/v) instead of mouthwash. The clotting time for each sample and blank was mentioned in minutes.

2.16. Statistical analysis

Results were expressed as a mean value with its standard deviation (mean ± S.D.) of each sample that is repeated three times (n = 3). Statistical analysis was performed with student's t-test and differences were considered as significant at p-values below 0.05.

3. Results and discussion

3.1. Isolation 16S rRNA and identification of dental caries bacterial

The study started with the isolation of three dental caries bacterial isolates from dental plaque patient. The microscopic examination showed that all the isolates had rod-shaped, endospore-forming bacterium and they were Gram-stain-positive. The isolates were previously, identified based on 16S rRNA as *Lysinibacillus cresolivorans, Lysinibacillus cresolivorans* and *Lysinibacillus boronitolerans* with 99% similarity Fig. 1 (A, B and C).

The second step in this research focused in trying to inhibit the isolates by natural products. All the isolates were cultured in sucrose medium and yielded glucan; the glucan was detected by paper chromatography. All the isolates yielded glucan with degree of variations. Plaque formation started by the firm adherence between the extracellular glucans and the tooth surface. Approximately, the dry weight of dental plaque contained about 20% of water-insoluble glucans (IG)

Table 2
LCMS/MS profiles of PGPM fraction.

Id.	Metabolites	$[M-H]^-$ (m/z)	MS^2 ion fragments (m/z)	t_R (min)	References
1	5-O-caffeoylquinic acid	353	173, 111, 191	0.98	Ambigaipalan et al. (2016)
2	Citric acid	191	111, 173	1.51	Mena et al. (2012)
3	Citric acid derivative	205	111, 173, 143	1.98	
4	Punicalin α/A	781	601, 721	2.05	Mena et al. (2012)
5	HHDP-hexoside	481	301, 275, 257	2.56	Mena et al. (2012)
6	HHDP-gallagyl-hexoside (punicalagin)/α	1083	302, 575, 601, 603, 781, 807	2.95	(Ambigaipalan et al., 2016; Fischer et al., 2011)
7	Pedunculagin I isomer	783	481, 602,721, 722, 765, 301	3.05	Mena et al. (2012)
8	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907, 783	3.09	Fischer et al. (2011)
9	Punicalin β/B	781	601, 721	3.29	Mena et al. (2012)
10	Galloyl-HHDP-DHHDP-hexoside(granatin B)	951	933, 907, 783	3.78/4.88	Fischer et al. (2011)
11	Digalloyl-gallagyl-hexoside	1085	765, 783, 633, 602, 451	5.88	Mena et al. (2012)
12	Castalagin, 2-O-galloyl-4,6(S,S) gallagoyl-D-glucose	933	915, 781, 763, 721, 601, 575	6.18	
13	HHDP-gallagyl-hexoside (punicalagin)/β	1083	302, 575, 601, 603, 781, 807	6.37	(Ambigaipalan et al., 2016; Fischer et al., 2011)
14	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907, 783	6.65	Fischer et al. (2011)
	Pedunculagin I isomer	783	481, 602,721, 722, 765, 301	6.88	Mena et al. (2012)
	Digalloyl-gallagyl-hexoside	1085	765, 783, 633, 602, 451	7.02	Mena et al. (2012)
15	digalloyl-HHDP-glucoside(punigluconin)	801	301, 347, 348, 649	7.55	Ambigaipalan et al. (2016)
16	Galloyl-HHDP-DHHDP-hexoside (granatin B)	951	933, 907, 783	7.74	Fischer et al. (2011)
17	Di(HHDP-galloylglucose)-pentose	1415	1397, 783, 933, 1113, 633	8.11	Mena et al. (2012)
18	HHDP-gallagyl-hexoside (punicalagin) isomer	1083	302, 575, 601, 781, 807	8.59	(Ambigaipalan et al., 2016; Fischer et al., 2011)
19	Punicalagin isomers	541	301, 532, 275	8.53	Mena et al. (2012)
20	HHDP-gallagyl-hexoside (punicalagin) isomer	1083	575, 601,721, 781, 807	9.14	(Ambigaipalan et al., 2016; Fischer et al., 2011)
21	HHDP-gallagyl-hexoside (punicalagin) isomer	1083	575, 601,721, 781, 807	13.03	(Ambigaipalan et al., 2016; Fischer et al., 2011)
22	Punicalagin-like	1109	1083, 781, 601	13.07	Mena et al. (2012)
23	Punicalagin isomers	541	301, 532, 275	13.12	Mena et al. (2012)
24	Di(HHDP-galloylglucose)-pentose	1415	1397, 783, 933, 1113, 633	13.22	Mena et al. (2012)
25	Digalloyl-gallagyl-hexoside	1085	765, 783, 633, 602, 451	13.57	Mena et al. (2012)
26	Valoneic acid dilactone	469	425	18.49	(Ambigaipalan et al., 2016; Fischer et al., 2011)
27	Galloyl-HHDP-hexoside (corilagin)	633	275, 301, 302, 463, 615	20.88	Mena et al. (2012)
28	Ellagic acid hexoside	463	300, 301	21.23	Ambigaipalan et al. (2016)
29	DigalloyltriHHDP-diglucose (sanguiin H10) isomer	1567	765, 935, 783, 915, 1209	21.84	Mena et al. (2012)
30	vanillic acid dihexoside	491	328, 329, 313	32.12	–
31	Ellagic acid	301	301, 257, 229, 185	37.27	Ambigaipalan et al. (2016)
32	Ellagic acid	301	301, 257, 229, 185	38.43	Ambigaipalan et al. (2016)

(Marotta et al., 2002).

3.2. Phytochemical studies of PGPC extract and PGPM fraction

The moisture content of PGP wastes was calculated to be 11.02% (w/w). The crude yield extract was determined to be 57.1% (w/w) and the methanol fraction 40.12%. Total carbohydrates in the PGPC extract were recorded 83% and the total phenolic was quantitated as 519.6 mg of gallic acid equivalent/g dry material. Rowayshed et al. (2013) reported that total carbohydrates in PGP was estimated to be 80% and Derakhshann et al. (2018) studied that total phenolic was ranged from 267 to 413 mg GAE/g. The polyphenolic components of the PGPC extract and its methanol fraction PGPM had been tentatively identified using HPLC-PDA-MS/MS that based on their molecular weights, fragmentation pattern, the spectral data from the PDA detector and all compounds fragmentations were compared with those published data as shown in (Tables 1 and 2). PGP contains different chemical components, which may possess the various pharmacological and toxicological activities. These components are identified as organic acids, ellagitannins and gallotannins, ellagic acid derivatives, catechin and procyanidins, anthocyanins and anthocyanidins, flavonols and summarized in Tables 1 and 2

3.3. UV–vis spectroscopy

The Ag nano-particle dispersions had brilliant colors and yellowish brown color in the reaction mixture. This result was an evident for the preparation of Ag nano-particles, due to the surface plasmon resonance (SPR) (Rivero et al., 2013). Fig. 2 (A and B) illustrated the UV–vis spectra of Ag nano-particles prepared by using variable amounts of PGPC extract and its PGPM fraction to 10 mL of 10^{-3} M $AgNO_3$ solution. The samples recorded an absorption in the visible region at 445–456 nm owing to the SPR band. The intensity of the SPR band rises with increasing the concentration. The growing intensity of the SPR band indicated that more Ag^+ ions were reduced to Ag nano-particles (Emam et al., 2017; Zayed et al., 2015). There was no obvious change in peak position for 10 days, except for the increase of absorbance. Increase of absorption proved that the amount of silver nanoparticles increases. The stable position of absorbance peak indicated that new particles did not aggregate. These spectra demonstrated that the silver nanoparticle colloidal solution could be remaining stable within one month.

3.4. TEM analysis

For the determination of the shape and size of Ag nano-particles the HRTEM technique was used. Fig. 3 (A&B) displayed the TEM image of the particle distribution in the prepared Ag NPs. It could be realized that the prepared nano-particles are mostly spherical in shape with particle size varies between 4 and 27 nm. Ag NPs PGPC appeared the smallest particle size 3–6 nm and the Ag NPs PGPM has intermediate particle size 18–22 nm. All the particle size had critical nano size (less than 100 nm) that may be reflected on their biological activities. The particles were apart from each other and this result proved the covering action of the plant extract in the preparation process.

3.5. Antimicrobial results

There are many studies have been reported that natural phenolics and their Ag NPs could be used as bactericidal agents (Al Jaouni and Selim, 2017). PGPC, PGPM, Ag NPs PGPC, Ag NPs PGPM were evaluated for the antimicrobial activity which expressed by measuring the

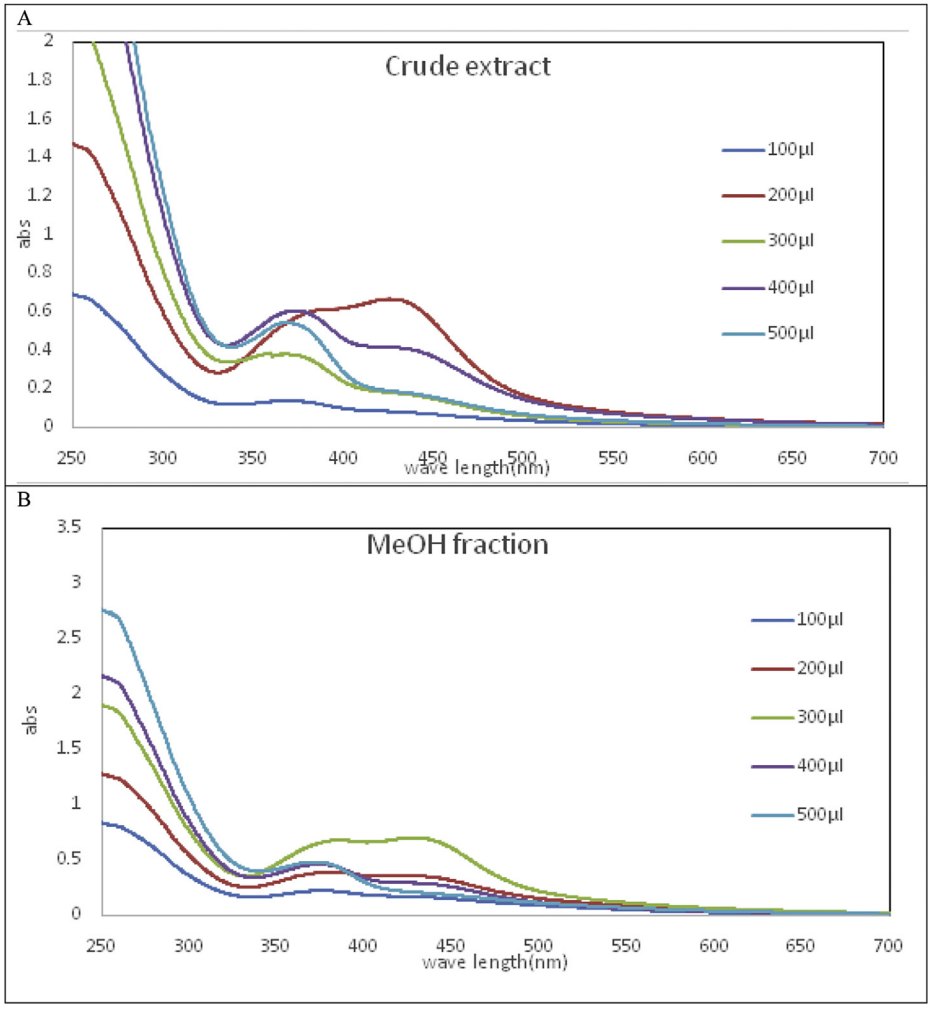

Fig. 2. The SPR band of Ag NPs recorded by UV–vis spectra as a function of varying addition of (A) PGPC extract and (B) PGPM fraction.

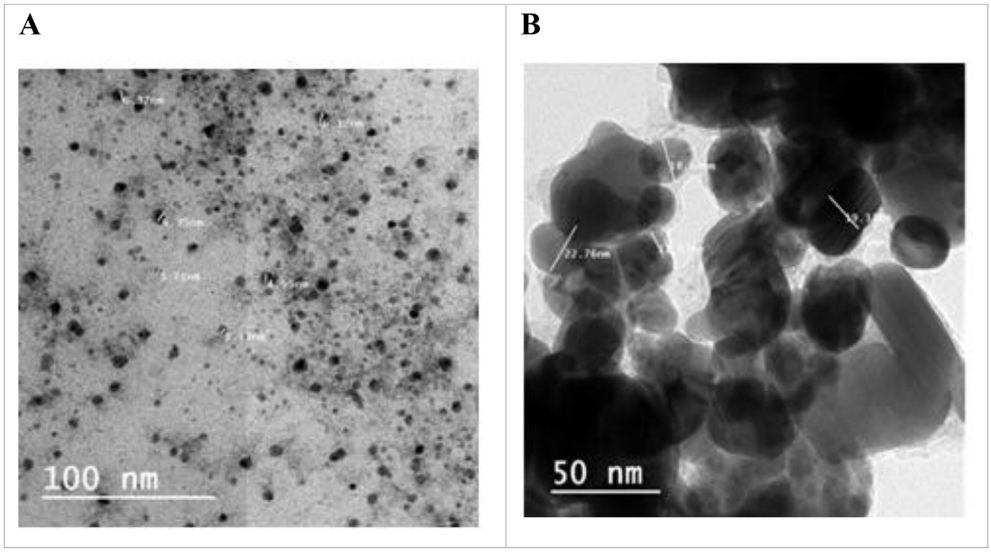

Fig. 3. TEM spectroscopy: (A) Ag NPs PGPC and (B) Ag NPs PGPM.

diameter of the inhibition zone (mm) against the pathogenic strain. The MeOH silver nanoparticles fraction from pomegranate peel Ag NPs PGPM in Table 3 showed the most active bactericidal compound with diameter zone ranged from 18.03 to 30.03 mm against the pathogenic isolates which had a role in tooth plaque formation and tooth decay. Also, the nano forms had promising antimicrobial activity against the *Streptococcus mutans* which play main role in dental plaque formation. From the recorded data, silver nanoparticles technique was applied to

Table 3
Antimicrobial activity of native and modified Ag NPs extract from PGP.

Samples	*Mean diameter of inhibition zone (mm)*			
	Streptococcus mutans	*Lysinibacillus cresolivorans*	*Lysinibacillus cresolivorans*	*Lysinibacillus boronitolerans*
PGPC	10.03 ± 0.15	15.03 ± 0.15	15.30 ± 0.26	20.16 ± 0.37
Ag NPs PGPC	16.20 ± 0.20	21.90 ± 0.10	28.16 ± 0.37	18.33 ± 0.35
PGPM	12.16 ± 0.15	15.03 ± 0.14	20.26 ± 0.25	25.06 ± 0.20
Ag NPs PGPM	18.03 ± 0.15	25.00 ± 0.10	30.03 ± 0.25	20.16 ± 0.20
Control	18.13 ± 0.16	20.10 ± 0.10	22.20 ± 0.26	20.40 ± 0.40

Control: Forcetex 10 µg for bacteria.

Table 4
MICantimicrobial activity of native and modified Ag NPs extract from PGP.

Samples	MIC (µg/mL)			
	Streptococcus mutans	*Lysinibacillus cresolivorans*	*Lysinibacillus cresolivorans*	*Lysinibacillus boronitolerans*
PGPC	150	150	200	200
Ag NPs PGPC	100	50	150	150
PGPM	100	100	150	150
Ag NPs PGPM	100	25	50	150
Control	100	100	100	100

Control: Forcetex 10 µg for bacteria.

Table 5
Antimicrobial activity of PGPC, PGPM mouthwashes and their modified nanoparticles.

Mouthwashes	Mean diameter of inhibition zone (mm)			
	Streptococcus mutans	*Lysinibacilus cresolivorans*	*Lysinibacillus cresolivorans*	*Lysinibacillus boronitolerans*
PGPC	12.50 ± 0.45	25.03 ± 0.15	16.80 ± 0 .81	14.83 ± 0.47
Ag NPs PGPC	20.1 ± 0.36	30.33 ± .035	27.00 ± 0.01	27.86 ± 0.41
PGPM	15.26 ± 0.37	25.23 ± 0.25	24.80 ± 0.52	20.23 ± 0.25
Ag NPs PGPM	18.03 ± 0.25	28.16 ± 0.15	29.60 ± 0.78	28.00 ± 0.40
Control	18.26 ± 0.33	20.93 ± 0.20	22.26 ± 0.30	19.93 ± 0.30

Control: Forcetex 10 µg for bacteria.

obtain a new natural bactericidal compound exhibited the highest antimicrobial activity. The capping agents, such as redox system had an essential role in Ag NPs yield. In addition, the concentrations and the nano sizes of the Ag NPs could have a fundamental role in improving its antimicrobial activity. This result in easy diffusion or penetration through the cell membrane of microorganisms and its growth inhibition (Panácek et al., 2006). Moreover, PGPC and PGPM have significant effect on gram positive and gram-negative bacteria pathogens as antimicrobial agents enhanced by nanoform.

Table 7
In vitro cytotoxicity activity % at100 µg/mL andLC_{50} µg/mL after 48 h.

Samples	Cytotoxicity% at 100 µg/mL	LC_{50}µg/mL
PGPC	63.46 ± 3.52	53.36 ± 2.09
PGPC mouthwash	28.33 ± 3.05	215.43 ± 5.66
Ag NPs PGPC	91.63 ± 3.72	35.60 ± 2.94
Ag NPs PGPC mouthwash	35.00 ± 2.00	185.16 ± 5.25
PGPM	59.56 ± 5.05	75.50 ± 2.78
PGPM mouthwash	28.33 ± 3.05	193.63 ± 3.46
Ag NPs PGPM	90.73 ± 3.16	44.76 ± 3.65
Ag NPs PGPM mouthwash	23.66 ± 2.08	134.80 ± 4.70

Table 8
In vitro anti-calculus and clotting time of the prepared mouthwashes.

Mouthwashes	Anti-calcus time per min				Percent%	Clotting Time (min)
	0.5	1	1.5	2		
PGPC	0.015	0.014	0.014	0.014	6.66	5
Ag NPs PGPC	0.014	0.013	0.012	0.012	19.99	3
PGPM	0.014	0.013	0.013	0.013	13.34	4
Ag NPs PGPM	0.010	0.0096	0.0072	0.0069	54.00	2

3.6. MIC assay

According to reutilization of PGP waste; originated from industrial processes, is converted to bioactive substance which contained high phenolic content and antioxidant properties. In this assay, the modified Ag NPs showed the highest antibacterial efficacy against the three bacterial pathogenic *Lysinibacillus cresolivorans*, *Lysinibacillus cresolivorans* and *Lysinibacillus boronitolerans* with MICs values ranged from 25 µg/mL and 200 µg/mL (Table 4). Menezes et al. (2006) and Foss et al. (2014) reported that hydroalcoholic extract showed high effective activity against dental plaque microorganism with MICs values of 15 µg/mL, and antifungal activity against dermatophyteshad MICs values of 125 µg/mL and 250 µg/mL. So it was concluded that modified nanoparticles showed a potent antimicrobial activity against dental plaque microorganisms.

3.7. Antimicrobial activity of mouthwashes

The results in Table 5 referred to the significant efficiency of the prepared mouthwashes as antimicrobial agents against *Streptoccous mutans* which considered the most pathogenic oral bacteria and main cause of dental caries. The Ag NPs PGPC and Ag NPs PGPM had inhibition zone ranging from 20.10 to 30.33 mm and 18.03–29.60 mm, respectively. The result demonstrated that both Ag NPs PGPC and Ag NPs PGPM mouthwashes have slightly significant antibacterial activity with diameter zone ranged from 18 to 30 mm against the isolated oral bacteria. This result proved that Ag NPs mouthwash was more effective agent rather than the crude extract. In this finding, Nóbrega et al. (2015) recorded that *Punica granatum* mouthwash was effective in reducing the oral *Streptococci* counting. Depending on the result of

Table 6
MIC of PGPC, PGPM mouthwashes and their modified nanoparticles.

Mouthwashes	MIC (µg/mL)			
	Streptococcus mutans	*Lysinibacillus cresolivoras*	*Lysinibacillus cresolivoans*	*Lysinibacillusboronitolran*
PGPC	150	100	100	100
Ag NPs PGPC	100	50	50	50
PGPM	150	100	100	100
Ag NPs PGPM	100	50	50	50
Control	100	100	100	100

Control: Forcetex 10 µg for bacteria.

antimicrobial activity and estimation of inhibition zone, MIC assay of PGPC, PGPM, Ag NPs PGPC, and Ag NPs PGPM mouthwashes was done against the pathogenic isolate. The result demonstrated that both Ag NPs PGPC and Ag NPs PGPM have the same significant antibacterial activity with MIC value 50–100 µg/Ml (Table 6).

3.8. Cytotoxicity studies

The prepared samples were examined for their cytotoxicity effect against the normal skin fibroblast (BJ-1). A dose of the most active samples was used for calculating their LC_{50} values (Table 7). In general, the cytotoxicity was high in Ag NPs PGPC, and Ag NPs PGPM, but reduced to great extent in mouthwash samples. These results suggested the mouthwash component had ability to reduce the cytotoxicity risk to great extent.

3.9. In vitro anti-calculus

Calculus is a form of hard-edged dental plaque. It is caused by precipitation of different minerals from residue food on the teeth and within the narrow sulcus. Calculus is composed of inorganic mineral 40–60% (calcium phosphate mainly) and organic components. *In vitro* anti-calculus assay was performed to estimate the dissolved calculus with different time using PGPC, PGPM, Ag NPs PGPC and Ag NPs PGPM prepared mouthwashes. The dissolved calculus was calculated accurately for all the prepared mouthwashes in different intervals time (0.5, 1, 1.5 and 2 min) as shown in Table 8. The data in Table 8 recorded that Ag NPs PGPM mouthwash has the highest percent 54% of dissolved calculus and Ag NPs PGPC mouthwash exhibited good percentage of the dissolved calculus 19.99%. The results concluded that Ag NPs PGPM mouthwash has significant effect in calculus dissolving in comparison with the PGPC extract.

3.10. Anti-hemorrhagic activity

The clotting time is the time required for the coagulation of the blood plasma under standard conditions. The normal clotting time was determined to be 8–15 min (Dayyal, 2016). The data recorded at Table 8 showed that the Ag NPs PGPM fraction mouthwash recorded the lowest clotting time equal 2 min. On the other side, the PGPC extract mouthwash recorded 5 min in comparison to the blank sample 8 min. From this results PGPC extract, PGPM fraction and their Ag NPs nano-forms considered as anti-hemorrhagic agents that used for treatment of bleeding disorders as mouthwash. These results compatible with Goshtasebi et al. (2015) that proved the anti-hemorrhagic activity of *punica granatum* L. flower by a double-blind trail.

4. Conclusion

This study aimed to fabricate cost-effective and efficient mouthwash from the *P. granatum* peel waste. The results recorded that the PGPC extract and PGPM fraction could be converted efficiently to nano-particles which played a significant role in mouthwash efficiency. Also, the prepared mouthwashes cytotoxicity was evaluated as a safe product. All the results recommended *P. grantum* peel native and nano-particles to be used as an effective antiplaque, anti-calculus and anti-hemorrhagic agents in pharmaceutical field. Accordingly, the mouth wash safety will be tested in another vivo study.

Conflicts of interest

No conflict of interest to be disclosed by authors.

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