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Inhibitory Effects of a Flavonoid-Rich Extract of *Pistacia vera* Hull on Growth and Acid Production of Bacteria Involved in Dental Plaque

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Abstract: The aim of present study was to investigate the antibacterial and the *in vivo* efficacy of aqueous, chloroformic and ethanolic extracts from *Pistacia vera* on bacteria involved in dental plaque. The growth inhibitory activity of the extracts were tested against *Strep. mutans*, *Strep. salivarius*, *Strep. sobrinus* and *Strep. Sanguis*. The bioautography and TLC analysis were used to provide relevant information on the chemical properties of extracts. Antimicrobial activity determined by agar well diffusion and determination of MIC values was measured by the liquid serial dilution culture method. Bactericidal and bacteriostatic, inhibition of glycolysis and sucrose-dependent adherence to smooth glass surface were tested using standard methods. The subjects used the mouthrinse and saliva samples were collected after 10 min, 1 and 3 h post-rinsing. The total streptococcal counts were measured before and after mouthrinsing. Aqueous, chloroformic and ethanolic extracts of *P. vera* hull inhibited the growth and acid production of tested bacteria. The result revealed that the ethanolic extract of *P. vera* hull displayed the most potent antimicrobial activity. *In vitro* studies had shown that ethanolic extract of *P. vera*, at concentrations of 2, 6 and 10% w/v, could inhibit the growth as well as the acid-producing ability of *Strep. mutans*. In *in vivo* study which was tested on three subjects shows that mouthrinse prepared by suspending 10% of ethanolic extract in PEG 300 (w/v) could reduced salivary bacterial count more than 55%, about 75% inhibition of adherence and inhibition of salivary glycolysis up to 3 h post rinsing.

Key words: *Pistacia vera* hull, mouthrinse, dental plaque, acid production, adherence

INTRODUCTION

Dental caries or tooth decay is the most important cause of tooth loss. It is characterized by a bacteria-induced progressive destruction of the mineral and organic components of the enamel and dentine, the two outer layers of the tooth. The two most common types of dental disease, dental caries and periodontal disease, are plaque-related infections. Dental plaque is required for caries development. Dental caries involves demineralization, cavitations and breakdown of calcified dental tissue and is caused by microorganisms that ferment dietary carbohydrates, notably sucrose, to produce acids; these acids initiate dissolution of the tooth enamel (Svensater *et al.*, 2003). The main bacteria in this group are *Streptococcus mutans*, *Strep. salivarius*, *Strep. sobrinus*, *Strep. sanguis* and *Actinomyces viscosus*.

Strep. mutans is generally known to be the principle causative of dental caries (O'Connor *et al.*, 2006). These bacteria metabolize carbohydrates and producing an adhesive polysaccharide such as dextran from the glucose moiety and lactic acid from the fructose moiety. The synthesis of sticky, insoluble glucan promotes the firm adherence of the organism to the tooth surface that contributes to the formation of dental plaque. Moreover, research indicates that the coexistence of *Strep. sobrinus* and *Strep. mutans* is an important factor in the development of dental caries (O'Connor *et al.*, 2006).

To achieve sufficiently low levels of plaque to support a healthy tooth several mechanical and chemical oral hygiene aids are necessary in addition to the toothbrush. Mechanical methods usually used in dental care services to remove plaques are time consuming and never completely effective (Gonzalez *et al.*, 2001).

Chemical approaches including use of antimicrobials, pH modifiers and many others alone or in combination with toothbrush and dental floss provide the best and reliable method in caries prevention (Xiao *et al.*, 2006). Strongly active substances like chlorhexidine gluconate 0.2% mouthrinse (CHX) are capable of reducing the percentage of cultivable microorganisms in the dental biofilm to 0.002%. But it should not be forgotten that some side-effects are present when CHX is used for extended periods of time such as staining of the tongue and of the dental biofilm, perturbation of the taste, burning sensations or mucosal erosion.

A large proportion of the population now prefers natural products and using the products with ingredients of plant origin is increasingly being offered. Therefore, a rinsing solution with inhibitory effect on plaque formation with anti-microbial activity will be very useful. Different plant derived phytopharmaceuticals with antimicrobial activity (Hayacibara *et al.*, 2005) have been used for oral cavity hygiene or treating of diseases. Some of these phytopharmaceuticals have been shown to be a good alternative to synthetic chemical substances for caries prevention (Hayacibara *et al.*, 2005).

Pistachio (*Pistacia vera* L.) is a member of the *Anacardiaceae* family. The genus *Pistacia* contains about 11 species, of which *P. vera* is by far the most economically important (Alma *et al.*, 2004). The *P. vera* tree is native of arid zones of Central and West Asia and distributed throughout the Mediterranean basin. *Pistacia* species have been generally used as traditional medicine for various diseases such as toothache, periodontal disease, blood clotting, gastralgia, dyspepsia, peptic ulcer, asthma, jaundice, diarrheic, throat infections, renal stones, astringent, anti-inflammatory, antipyretic, antibacterial and antiviral (Magiatis *et al.*, 1999; Marone *et al.*, 2001; Duru *et al.*, 2003; Ozcelika *et al.*, 2005). Antimicrobial, anti-inflammatory and insecticidal activities of essential oils and crude extracts of leaves and gums of *Pistacia* species (specifically, *P. lentiscus*) have been reported previously (Magiatis *et al.*, 1999; Grassmann *et al.*, 2000; Traboulsi *et al.*, 2002; Kordali *et al.*, 2003). The chemical composition (Magiatis *et al.*, 1999) and antimicrobial activities (Nguefack *et al.*, 2004) of essential oil from the leaves of *P. vera* have been reported. In Iran, the role of *P. vera* in treatment of diarrhea has been known for many years. The aim of this study is to evaluate the effects of extract obtained from *P. vera* hull, used as a mouthrinse, in reduction of dental plaque formation, anti-adherence and antiglycolytic of bacteria.

MATERIALS AND METHODS

Plant material: In this study, Pistachio fruits [*P. vera* L.] were collected on August, 2005 from Sarakhs mountains, Khorasan province, northern-east part of Iran, with an altitude of 700 m. The plant was authenticated by Herbarium, Faculty of Pharmacy, Medical Sciences, University of Tehran, Tehran, Iran. Hull were pilled off, washed with distilled water and dried in shade at room temperature to constant weight.

Preparation of extracts: The ethanolic and chloroformic extracts of the hull (78 g) of pistachio was obtained by solvent-distillation method using a Soxhlet apparatus for 3 h. The solvent evaporated at reduced pressure to constant weight and the yields of the oily residue were 4.5 and 4 g, respectively. Aqueous extract of pistachio's hull was obtained by percolation method (50 g, 500 mL distilled water) with the aid of mechanical shaker for 48 h and clarified by centrifugation at 750 g for 15 min. Non-alcoholic chlorhexidine gluconate 0.2% mouthrinse (CHX, Behvazan Co., Iran) was purchased from local market and used as reference mouthrinse.

Test bacteria: The growth inhibitory activity of the aqueous, chloroformic and ethanolic extracts was tested against 4 strains of bacteria (*Strep. mutans* ATCC 35668, *Strep. salivarius* 1448, *Strep. sobrinus* 1601 and *Strep. sanguis* 1449), which prepared from (PTCC or ATCC strains) and cultured in standard condition. These microorganisms were provided from Microbiology Laboratory, Razi Vaccine and Serum Institute, Tehran, Iran.

Thin layer chromatography studies: The study was carried out to aid in the selection of the appropriate solvent system for bioautography and also to provide relevant information on the chemical properties of extracts. Each extract was reconstituted in minimal amount of ethanol (96%) before spotting on the chromatography plates. Chromatography plates used were precoated with silica gel (0.2 mm Kieselgel 60 F254, Merck) and glass-backed as the adsorbent. The plates were placed in chromatographic tanks containing solvent systems of varying polarities. The developed plates, after air-drying, were examined under the UV lamp at wavelengths of 254 and 366 nm. The plates sprayed with vanillin/sulphuric acid were heated at 100°C until color developed.

Bioautography studies: The bioautography procedure of (Rohalison *et al.*, 1991) was employed. The samples were

loaded on the plates in bands. They were developed in duplicates in selected solvent systems for each of the *P. vera* hull extracts. After air drying overnight, each of the plates was placed in a humid chamber and overlaid with 10 mL molten nutrient agar seeded with 0.2 mL of *Strep. mutans* ATCC 35668. Adequate humidity was maintained by placing moist cotton buds at the corners of the plates. The overlaid plates were left for 30 min after which they were incubated at 37°C for 24 h. The cultures were sprayed with an aqueous solution of a dehydrogenase indicator-2.5 mg mL⁻¹ thiazolyl blue (methyl thiazolyl tetrazolium chloride). The plates were further incubated at 37°C for 4 h and thereafter sprayed with absolute ethanol to kill the test organisms in order to conserve the bioautography plates. The plates were allowed to air dry and covered with plastic plates. A reference plate (not overlaid with the culture medium) sprayed with vanillin/sulphuric acid was compared with each of the bioautography plates to ascertain the location of the active constituents of the extracts.

Phytochemical analysis: TLC analysis was carried out on Kieselgel 60 F254 (precoated 0.2 mm thick plastic plates, Merck, Germany) using the mobile phase 1-butanol/acetic acid/water (4:1:1.5, V/V). Visualisation of flavonoids and phenolic acids was achieved by spraying the sheet with 1% methanolic solution of diphenylboric acid aminoethyl ester followed by 5% ethanolic solution of polyethylene glycol 4000. The chromatogram was evaluated under UV light at 365 nm (Cvetnić and Vladimir-Knezević, 2004). For reference purposes, quercetin, flavon and rutin were used. Determination of the flavonoid in the ethanolic extract was performed according to previously reported method (Cvetnić and Vladimir-Knezević, 2004). After acid hydrolysis, the formed flavonoid aglycones were spectrophotometrically determined at 425 nm by creating a complex with AlCl₃. The content of flavonoids in the extract was calculated as hyperoside. The measurements were carried out using a Shimadzu 160-A UV-Visible spectrophotometer.

Antimicrobial activity determined by agar well diffusion:

Antimicrobial activities of the extracts of *P. vera* were determined using determined by the agar well diffusion method as will be described below. *Streptococcus* sp. were cultured in Brain Heart Infusion (BHI, Difco Laboratories) agar (sterilized in a flask and cooled to 45-50°C) at 37°C for 24 h an aerobically (95% N₂, 5% CO₂). Antibacterial activity was assayed by measuring the diameter of zone of inhibition against *Strep.* sp. seeded in Mitis salivarius (MS, Difco) agar plates. The plates were prepared by inoculating 15 mL of MS agar with 0.1 mL (~1.5×10⁷ colony forming unit, cfu) of *Strep.* sp.

grown in BHI at 37°C for 24 h. Assay samples (100 FL of each extract) with appropriate dilution were add to well (3×5 mm diameter) and the plates were incubated an aerobically by gas-substitution at 37°C for 24 h. Chlorohexidine mouthrinse (0.2%) was used as a positive control. After 24 h, inhibition zones appearing around the cylinder were measured and recorded in mm (Hirasawa *et al.*, 1999).

Determination of MIC values: The MIC of the pistachio hull extracts was measured by the liquid serial dilution culture method using 10 mL of sterile 5% sucrose-Trypticase Soy Broth (TSB). The ethanolic and aqueous extracts were diluted with water. The chloroformic extract was diluted with chloroform and mixed to medium with aid of sonication (50w, 10s). Bacteria (1×10⁶ cfu mL⁻¹) were added to each culture tube containing serially diluted test extract or control and incubated for 24 h at 37°C under aerobic condition. The lowest concentration of the extracts that inhibited growth was noted.

Determination of bactericidal activity: To estimate whether inhibition of growth was bactericidal or bacteriostatic, 10 times MIC of the pistachio hull extracts were used in the experiment. Samples were collected over an extended period. The stocks concentration used were 1 mg mL⁻¹ of chloroformic and ethanolic extract and 100 mg mL⁻¹ of aqueous extract for *Streptococcus* species. These samples were diluted and inoculated onto a plate at each appropriate time. After incubation, the number of colonies was counted. Further, to investigate the effect of the extracts on non-multiplying bacterial cells, resting bacterial cells were prepared. The growing cells were harvested, washed three times with 50 mM tris-HCl buffer (pH 7.3, TB) and used for determination.

Influence of heat treatment: The *P. vera* hull extracts were treated at 60°C for 30 min and 100°C for 5 min. After heat treatment, samples were centrifuged (1000 g, 15 min) and supernatants were used for determination of antibacterial activity. The antibacterial activity of extract was compared with that of non-treated pistachio hull extracts.

Inhibition of glycolysis: The effects of the extracts on acid production by *Strep. mutans* was studied by a reported method (Köhler *et al.*, 1981; Corner *et al.*, 1990) with slight modification. 2 ML of fresh clarified human saliva, was added 0.1 mL of 5% glucose and 0.1 mL of various concentrations of the ethanolic *P. vera* extracts (2, 6 and 10%). Control consisted of the saliva/glucose mixes containing 0.1 mL of PEG 300 (20% v/v). The pH of the samples were recorded immediately after preparation and at 10, 15, 30, 45, 60, 90, 120 min and 24 h.

Effect on sucrose-dependent adherence to smooth glass surface:

To assess the adherence of growing cells of *Strep. sobrinus* 1601 to a glass surface, organism was grown at 37°C at an angle of 30° for 18 h in test-tubes (Köhler *et al.*, 1981). Individual 18-24 h colonies from brain-heart infusion agar plates were suspended in 5.0 mL of sterile 145 mM NaCl and the suspension adjusted to 0.5 on McFarland scale. A portion of the suspension was mixed with TSB (1:100 dilution, v/v) containing 29 mM sucrose and then 2.48 mL were transferred to a test-tube. Subsequently, 20 FL of 2-fold dilution series of the ethanolic extract (2, 6 and 10%), chlorhexidine (0.2%) and water as a control were inoculated, gently stirred and then incubated. After incubation, the adherence cells were washed and suspended using the procedures outlined by Köhler *et al.* (1981). The amount of adherent cells was measured at 550 nm (OD₅₅₀). Five replicate were made for each concentration of the tested extract.

Study in vivo: Three young men, between the ages 22-30 years, with 20 or more natural teeth free of dental caries were recruited for this study. The protocol for study was approved by the local ethical review committee. The procedure, possible discomfort, or risk was fully explained to the volunteers and their written consent obtained. Subjects kept on their usual oral hygiene routines and no attempts were made to change or standardize or eating habits. We preferred to collect salivary rather than plaque samples, because salivary microbial counts are more consistent than plaque counts (Mundorff *et al.*, 1990).

Non-stimulated saliva samples (2 mL) were collected prior to study and served as the control and were placed immediately on ice. Mouth-rinse was prepared by suspending the ethanolic extract of *P. vera* in PEG 300 (20% v/v) and distilled water to give a final concentration of 10% of extract. The subjects were asked to rinse their mouths with 10 mL of mouthrinse for 1 min and do not rinsed their mouths with water afterwards. Further saliva samples were collected after 10 min, 1 and 3 h post-rinsing. Subjects were not allowed to eat or drink between sample collections. Placebo rinses was prepared using PEG 300 (20% v/v) in distilled water. Each saliva sample was immediately diluted 103 times with sterile saline and streaked on BHI Agar plates to determine the total saliva bacteria. Mitis salivarius agar (Difco) supplemented with sucrose (200 g L⁻¹) and bacitracin (0.2 units mL⁻¹) for the count of oral *streptococci*. The plates were incubated into jars at 37°C for 24 h. After incubation, the colonies were counted using a stereomicroscope. Among colonies grown on mitis salivarius agar supplemented with sucrose, only those with the morphology of mutans *Streptococci* were considered.

Statistics analysis: Data obtained for acid production and inhibition of bacterial growth were analyzed. Data due to the mouthrinse were analyzed, for the inhibition of bacterial growth by comparing the prerinsing values with the post-rinsing values using paired t-test. For *in vitro* glycolysis, the data from the test at various time intervals were compared with those obtained for control at the same times interval using t-paired test. All statistical analysis was done in SPSS version 10.0. All values were considered significant with $p < 0.05$.

RESULTS

Antibacterial activity was determined by measuring the diameter zone inhibition. The ethanolic extract showed more inhibitory effect than aqueous and chloroformic extract (Table 1) on all test bacteria and used for rest of this study. CHX always have highest inhibitory zone.

Minimum inhibitory concentration: The MIC values of the ethanolic, chloroformic and aqueous extracts varied from 1.25-2, 3.25-3.75 and 50-75 mg mL⁻¹. The results show that ethanolic extract of *P. vera* hull had the most inhibitory effect rather than other extracts on *Streptococcus* species (Table 2).

Minimum bactericidal concentration: Table 3 shows the antibacterial effects of the three extracts on growing bacterial cells and resting bacterial cells of *Strep. mutans* ATCC 35668. The growth or survival of both the growing and resting cells treated with ethanolic extract decreased gradually with the incubation time. The action of ethanolic extract was bactericidal while chloroformic and aqueous extracts showed more bacteriostatic activity against *Strep. mutans* ATCC 35668.

Bioautographic study: This test revealed that among the ethanolic, chloroformic and the aqueous extract of the *P. vera* hull extract only the ethanolic extract demonstrated activity while no activity was observed from the aqueous and chloroformic extract. The purple zone of inhibition is shown against a pink background. The ethanolic extract exhibited very weak activity at the origin and a strong activity at R_f value of 0.72 (Fig. 1). The presence of quercetin in the ethanolic extract was confirmed by the TLC method. The content of flavonoid was 0.105%.

Effect of heat treatment on antibacterial activity: A 17% reduction was observed in the antibacterial effect against *Strep. mutans* ATCC 35668 with moderately heat-treated extract (60°C, 30 min). A 40% reduction was observed with strongly heat-treated (100°C, 5 min).

Table 1: Mean area of the zone of microbial growth inhibition provided by aqueous, ethanolic and chloroformic extracts of *P. vera* and chlorhexidine gluconate

Microorganisms	Treatments*			
	Aqueous	Ethanolic	Chloroformic	Chlorhexidine
<i>Streptococcus mutans</i>	18±0.9	46±0.3	34±0.7	52±0.4
<i>Streptococcus salvarius</i>	19±0.7	48±0.4	34±0.7	54±0.5
<i>Streptococcus sanguis</i>	21±1.0	46±0.6	34±0.8	54±0.7
<i>Streptococcus sorbinus</i>	22±0.5	46±0.4	28±0.5	48±0.6

*: The antimicrobial test was done using the agar-well diffusion method on BHI agar and incubated for 24 h at 37°C. Values are given in mm and expressed as mean±SEM (n = 5)

Table 2: Minimal Inhibitory Concentration (MIC) of aqueous, chloroformic and ethanolic extracts of *P. vera*

Microorganisms	Treatments (mg mL ⁻¹)		
	Aqueous	Chloroformic	Ethanolic
<i>Streptococcus mutans</i>	≥ 75	3.75	2.0
<i>Streptococcus salvarius</i>	50	3.75	1.75
<i>Streptococcus sanguis</i>	50	3.50	1.25
<i>Streptococcus sorbinus</i>	≥ 75	3.25	1.25

Table 3: Antibacterial effect of *P. vera* hull extracts against *Strep. mutans* ATCC 35668 at growing stage

<i>P. vera</i> hull extracts	Time (h)				
	0	2.5	5	10	24
Aqueous	10 ⁶ *	1.2×10 ⁶	1.3×10 ⁶	1.3×10 ⁶	1.4×10 ⁶
Chloroformic	10 ⁶	9.0×10 ⁵	7.5×10 ⁵	7.2×10 ⁵	5.6×10 ⁵
Ethanolic	10 ⁶	10 ⁵	10 ⁴	8.0×10 ³	5.0×10 ³
Distilled water	10 ⁶	9×10 ⁶	10 ⁷	5.0×10 ⁷	10 ⁸

*: Colony forming units (cfu mL⁻¹)

Table 4: Temporal effects of the 2, 6 and 10% ethanolic extract mouth-rinse of *P. vera* on *in vitro* salivary glycolysis inhibition

Extract conc.	pH of the before and post-rinsing saliva samples collected at various time intervals (min)								
	0	10	15	30	45	60	90	120	140
Saliva sample + Glucose	5.94±0.02	5.91±0.01	5.46±0.03	5.19±0.03	4.92±0.05	4.87±0.07	4.83±0.08	4.72±0.09	5.20±0.05
2% + Glucose	5.84±0.03	5.79±0.01	5.53±0.04	5.26±0.10	5.07±0.04	4.98±0.04	4.89±0.05	4.70±0.10	5.09±0.03
6% + Glucose	5.39±0.02	5.38±0.02	5.29±0.06	5.14±0.03	5.02±0.04	4.95±0.01	4.87±0.04	4.71±0.03	4.85±0.07
10% + Glucose	5.44±0.05	5.43±0.03	5.32±0.04	5.13±0.03	5.00±0.03	4.81±0.03	4.86±0.01	4.67±0.01	4.93±0.02
PEG + water + Glucose	5.85±0.02	5.80±0.07	5.44±0.05	5.13±0.07	5.03±0.05	4.89±0.04	4.87±0.05	4.72±0.05	5.12±0.06

Effect on acid production by salivary bacteria: The result of *in vitro* salivary glycolytic assay revealed that at concentrations of 2% of the ethanolic extract had no significant inhibition on acid production ($p < 0.05$) when compared to vehicle (PEG 200v/v and water). In glycolytic reaction, the pH values did not changed significantly ($p < 0.05$) at higher concentrations of extract (6 and 10%) over a period of 120 min (Table 4). Six percent solution of the extract showed the good inhibitory results on acid production which is comparable to 10% extract ($p < 0.05$). The significantly lower pH values of all the samples as compared with the control values are due to the low pH of the extract itself (Table 4).

Inhibition of adherence of strep. Sobrinus: As shown in Fig. 2, inhibition of *in vitro* adherence of *Strep. sobrinus* to glass was evident when the cells were grown in BHI broth containing sucrose and various concentrations of the extract of *P. vera*. At a concentration of 10% the extract showed about 75% inhibition of adherence while CHX (0.2%) produced an inhibition greater than 94%.

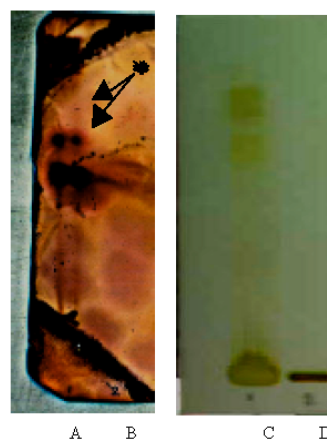


Fig. 1: Bioautography of crude extracts of *Pistacia vera* hull. Test organisms: *Strep. mutans* ATCC 35668; Solvent systems Ethyl acetate/Chloroform (70:30). (A) Ethanolic extract of *P. vera* hull, (B) Chloroform extract of *P. vera* hull, (C) Chromatogram reference for A, (D) Chromatogram reference for B. * Zones of inhibition are shown by purple haloes against a pink background

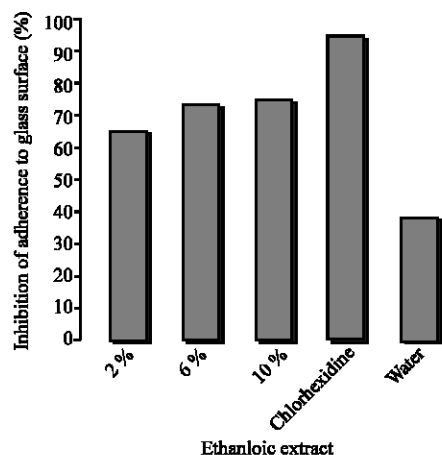


Fig. 2: Effect of various concentrations of ethanolic extracts of *P. vera* hull, chlorhexidine and water on the adherence of growing *Strep. sobrinus* 1601

Table 5: Temporal effects of mouth-rinses with 10% ethanolic extract of *P. vera* on Total Salivary Bacterial Counts (TSBC)

Time (min)	Reduction of TSBC mouthrinsing (%)
10	75.34±6.94
60	64.25±7.70
180	57.10±8.37

Study in vivo efficacy of mouth rinses: Mouthrinsing with a 10% solution of the extract of *P. vera* nut shells showed a significant reduction in total *Streptococcal* count at 10 min, 1st and 3rd h of post rinsing ($p < 0.05$). Rinsing with the vehicle showed no significant difference in the salivary bacterial count at any time interval (Table 5).

DISCUSSION

Pistacia species have a wide range of uses in food industries, eczema treatment, paralysis, diarrheic, throat infections, renal stones, jaundice, asthma and stomachache and as astringent, anti-inflammatory, antipyretic, antibacterial, antiviral, pectoral and stimulant (Duru *et al.*, 2003). Resin is also traditionally used as chewing gum, against lip-dryness, some stomach diseases and antiseptic for respiratory system. Flavonoids, triterpenoids, phenolics and essential oils are the major constituents of *P. vera*. Investigation on *Pistacia* species has also revealed that crude extracts, essential oils and some triterpenoid constituents exhibit anti-inflammatory, antifungal activities (Duru *et al.*, 2003).

In this study, we assessed the antibacterial activity of *P. vera* hull extracts on oral bacteria with the aim of preventing dental caries. In this microbial analyses, the ethanolic extracts of *P. vera* showed stronger inhibitory activity compared to chloroform and aqueous extracts.

Ethanolic extract of *P. vera* has been demonstrated to manifest good antimicrobial activity, as evidenced by the MICs of obtained against a *Strep. sp.* But no significant difference was seen between the inhibitory growth of *Strep. mutans* with *Strep. sanguis*, *Strep. sobrinus* and *Strep. salivarius*. The weak effect of aqueous extract was predictable. The terpenoids have hydrophobic properties causing low solubility in aqueous media. Either the ethanolic extract or chlorhexidine showed better antibacterial effect on *Strep. salivarius*, probably due to exhibition of the same antibacterial mechanisms.

To determine whether the observed inhibition of bacterial growth by ethanolic pistachio hull extract is bactericidal or bacteriostatic, viable cell method against *Strep. mutans* was used. The ethanolic extract killed more than 75% of both growing and resting cells of *Strep. mutans* ATCC 35668 within 2 h. With respect to *Strep. mutans*, the chloroform extract possessed bacteriostatic activity. While aqueous extract showed weak bacteriostatic activity, the action of ethanolic extract was bactericidal. In contrast, the bacterial cells were not affected by the aqueous extract and the chloroformic extract showed more bacteriostatic activity against *Strep. mutans* ATCC 35668. The antibacterial effect of ethanolic hull extract can be attributed to the presence of phenolic compounds in the extract and similar activity has been reported previously (Kubo *et al.*, 1993). Present data in agreement with other reports proposing that *P. vera* have anti-bacterial activity (Alma *et al.*, 2004). As stated previously, the aqueous extract of *P. vera* was less efficient than the alcoholic or chloroformic extracts in inhibiting growth of bacteria. As ethanol solubilized significantly higher amounts of compounds such as tannins, flavonoids and essential oils as compared with the aqueous extraction, we anticipated a greater suppression of plaque formation on the surface of the tooth with the alcoholic extract. It seems that the low efficiency of the aqueous extract is due to low level of extraction of non-polar active materials.

The relative heat stable nature of hull extract against *Strep. mutans* revealed that the composition of extract probably less heat sensitive or volatile. The inhibition of acid production in the saliva samples after applying the 10% extract may be due to the antibacterial effect of the extract on the salivary bacteria *in situ* at that concentration. This could be concluded from the inability of the extract to inhibit acid production in the *in vitro* salivary glycolytic assay at low concentrations ($< 1\%$). At concentration of 2% the pH values change significantly throughout the glycolytic reaction showing absence of anti glycolytic effect. At higher concentrations of the extract (10%) the pH values did not change significantly throughout the glycolytic reaction. The mechanism of the

observed long term effect upon the acidogenicity of the saliva on mouthrinsing with the extract could therefore be related to the extracts bactericidal or bacteriostatic effect. On the other hands, the hull extract had an immediate effect on the salivary bacteria and this effect continued up to 3 h.

In the sucrose-dependent adherence study, glass surface was used to represent the hard surface of the tooth (Fathilah and Rahim, 2003). Bacterial adherence to glass surface is the model system chosen because the adherence is mediated by glucan as well as the *in vivo* situation and the glass adherence assay is still used in some recent studies (Koo *et al.*, 2000; Nostro *et al.*, 2004). The adherence of *Strep. mutans* to the glass surface was markedly inhibited by sub-MIC concentrations of the extract. It would be possible that the bioactive compound(s) such as flavonoids, tannins in the *P. vera* hull extract are responsible for the bacterial adherence inhibition. Flavonoids are known to have anti-GTase activity (Iio *et al.*, 1984). This enzyme is responsible for the conversion of sucrose to sticky insoluble glucan, which promotes the firm adherence of *Strep. mutans* to the surface of the tooth.

At concentrations of 10%, the extract had an immediate effect on the salivary bacteria and this effect was retained for 3 h. For an agent to work successfully in the oral cavity, it should have an immediate effect that is sustained over time. This activity is also associated with inhibition of adherence to glass surface. This inhibition of bacterial attachment prevents adherence of *Strep. sobrinus* to glass surface by a mechanism thought to involve dextran formation from sucrose. At a concentration of 10%, the extract was found to be as effective *in vitro* as CHX. Thus, the extract could successfully prevent plaque formation on the surface of the tooth, as it inhibited the sucrose-induced adherence, this process that foster the colonization of the organism on the surface of the tooth. The *in vitro* plaque assay revealed that as the concentration of the extract increased, the inhibition of adherence to the saliva coated tooth decreased. The sustained antibacterial and antiglycolytic effect observed in the oral cavity on mouthrinsing with the extract suggested that the extract had a good substantivity in the oral cavity. Based on results obtained from the present study, it is evident that the flavonoid and other phytoconstituents present in the hull extract are involved in the bactericidal and bacteriostatic activity against *Strep. mutans* at different concentrations. In addition, preliminary studies suggest that *P. vera* extracts does not produce staining (Fardal and Turnbull, 1986), a significant side effect of CHX. A potent agent for use in the oral cavity that does not stain would be a valuable aid

in oral hygiene. Thus, when considering the overall effect of the extracts on *Strep. mutans*, it can be said that the use of the hull extract of *P. vera* as a mouth rinse will have an effect on the growth, adherence and acid-producing ability of *Strep. mutans*.

We now have a scientific reason to justify the use of these *P. vera* as an aid to maintain oral hygiene. The results of this study reveal that the *P. vera* hull as a by-product can be a valuable source for pharmaceutical preparations such as mouthrinse.

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