Original Article

In Vitro Assessment of Antibacterial Activity of Pomegranate Vinegar and Rose Water Compared with Persica Mouthwash against Oral Bacteria

F. Ramezanalizadeh [1], M. Rabbani [2], M. Khoroushi [3], A. Aliasghari [4]

[1] Graduate Student, Department of Microbiology, School of Biology, Isfahan University of Medical Sciences, Isfahan, Iran
[2] Associate Professor, Department of Microbiology, School of Biology, Isfahan University of Medical Sciences, Isfahan, Iran
[3] Professor, Department of Operative Dentistry, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran
[4] Postgraduate Student, Department of Microbiology, School of Biology, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background and Aim: Mutans streptococci are the main cause of tooth decay. Application of natural materials as mouthwash has been effective in reducing the bacterial count. This study aimed to assess the antimicrobial effects of rose water and pomegranate vinegar in comparison with Persica mouthwash on two oral bacteria responsible for tooth decay.

Materials and Methods: Strongly adherent strains of *Streptococcus mutans* and *Streptococcus sobrinus* were selected for this in vitro study. Antimicrobial effects of pomegranate vinegar and rose water on microbial count in the biofilm and adhesion potential of bacteria were evaluated by microtiter plate method. Also, the well-plate technique was used to assess the effect of rose water and pomegranate vinegar in comparison with Persica mouthwash on bacterial growth and proliferation. The obtained resulted were analyzed by one-way ANOVA and Tukey's post hoc test using Graph Pad Prism version 5 software. Level of significance was set at P=0.05 with 95% confidence interval.

Results: Pomegranate vinegar, rose water and Persica decreased plaque formation by S. *mutans* by 93%, 80% and 68%, respectively. These values for the *S. sobrinus* were 92%, 57% and 48%, respectively (all Ps<0.001). Pomegranate vinegar was more effective than the other two materials (P<0.001). However, none of these materials eliminated the biofilm. Pomegranate vinegar and Persica mouthwash inhibited the growth of the afore-mentioned bacteria.

Conclusion: Within the limitations of this study, it seems that pomegranate vinegar and rose water have the potential to prevent or control the proliferation of S. mutans and S. sobrinus.

Key Words: Dental Caries, Mouthwashes, Streptococcus mutans, Streptococcus sobrinus

- **Cite this article as:** Ramezanalizadeh F, Rabbani M, Khoroushi M, Aliasghari A. In Vitro Assessment of Antibacterial Activity of Pomegranate Vinegar and Rose Water Compared with Persica Mouthwash against Oral Bacteria. J Islam Dent Assoc Iran. 2015; 27(3):150-157.

Corresponding author: M. Rabbani, Associate Professor, Department of Microbiology, School of Biology, Isfahan University of Medical Sciences, Isfahan, Iran

m.rabbani@biol.ui.ac.ir

Received: 19 March 2015
Accepted: 15 May 2015

Introduction

Increased public demand for natural substances for treatment of diseases has resulted in a search for herbal medications. There are approximately 500,000 plant species worldwide; out of which, only 1% have been photo-chemically evaluated and there is a great potential for finding bioactive materials [1]. Several articles are available on the

antimicrobial efficacy of plant extracts; however, the effects of medicinal plants on oral pathogenic microorganisms have been less commonly evaluated.

Tooth decay is a common infectious disease with higher prevalence among low socioeconomic groups [2]. Treatment of caries is time consuming and costly. Considering the high prevalence of caries, increased bacterial resistance to antibiotics, adverse effects of chemical and synthetic drugs as well as their high cost, there is a clear need for natural, cost effective alternatives for prevention of caries. The currently available chemical medications change the oral microbiota and have complications such as diarrhea, vomiting and tooth staining [1,3]. *Streptococcus mutans* and *S. sobrinus* are the main causes of caries due to their high potential for biofilm formation. These bacteria adhere to tooth enamel and produce glucans following the consumption of sucrose in foods and result in accumulation of glucans in the biofilm and eventually cause enamel destruction viaacid production [4]. Since bacterial adhesion is the first step for bacterial prolifertaion and tooth decay, adhesion is the new target for the modalities aiming to inhibit bacterial colonization [3].

In this regard, medicinal plants have also been evaluated. Studies in this regard are divided into two groups. Group one studies assess the activity of natural compounds, plant extracts and pure photochemical agents against oral pathogenic microorganisms. Group two studies assess the efficacy of these products for inhibition of oral biofilm formation via decreasing microbial attachment to tooth surfaces [5]. Materials that have a potential to inhibit bacterial adhesion and growth would be ideal for prevention of tooth caries.

Rose water and vinegar have been used in the Iranian traditional medicine to prevent tooth decay. Rose water is derived from Rosa damascena Mill. From the Rosacea family [6].

Rose water is produced in Iran in large amounts and is exported to different countries worldwide. It has several applications and is used for different conditions such as skin lesions [7]. It is also used in cosmetic industries and for the production of moisturizing lotions or cleansers [8]. If its antimicrobial efficacy is confirmed, it can be effectively used in mouthwashes for caries prevention. Thus, this in vitro study aimed to assess the effect of rose water on the most common cariogenic bacteria.

Vinegar has also been used in the Iranian traditional medicine. Its production dates back to 4000 B.C. Its production process is sort of an incomplete oxidation reaction. In the process of vinegar production, yeasts first convert sugar to alcohol and then acetobacters convert alcohol to citric acid [9]. Vinegar increases the accumulation of glycogen, prevents hypertension, stimulates the uptake of calcium and decreases total serum cholesterol and triacylglycerol as indicated in animal studies [10]. Use of vinegar for infection control and treatment of acute conditions dates back to the time of Hippocrates, who recommended vinegar for wound cleansing and faster healing [9]. Different types of vinegars are available. This study aimed to assess the antimicrobial effect of pomegranate vinegar on oral bacteria. The effects of pomegranate extract on oral bacteria have been previously evaluated but no study has evaluated the effect of pomegranate vinegar on oral bacteria.

In another study, Brazilian researchers evaluated the effect of pomegranate extract on adhesion of oral bacteria (which is the first step in development of caries) [11].

Persica mouthwash contains Salvadora, Persica, mint and yarrow extracts. Several studies have reported antimicrobial, antiplaque and anti-cariogenic effects of Salvadora (Miswak) [12]. Since Persica mouthwash contains natural substances and is commercially available in the market, it was used for the purpose of comparison in the current study.

Rose water and vinegar have been recommended for caries prevention in the Iranian traditional medicine. Since the antimicrobial effects of these two substances on oral streptococci have not been evaluated before, this study aimed to assess the antimicrobial effects of these substances on cariogenic oral microorganisms in comparison with Persica (a commonly used botanical mouthwash).

Materials and Methods

In this in vitro study, five strains of S. mutans were isolated from the dental caries and dental plaque of candidates using a sterile curette. Standard strains of S. mutans (ATCC35668) and S. sobrinus (ATCC27607) were also obtained from the Iranian Scientific and Industrial Research Organization in lyophilized form.

A suspension was prepared of the lyophilized powder. All strains were cultured in blood agar and Mitis salivarius agar (MSA) and incubated at 37°C and 5% CO2 for 24 hours. Standard strains were cultured, exposed to bacitracin and optochin discs, Gram stained and subjected to catalase test for final confirmation.

Determining the quantity of biofilm formation by the bacteria using microtiter plate method:

The microtiter plate method is based on colorimetry and is used to determine the biofilm formation potential of bacteria and assessment of the effect of antimicrobial agents on the biofilm. This method requires a small culture medium, is not time consuming and can be used for assessment of the antimicrobial efficacy of a wide range of antimicrobial agents with different concentrations and in combination with each other [13,14].

In order to assess the biofilm formation potential of the bacteria, 18-24 hour culture of the bacteria in Tryptic soy broth (TSB) supplemented with 1% sucrose and 5% CO2 was prepared. This microbial suspension was diluted with sterile TSB to obtain 0.5McFarland turbidity. Of this suspension, 250µL was transferred to eight wells of a polystyrene 96-well plate. The control wells only contained sterile culture medium. After 24 hours, the content of the wells was removed and each well was rinsed with 300µL of sterile saline. Next, bacteria attached to the walls and bottom of the wells were fixed with 250µL of 96% ethanol. After 15 minutes, the contents of the wells were removed and the plates were dried at room temperature. Then, they were stained with 2% crystal violet for five minutes and after washing the excess dye with water, the plates were dried at room temperature. Next, 200 µL of 33% glycol acetic acid was added to each well and the optical density (OD) of the crystal violet present in the solvent was read by an ELISA reader at 492nm wavelength. Bacterial strains were then classified based on their OD as follows [15]:

OD: The mean light absorbance of bacteria

ODc: The mean light absorbance of control wells

$OD \leq ODc$: No adhesion

$ODc < OD \leq 2OD$: Poor adhesion

$2ODc < OD \leq 4ODc$: Moderate adhesion

$4ODc < OD$: Strong adhesion

Strains with strong adhesion, which had greater biofilm formation potential than others were chosen for the next step.

Determination of the efficacy of rose water, pomegranate vinegar and Persica mouthwash for elimination of biofilm:

Microtiter-plate method was used for this purpose. After preparation of 18-20 hour culture, 0.5 McFarland standard suspensions were prepared of the strains with strong adhesion in TSB supplemented with 1% sucrose. The suspensions were then diluted 1/100 in sterile TSB and all wells of a polystyrene 96-well plate were filled with 250µL of this suspension. Control wells only contained aqueous medium. After inoculation, the plates were covered and incubated at 37°C and 5% CO2 for 24 hours. Then, the wells were emptied and washed with 300µL of sterile saline; 250µL of vinegar, rose water and Persica mouthwash were added using 0.2 um Millipore syringe filter for one hour.

All eight wells in each row were treated the same and the antimicrobial agents were refreshed every 20 minutes. The control well rows only contained biofilm. After one hour, antimicrobial agents were removed by washing the wells. Next, the wells were stained with 200µL of 2% crystal violet for five minutes and after rinsing, they were filled with 200µL of 33% glycol acetic acid. In the next step, they were shaken on a shaker for 15 minutes and their OD was read at 492nm wavelength by an ELISA reader. Assessment of the efficacy of these materials was done by calculating the percentage of reduction in biofilm via OD of treated and control wells using the formula below: [16]

$$\text{Percentage of biofilm reduction} = \frac{(C-B)-(T-B)}{(C-B)} \times 100$$

Where C is the mean OD of positive control wells, B is the mean OD of negative control wells and T is the mean OD of treated wells.

Effect of rose water, pomegranate vinegar and Persica mouthwash on attachment of bacteria with strong adhesion:

Overnight culture of strongly adherent streptococci in TSB medium supplemented with 1% sucrose was done. To assess the effect of these materials on bacterial adhesion, two methods were used:

In the first method, 200µL of a mixture with similar portions of antimicrobial agent and bacteria was transferred to the wells while the control wells only contained microbial suspension. In the second method, 100µL of rose water, pomegranate vinegar and Persica mouthwash were poured into wells and streptococcal suspension was added 30 minutes later. In the control wells, first 100µL of phosphate buffered saline (PBS) and after 30 minutes, 100µL of streptococcal suspension were added. After 24 hours of incubation, the solutions and nutrients were removed from the wells and after three times of rinsing with PBS, staining was performed using 2% crystal violet for five minutes. After addition of 33% acetic acid, OD of the solvent was read using an ELISA reader.

Assessment of the effect of rose water, pomegranate vinegar and Persica on bacterial growth and proliferation:

The well-plate technique was used to assess the effect of antibacterial agents in this respect [7]. After 18-20 hour culture of S. mutans and S. sobrinus in brain heart infusion broth, a microbial suspension with 0.5 McFarland standard concentration was prepared of the bacteria and cultured on Mueller Hinton agar supplemented with 5% defibrinated sheep blood using a sterile swab in three directions with a 60° angle.

Inoculated plates were placed on a smooth surface for three to five minutes and then, equal wells measuring 6mm in diameter were created in the medium using the tip of a sterile Pasteur pipette. One drop of melted Mueller Hinton agar was poured into each well in order to seal the bottom of the wells. Next, 100 µL of the antimicrobial agents were added to each well. Distilled water was used as the negative control. The plates were refrigerated for one hour in order for the antimicrobial agents to be able to diffuse in the medium before bacterial proliferation. The plates were incubated for 16-20 hours and the diameter of the growth inhibition zone was then measured.

The data were analyzed using SPSS version 16. One-way ANOVA and Tukey's post hoc test were used for analysis of the data related to S. mutans, and the Kruskal Wallis test and Mann Whitney U test were used to analyze the data regarding S. sobrinus. Level of significance was set at P=0.05.

Results

Assessment of the quantity of biofilm formation by streptococci:

To assess the effect of rose water, pomegranate vinegar and Persica on bacterial adhesion, first the quantity of formed biofilm was assessed in order to select strains with greater adhesion to assess the effect of these antimicrobial agents on adhesion of bacteria with strong adhesion. Diagram 1 shows the mean of three measurements of OD of bacteria. Standard S. mutans and S. sobrinus strains (bacteria #1 and 2) were the strongly adherent strains. Bacteria #4-7 isolated from the mouth of candidates had poor adhesion and bacterium #3 was non-adherent.

Effect of understudy materials on S. mutans and S. sobrinus biofilm:

The results showed that rose water, pomegranate vinegar and Persica were able to prevent biofilm formation by these bacteria. The percentage of reduction in biofilm formation in presence of pomegranate vinegar, rose water and Persica was 0.089%, 0.38% and 0.089%, respectively for S. mutans and 0.061%, 0.37% and 0.062%, respectively for S. sobrinus; no statistically significant difference was noted in this respect (P=0.514).

Effect of understudy materials on strongly adherent bacteria:

The effects of rose water, pomegranate vinegar and Persica on adhesion of S. mutans and S. sobrinus are presented in Table 1. All three materials significantly decreased the adhesion of S. mutans and S. sobrinus compared to the control group (P<0.001). As seen in Table 1, rosewater resulted in 80 and 56% reduction in adhesion of S. mutans (in the first and second methods, respectively) and 57% and 60%,

respectively for the adhesion of S. sobrinus, which indicate no significant difference in the two methods for the effect of rose water on S. sobrinus (P=0.451). Also, the effect of rosewater on adhesion of S. mutans in the second method had no significant difference with that of Persica (P=0.319). However, the second method was effective for S. sobrinus (P=0.003).

Pomegranate vinegar inhibited the adhesion of both bacteria and had the greatest effect on bacterial adhesion compared to other materials in both methods (P<0.001); no significant difference was observed between the two methods in this respect (P=0.514)

Persica significantly decreased bacterial adhesion compared to the control group (P<0.001). No significant difference was found between the two methods for S. sobrinus, and both methods decreased the adhesion of both bacteria to the same extent (P=0.105).

Effect of medicinal plants on growth of streptococci:

Table 2 shows the mean diameter of the growth inhibition zones of S. mutans and S. sobrinus. As seen in Table 2, rosewater could not inhibit the growth of S. mutans and S. sobrinus and caused no growth inhibition zone. Pomegranate vinegar created considerable growth inhibition zones with 16 and 18mm diameters around S. mutans and S. sobrinus, respectively. The growth inhibition zone of S. mutans was 11mm in presence of Persica, which was significantly smaller than that caused by pomegranate vinegar (P<0.001); this value was 14mm for S. sobrinus, which was also smaller than that caused by pomegranate vinegar (P=0.003).

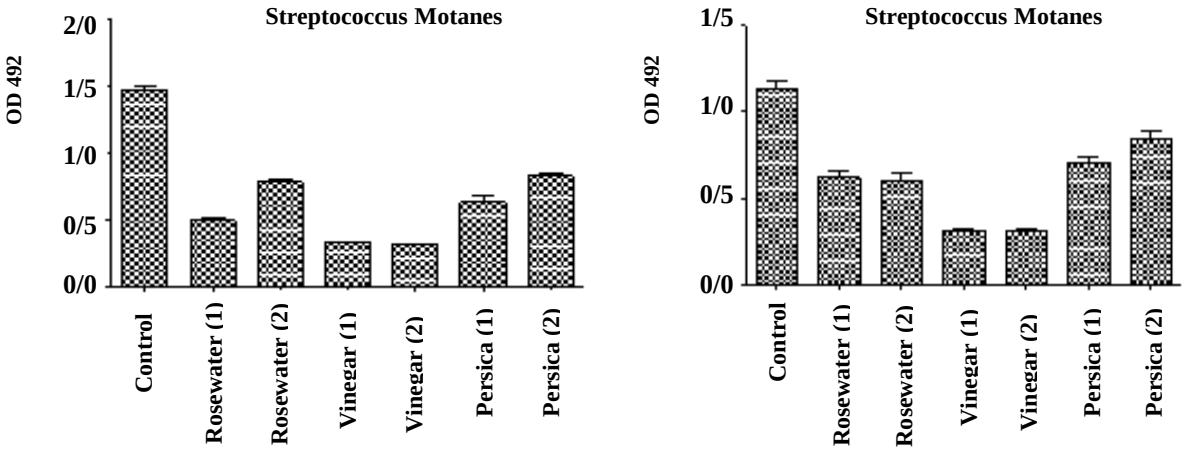

Diagram 1. The trend of reduction in biofilm formation in presence of rose water, pomegranate vinegar and Persica mouthwash

Table 1. The percentage of reduction in adhesion of S. mutans and S. sobrinus in presence of rosewater, pomegranate vinegar and Persica

Material	Bacteria	Percentage of reduction in adhesion (method 1)*	Standard deviation	Percentage of reduction in adhesion (method 2)**	Standard deviation
Rosewater	S.mutans	80	0.06	56	0.08
	S.sobrinus	57	0.10	60	0.16
Pomegranate vinegar	S.mutans	93	0.01	95	0.02
	S.sobrinus	92	0.03	93	0.03
Persica	S.mutans	68	0.11	52	0.06
	S.sobrinus	48	0.10	32	0.12

*Antimicrobial agents and bacteria were transferred to wells simultaneously
**Antimicrobial agents were transferred to wells 30 minutes earlier than the bacteria

Table 2. The mean diameter of growth inhibition zones for S. mutans and S. sobrinus in presence of pomegranate vinegar, rose water and Persica

Material	Bacteria	Mean diameter of growth inhibition zone in well-plate method (mm)	Standard deviation
Pomegranate vinegar	S.mutans	16	0.89
	S.sobrinus	18	0.95
Rose water	S.mutans	0	0
	S.sobrinus	0	0
Persica	S.mutans	11	0.79
	S.sobrinus	14	1

Discussion

Several studies have assessed the antimicrobial efficacy of Persica herbal mouthwash for reduction of dental plaque. This material has antimicrobial effects on oral pathogenic bacteria [12]. The current study showed the efficacy of Persica in decreasing the adhesion of oral streptococci; this result is in agreement with that of other studies on the effect of this mouthwash on oral bacteria. On the other hand, inability of Persica to eliminate biofilm or inhibit the growth and proliferation of oral bacteria was noted as well.

The antimicrobial effects of vinegar have been previously confirmed. Ismael evaluated the effect of several types of vinegar on the biofilm of Streptococcus pyogenes isolated from patients and showed that vinegar eliminated a minimum of 90% of the biofilm [17]. Komiyama et al, in 2010 evaluated the antimicrobial and disinfecting effects of 0.12% chlorhexidine, 0.50% white vinegar and two other materials on S. mutans, S. pyogenes, Staphylococcus aureus and Candida albicans on toothbrush and showed that vinegar decreased the count of S. mutans, S. pyogenes and S. aureus [18].

A few studies are available on the antimicrobial effects of compounds derived from pomegranate on oral pathogenic microorganisms.

Loo et al, in 2010 reported the effect of lactic acid on oral bacteria [19]. Pomegranate extract contains about 40% lactic acid. Also, Vasconcelos et al, in 2006 showed the antimicrobial effects of pomegranate extract on S. mutans [11].

Subramaniam et al. compared the antimicrobial effects of pomegranate extract with those of aloe Vera and sorbitol and showed that pomegranate

extract had greater antimicrobial efficacy compared to the other two against S. mutans [20]. The current results showed that pomegranate vinegar significantly inhibited the adhesion and proliferation of bacteria; however, after biofilm formation, it could not eliminate it. Vinegar may be suitable for elimination of oral bacteria since it can inhibit adhesion and proliferation of bacteria at the same time. An important issue with regard to vinegar is its acidic pH. Although mutans streptococci are among the acid-producing bacteria (acid production is a mechanism for them to compete with other pathogenic microorganisms in the oral cavity), they cannot survive vinegar exposure. Iranian traditional medicine has also pointed to the anticariogenic effects of vinegar; however, this property of vinegar has been neglected due to the existing concerns regarding the adverse effects of acids on tooth enamel. But, further studies are required to focus on the constituents of vinegar and purify the extract effective on cariogenic bacteria.

Search of the literature yielded no study on the effect of rose water on plaque producing oral bacteria. Thus, the current study was the first to assess the effect of rose water on oral bacteria. The results showed that rose water had a significant effect on decreasing the adhesion of bacteria but had no effect on their growth and could not eliminate biofilm after its formation. This finding shows that the mechanism of rose water in decreasing biofilm formation is different from its bactericidal and growth inhibition mechanisms; however, a definite conclusion in this regard requires further studies.

The results of this study showed that pomegranate vinegar, Persica and rosewater were all effective against adhesion of oral bacteria; however, they were not effective for elimination of biofilm. This finding may be due to strong adhesion of dental biofilm due to the activity of mutans streptococci. They first attach to plates by sucrose-independent adsorption and then sucrose-dependent adherence occurs following the synthesis of insoluble glucans [21]. Therefore, these colonization mechanisms may be sointense that they prevent the effect of antimicrobial agents on elimination of biofilm. Future studies are required to further assess the effects of vinegar and rose water in effective formulations on dental caries in clinical trials.

Conclusion

Considering the optimal antimicrobial effects of pomegranate vinegar on proliferation of cariogenic streptococci and the optimal efficacy of pomegranate vinegar and rose water in decreasing the adhesion of these bacteria, future studies are recommended to assess the application of these materials in different pharmaceutical formulations for caries prevention.

Acknowledgement

The authors would like to thank the Biology Group of Isfahan University of Science for their scientific cooperation and the Research Deputy of Isfahan University of Medical Sciences for financially supporting this project.

References

1. Palombo EA. Traditional medicinal plant extracts and natural products with activity against oral bacteria: potential application in the prevention and treatment of oral diseases. Evid Based Complementary Altern Med. 2011;2011: 680354.

2. Curzon ME, Preston AJ. Risk groups: nursing bottle caries/caries in the elderly. Caries Res. 2004 Jan;38(1):24-33.

3. Lamont RJ, and HF. Jenkinson. Oral microbiology at a glance. Wiley Black well publication,England. 2010,Chapter 13.

4. Taubman MA, Nash DA. The scientific and public-health imperative for a vaccine against dental caries. Nat Rev Immunol. 2006 Jul; 6(7): 555-63.

5. Steinberg D, Feldman M, Ofek I, and Weiss, EI. Effect of a high-molecular weight component of cranberry on constituents of dental biofilm. J Antimicrob Chemother. 2004 Jul; 54(1):86-89.

6. Mirz M, Najafpour Navae M. The effect of distillation method on extracted compounds from rose water. IJMAPR. 2007 fall;23(3):375-381.

7. Tofighi Z, Molazem M, Doostdar B, Taban P, Shahverdi AR, Samadi N, et al. Antimicrobial activities of three medicinal plants and investigation of flavonoids of Tripleurospermum

disciforme. IJPR. 2015 Jan; 14(1):225-231.
8. Naquvi KJ, Ansari SH, Ali M and Najmi AK. Volatile oil composition of Rosa damascena Mill. (Rosaceae) J Pharmaco and Phytochemist. 2014 Dec; 2(5):177-181.
9. Johnston CS, Gaas CA. Vinegar: medicinal uses and antiglycemic effect. MedGenMed. 2006 May; 30,8(2):61.
10. Setorki M, Asgary S, Eidi A, Rohani AH, Khazaei M. Acute effects of vinegar intake on some biochemical risk factors of atherosclerosis in hypercholesterolemic rabbits. Lipids Health Dis. 2010 Jan 28;9:10.
11. Vasconcelos LC, Sampaio FC, Sampaio MC, Pereira MS, Higino JS, Peixoto M H. Minimum inhibitory concentration of adherence of Punica granatum Linn (pomegranate) gel against S. mutans, S. mitis and C. albicans. Braz Dent J. 2006 Nov;17(3):223-7.
12. Firas A. AL-BAYATI, Khudir D. Sulaiman. In Vitro Antimicrobial activity of Salvadora persica L. Extracts. against some isolated oral pathogens in Iraq. Turk J Biol. 2008 Feb; 32(1): 57-62.
13. Stepanovic S, Vukovic D, Dakic and I, Savic B. A modified microtiter plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods. 2000 Apr; 40 (2):175-9.
14. Pitts B, Hamilton MA, Zelver N, and Stewart PS. A microtiter-plate screening method for biofilm disinfection and removal. J Microbiol Methods. 2003 Aug;54(2):269-76.
15. O'Toole GA, Kolter R. Initiation of biofilm formation in Pseudomonas fluorescensWCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol. 1998 May; 28(3):449-61.
16. Sonak S, and Bhosle NB. A simple method to assess bacterial attachment to surfaces. Biofouling. 1995 Jan; 9(1):31-38.
17. Ismael NF. Vinegar as Anti-bacterial Biofilm formed by Streptococcus pyogenes Isolated from Recurrent Tonsillitis Patients, in vitro. J of Biologi Sci. 2013 Sept; 6(3):191-197.
18. Komiyama EY, Back-Brito GN, Balducci I and Koga-Ito CY. Evalution of alternative methods for the disinfection of toothbrushes. Braz Oral Res. 2010 Jan-Mar;24(1):28-33.
19. Loo WT, Jin L, Cheung MN, and Chow L W. Evaluation of Ellagic acid on the activities of oral bacteria with the use of adenosine triphosphate (ATP) bioluminescence assay. African J Biotech. 2010 June;9(25):3938-3943.
20. Subramaniam P, Dwivedi S, Uma E, Girish Babu KL. Effect of pomegranate and aloe vera extract on streptococcus mutans: An in vitro study. Original Res. 2012 July;3(3):99-105.
21. Shimazaki Y, Mitoma M, Oho T, Nakano Y, Yamashita Y, Okano K, et al . Passive Immunization with Milk Produced from an Immunized Cow Prevents Oral Recolonization by Streptococcus mutans. Clin Diagn Lab Immunol. 2001 Nov; 8(6):1136-9.